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CANINE MAMMARY TUMORS:
QUANTITATIVE MORPHOLOGICAL AND GENETIC STUDIES

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# CANINE MAMMARY TUMORS:

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MARTA SUSANA AMARO DOS SANTOS
TESE DE DOUTORAMENTO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS
ABEL SALAZAR DA UNIVERSIDADE DO
PORTO EM
PATOLOGIA E GENÉTICA MOLECULAR

Marta Susana Amaro dos Santos

# CANINE MAMMARY TUMORS: QUANTITATIVE MORPHOLOGICAL AND GENETIC STUDIES

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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**Preambule**

This Thesis includes material published or submitted for publication as:

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In addition to those listed above, other chapters (6, 7 and 8) of the Thesis are being prepared to be submit as full articles to international journals devoted to Veterinary Pathology and Oncology.

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**Abbreviations and symbols:**

$\beta$ – beta coefficient

$\kappa$ – kappa statistics

mm – millimeter

µm – micrometer

$\kappa_u$ – unweighted kappa

$\kappa_w$ – weighted kappa

$\bar{v}_N$ – number-weighted mean nuclear volume

$\bar{v}_V$ – volume-weighted mean nuclear volume

2D – two-dimensional

3D – three-dimensional

aCGH – array comparative genomic hybridization

AgNOR – argyrophilic nucleolar organizer region

BAC – bacterial artificial chromosome

C – percentage of concordance

CE – coefficient of error

CGH – comparative genomic hybridization

CMC – canine mammary carcinomas

CMT – canine mammary tumors

CNA – copy number aberrations

CV – coefficient of variation

DFI – disease-free interval

DNA – deoxyribonucleic acid

FISH – fluorescence *in situ* hybridization

NHG – Nottingham histological grade

NPI – Nottingham Prognostic Index

$N_V$ (nuclei, tumor) – nuclear numerical density

OS – overall survival

PCNA – proliferating cell nuclear antigen

PSI – point sampled intercepts

rMC – systematic random mitotic count

ROC – receiver operating curve

SD – standard deviation

SE – standard error

sMC – selective mitotic count

TNM – tumor-node-metastasis

$V_V$ (nuclei, tumor) – nuclear volume density

WHO – World Health Organization

**Resumo**

Apesar da evolução do conhecimento sobre aos tumores mamários da cadela nas últimas décadas, a definição do prognóstico e de protocolos de tratamento representam ainda desafios atuais, tanto para patologistas como para clínicos. Neste sentido, a avaliação de fatores prognósticos em tumores mamários malignos da cadela (TMMC) assume grande importância clínica. Nesta Tese estudou-se a relação entre a sobrevivência dos animais e o grau histológico, incluindo uma avaliação detalhada dos parâmetros constituintes do grau (formação tubular, pleomorfismo nuclear e contagem de figuras de mitose). Neste âmbito, foram também testadas novas abordagens quantitativas para avaliação de potenciais características morfológicas de prognóstico nos TMMC. Paralelamente estimou-se, pela primeira vez, a reprodutibilidade do método de determinação do grau histológico em TMMC.

Para a realização destes estudos foram selecionados casos de TMMC espontâneos, a partir de uma série de cadelas seguidas durante um período de dois anos após a cirurgia.

Nestes animais, o grau histológico de Nottingham (originalmente concebido para carcinomas mamários da mulher) tinha valor prognóstico em análise estatística univariada. Globalmente, a determinação do grau apresentava moderada reprodutibilidade entre observadores, sendo o pleomorfismo nuclear o parâmetro menos reprodutível. Ainda assim, este foi o único parâmetro com relação estatisticamente significativa e independente com a sobrevivência. Já os restantes parâmetros do grau, formação tubular e contagem de figuras de mitose, não tinham individualmente valor prognóstico.

Relativamente a novas abordagens quantitativas aplicáveis aos TMMC, testou-se a avaliação do pleomorfismo nuclear através da quantificação do “volume nuclear médio pesado pelo volume”. Este parâmetro estereológico foi significativamente menor em tumores benignos e, permitiu distinguir duas classes em termos de pleomorfismo do volume nuclear nos tumores malignos. Relativamente à formação tubular, e admitindo que este parâmetro do grau poderia estar relacionado com a celularidade tumoral, foi aplicado um método estereológico (*disector* ótico) para determinar a densidade numérica nuclear das células tumorais. Este método foi aplicado pela primeira vez no estudo dos TMMC e demonstrou que a densidade numérica nuclear era menor nos

tumores mais agressivos, correlacionando-se negativamente com o volume nuclear médio pesado pelo volume. Estes resultados sugerem que parâmetros quantitativos relacionados com o pleomorfismo nuclear e com a celularidade poderão ter valor prognóstico nos TMMC e tal deverá ser explorado em estudos futuros.

Quanto às contagens de figuras de mitose, foi testado um método de contagem em campos selecionados através de amostragem aleatória e sistemática do tecido tumoral, associada à correção das contagens para a quantidade de epitélio maligno presente em cada campo. No entanto, a contagem de mitoses segundo este método não apresentava vantagens para a definição do prognóstico dos TMMC.

Ainda no seguimento da otimização da determinação do prognóstico na cadela, testou-se a utilização de uma fórmula de índice multifatorial de prognóstico, a qual integrava o grau histológico, o tamanho tumoral e a evidência de invasão vascular e/ou de metástases nos gânglios linfáticos regionais. Esta fórmula foi adaptada do índice de prognóstico de Nottingham, utilizado na Medicina Humana, e demonstrou uma boa capacidade discriminativa dos tumores mais agressivos, parecendo constituir uma ferramenta útil para a definição do prognóstico nos TMMC. Estudos prospetivos são necessários para validar o uso deste índice de prognóstico nos TMMC.

Com o objetivo de estabelecer novos parâmetros morfológicos com potencial valor prognóstico para os TMMC, foram avaliadas diversas variáveis morfológicas em preparações histológicas de rotina. A presença de invasão vascular, percentagem de necrose, de diferenciação escamosa e de formas nucleares anormais, assim como a presença de nucléolos grandes e atípicos, emergiram como fatores de prognóstico. De acordo com os nossos dados preliminares, o contributo de cada fator poderá ser calculado e integrado numa fórmula de um índice de graduação prognóstica específico para os TMMC.

Reconhecendo que as características morfológicas dos tumores podem ter subjacentes um espetro de alterações genéticas, procedeu-se à caracterização genómica de uma série de TMMC através do método da hibridização comparativa genómica em *arrays*. Os perfis genómicos dos TMMC apresentavam uma grande variedade de alterações cromossómicas, incluindo ganhos, deleções e perdas em diferentes cromossomas, em especial nos

cromossomas 9, 22, 27, 34 e X. Na série analisada foram incluídos tumores múltiplos síncronos da mesma cadela, no sentido de determinar a sua relação clonal. Ainda que face à reduzida amostragem os resultados possam ser considerados preliminares, estes já apontam para que as lesões síncronas possam constituir entidades neoplásicas independentes do ponto de vista genético.

Em termos gerais, demonstrou-se nos estudos desta Tese que parâmetros morfológicos quantitativos podem ser incluídos na avaliação do prognóstico dos TMMC e que uma maior utilidade clínica do grau histológico poderá passar por refinamentos no método e nos componentes a avaliar. Complementarmente à abordagem morfológica verificou-se que a heterogeneidade clinico-histológica reconhecida dos TMMC poderá refletir uma grande variabilidade em termos de bases genéticas dos tumores. Deste modo, a relação das alterações genómicas com o espetro clínico da doença deverá ser alvo de novos estudos mais alargados.

**Summary**

The body of evidence regarding the biology of canine mammary tumors has increased over the decades, however the clinical management of female dogs affected by the disease is still challenging for both clinicians and pathologists. It is well recognized that searching of new prognostic factors is of uttermost clinical importance. Herein, a comprehensive evaluation of the prognostic value of the histological grade and its parameters (tubule formation, nuclear pleomorphism and mitotic count) in malignant canine mammary tumors (CMT) was performed. Moreover, quantitative methods were introduced to evaluate the potential prognostic utility of morphological parameters. Additionally, the repoduciblity of the histological grading method was evaluated, for the first time, in CMT. For these purposes, spontaneous malignant CMT, surgically removed and belonged to a series of female dogs followed over two years, were selected.

When applied to malignant CMT, the human Nottingham grading method presented prognostic value in univariable analysis and a moderate reproducibility, being nuclear pleomorphism the least reproducible parameter. Nuclear pleomorphism classification showed a significant and independent association with survival, while tubule formation and mitotic count scores did not provide prognostic information.

As to the quantification strategies, a stereological estimator of the nuclear size pleomorphism named volume-weighted mean nuclear volume was significantly lower in benign tumors, and it allowed the identification of two classes of nuclear size pleomorphism in malignant cases. Considering that tubule formation could be related with the level of tumor cellularity, a stereological method (optical disector) was applied to estimate the nuclear numerical density, a cellularity-related parameter. This method was used for the first time in the study of malignant CMT and rendered interesting results: the nuclear numerical density was lower in cases that progressed and this parameter was negatively correlated with the volume-weighted mean nuclear volume. Accordingly, quantitative estimations of nuclear pleomorphism and cellularity-related parameters could have prognostic value in CMT and this deserves further evaluation.

As regarding to mitotic count, a new counting method was investigated. Mitotic figures were counted in systematic random sampling fields, considering the whole tumoral section and correcting for the area of the malignant epithelium present in each field. However, this approach did not improve the prognostic value of the mitotic counts.

Seeking for improvements in prognostic definition of malignant CMT, a multifactorial index formula, which included the histological grade, the tumor size, and presence of vascular invasion and/or regional lymph node metastases, was tested. The formula was adapted from the human Nottingham Prognostic Index (NPI) and demonstrated a good predicting capacity of a clinical aggressive tumoral behavior. The adaptation of the NPI to CMT seemed to be a valuable tool for prognosis definition, pending this evidence for validation in prospective studies.

Aiming to find new potential grading parameters in malignant CMT, different morphological characteristics were evaluated in routine slides. The presence of vascular invasion, percentage of necrosis, squamous differentiation, atypical nucleoli and abnormal nuclear forms appeared as suitable prognosticators in malignant CMT. All these features could be easily assessed during histopathological examination and, according to our preliminary evidences the value of each parameter could be computed and integrated in a canine-specific prognostic index formula.

Considering that the tumoral morphological features could be underpinned by the genetic changes, we also evaluated the genomic aberrations in malignant CMT using array comparative genomic hybridization. The genomic profiles of malignant CMT revealed a wide range of genomic aberrations affecting different chromosomes, with gains, losses and deletions. Chromosomes 9, 22, 27, 34 and X were more frequently affected. By analyzing a small set of synchronous multiple tumors of the same female dog, preliminary evidence pointed to a clonal genetic independence of the lesions.

Overall, the studies of this Thesis showed that quantitative methodologies could be valuable for prognosis definition in malignant CMT and that the clinical utility of the histological grade could be improved if adjustments regarding the method and the parameters were considered. Moreover, the works suggested that the clinicopathological variability of malignant CMT seemed to be closely related to

genetic heterogeneity, and supported that influences of genomic aberrations in the clinical spectrum of the disease deserve more and large rigorous studies.

# CHAPTER 1

## CANINE MAMMARY TUMORS – FACES AND SHADOWS OF A COMPLEX DISEASE INTRODUCTION AND AIMS

# 1. Introduction

## 1.1 The canine mammary gland and its tumors

The canine mammary gland is a unique organ, undergoing extensive remodeling and differentiation during the entire reproductive life of female dogs (Santos *et al*., 2010a). In each estrous cycle, waves of epithelial and mesenchymal proliferation, ductal branching and alveoligenesis are followed by intense regressive changes. All these are strictly coordinated with hormonal levels of estrogen and progesterone (Rehm *et al*., 2007; Santos *et al*., 2010a). Unfortunately, these profound physiological and morphological activities of canine mammary gland are linked to a high risk of neoplastic transformation. In fact, mammary neoplasia is the most common form of cancer in intact female dogs (Sorenmo, 2003). According to the large European cancer registries databases, the incidence ratio of mammary gland tumors varies from 111 per 10,000 to 200 per 100,000 dog-years of risk, with the median age of first diagnosis being around seven years old (Dobson *et al*., 2002; Egenvall *et al*., 2005). In general, the development of mammary tumors in dogs less than five years old is rare and, in most cases, only benign tumors are diagnosed at that age (Kurzman and Gillbertson, 1986). There are differences between breeds regarding the risk for developing mammary tumors, suggesting a genetic influence in the canine mammary tumorigenesis (Sorenmo, 2003; Egenvall *et al*., 2005; Sorenmo *et al*., 2011). However, nowadays there is little evidence that canine mammary tumors (CMT) have a specific inheritance or are associated with germ line mutations (Nieto *et al*., 2003; Rivera and von Euler, 2011); this contrasts with human breast cancer cases (van der Groep *et al*., 2011). Rivera *et al* (2009) reported that BRCA1 and BRCA2 germ line mutations were associated with an increased risk for developing CMT in particular breeds of dogs, but larger studies about their importance and role in the risk of disease are still missing (Rivera and von Euler, 2011). In this vein, the majority of CMT appear to correspond to sporadic cases (Nieto *et al*., 2003).

The pioneer work of Schneider *et al.* (1969) demonstrated a chief role of sex hormones in the neoplastic process and, nowadays, this is consensual (Gobello and Corrada, 2001; Lana *et al*., 2007; Smith, 2014). Therefore, it is not surprising that an increasing emphasis has been given to the expression of

estrogen and progesterone receptors in benign and malignant CMT (*e.g.*, de las Mulas *et al*., 2005; Mainenti *et al*., 2014; Peña *et al*., 2014). In the dog, the mammary expression of growth hormone (regulated by the progesterone) and growth hormone receptor (which are important for the normal cyclic epithelial changes), is altered in tumors and apparently, this endocrine pathway could also contribute to the tumorigenesis (van Garderen and Schalken, 2002).

The heterogeneity, at clinical and at morphological level is, eventually, the most idiosyncratic characteristic of CMT (Sorenmo *et al*., 2011). There is no typical clinical presentation for CMT — animals of different ages can present as asymptomatic or symptomatic, with a single or multiple nodules, small or large, slow or rapidly growing tumors (Perez-Alenza *et al*., 2000; Hellmén, 2005). Moreover, the coexisting mammary nodules have frequently different histopathological classification (Sorenmo *et al*., 2009). The presence of multiple palpable nodules variably sized in the mammary glands with a synchronous or metachronous development is more common in female dogs than in other mammals (Sorenmo *et al*., 2009).

In a microscopical perspective, CMT are also highly heterogeneous. This is somehow reflected by the increased number of classification systems published so far and by the existence of some level of disagreement between those classifications (Hampe and Misdorp, 1974; Monlux *et al.*, 1977; Moulton *et al*., 1990; Benjamin *et al*., 1999; Misdorp *et al*., 1999; Misdorp, 2002; Goldschmidt *et al*., 2011). In recent years, the official classification of the World Health Organization (WHO) has been widely adopted by the veterinary diagnostic pathology community and this is highly recommended for the sake of parallelism between studies (Sleeckx *et al*., 2011; Matos *et al*., 2012). CMT can be composed of luminal epithelial cells only (simple tumors), or associated with myoepithelial cells (complex tumors) and with mesenchymal cells, like cartilage or bone tissue (mixed tumors) (Misdorp *et al*., 1999; Misdorp, 2002). The frequent biphasic appearance of the tumors is a unique feature of CMT (Rasotto *et al*., 2014). In veterinary pathology myoepithelial cells (either resting or proliferating) could be part of an invasive carcinoma (Sánchez-Céspedes *et al*., 2011; Peña *et al*., 2014). In contrast, in human pathology, the presence of a myoepithelial cell layer (assessed by immunohistochemistry) assists in the

differential diagnosis between benign and malignant tumors (*e.g.*, intraductal papilloma *versus* papillary carcinoma and for distinguishing invasive from *in situ* carcinomas) (MacGrogan *et al*., 2012; O'Maley *et al*., 2012; Pinder *et al*., 2012). A special attention has been given to the histogenesis of mixed CMT with the great majority of studies using imunohistochemical methods and aiming to clarify the origin of the mesenchymal metaplastic components, such as cartilage or bone (Gärtner *et al*., 1999; Tateyama *et al*., 2001; Ramalho *et al*., 2006). Despite some debate on the origin of these tissue within the mixed CMT still prevails, the recent literature appears to reinforce the putative role of myoepithelial cells in the mesenchymal metaplastic differentiation (Goldshmidt *et al*., 2011; Rasotto *et al*., 2014).

About 30% of excised tumors are malignant (Misdorp, 2002). These malignant CMT can spread to distant organs and cause the death of the animal. Metastases affect mainly the lungs, liver and spleen, in a decreased order of frequency (Clemente *et al*., 2010a). The inflammatory carcinoma is a highly aggressive tumor that presents a slightly different metastatic pattern, since it has a predilection for the genitourinary system (Clemente *et al*., 2010a).

### 1.2 Canine mammary tumors – malignancy and prognosis

The prevalence of metastases-associated death after two years of follow-up ranges from 20 to 44% (Hellmén *et al.*, 1993; Peña *et al.*, 1998; Karayannopoulou *et al.*, 2005; Sassi *et al.*, 2010; Peña *et al.*, 2013). The prediction of the metastatic capacity of malignant tumors is the unresolved chief question in CMT and a limitative factor for implementing new therapeutic options. The remarkable clinicopathologic heterogeneity of malignant CMT probably accounts for the limited available therapeutic options (Goldshmidt *et al*., 2011; Klopfleisch *et al*., 2011, Sorenmo *et al*., 2011).

Traditionally, surgery is considered the gold standard therapy for CMT (Sorenmo, 2003; Sorenmo *et al*., 2011). However, the extension and type of the surgical procedure has been debated for long among veterinarians (Lana *et al*., 2007). In general, it is recommended to perform a radical mastectomy (all chain mastectomy), instead of multiple nodulectomies or local mastectomies, especially in animals bearing multiple tumors (Sleeckx *et al*., 2011). Proponents

of radical mastectomy argue that this approach is faster and reduces the risk of new tumors, thus avoiding the need of a second surgery (Stratmann *et al*., 2008). Recently, is has been stressed that the focus should be placed in the completeness of surgical margins, as this factor is associated with an improved survival of dogs with malignant tumors (Tran *et al*., 2014).

The prognosis of malignant CMT still remains a challenge to both pathologists and clinicians. In recent years, relevant literature has been published addressing consensual recommendations in veterinary oncology, some of them specifically referring to CMT (Webster *et al*., 2011; Matos *et al*., 2012; Nguyen *et al*., 2013). According to those guidelines, prognostic studies should completely describe the selection of the sample and the methodology of the assessments. The pathological evaluation of possible prognostic markers should be done by at least two observers and, whenever possible, multivariable analysis should be performed as this statistical evaluation eliminates possible confounders (Webster *et al*., 2011). Unfortunately, these procedures were not always followed in CMT studies; the high variability in study designs is nowadays evident, jeopardizing a straightforward identification of robust prognostic factors in CMT (Matos *et al*., 2012). The search for prognostic factors in CMT is already beyond the traditional clinicopathologic parameters (Dagli, 2008). Undoubtedly, this represents a promise way to reach new therapeutic targets, but at the same time, it is important to keep in mind that histopathological evaluations are simple and cost-effective methods for assessing prognosis, as occurs in human breast cancer (Pinder *et al.,* 1995; Rakha *et al*., 2010). Fortunately, the amount of evidence collected for some clinicopathologic factors is large and their prognostic role is already well established in CMT (Sorenmo *et al*., 2011; Sleeckx *et al*., 2011). Probably, the two most important prognostic factors are tumor size and lymph node metastases (Sleeckx *et al*., 2011). Both are associated with distant metastases for the WHO staging of the disease, also known as the tumor-node-metastasis (TNM) system (Rutteman *et al*., 2001).

Tumor size is defined as the largest diameter determined either by clinicians or pathologists (Peña *et al.*, 1998; de las Mulas *et al.*, 2005; Sorenmo *et al.*, 2011) and there is a general agreement that it has prognostic significance (Sorenmo

*et al*., 2011). Some studies suggested that a single cut-off of 3 cm, rather than the two cut-offs of the WHO staging (3 and 5 cm), is more suitable for prognostic purposes (Kurzman and Gilbertson, 1986; Philibert *et al*., 2003; Santos *et al*., 2013). However, Chang *et al.* (2005) using a multivariable analysis found that dogs with tumors larger than 5 cm had a significant worse prognosis. Despite some discrepancies regarding the threshold, several studies supported the importance of tumor size in prognosis definition and its inclusion in the stage of the disease (Ferreira *et al*., 2009; Sorenmo *et al*., 2011).

The presence of regional lymph node metastases is also relevant for staging the disease. However, the number of positive nodes, which is of utmost importance in human breast cancer stage (Senkus *et al*., 2013), is usually disregarded in CMT (Rutteman *et al.*, 2001). Lymph node assessment in female dogs is primarily performed by physical examination (Sorenmo *et al*., 2011), which constitutes another difference from breast cancer patients. These latter are usually submitted to diagnostic imaging of regional lymph nodes (usually by ultrasonography) and, in most centers of developed countries, a sentinel lymph node procedure during the surgery is also performed (Senkus *et al*., 2013). Even if this is the state of the art in women's breast cancer, such procedures are not routinely performed by veterinarians (Webster *et al.*, 2011). In female dogs bearing mammary nodules, the draining lymph nodes are carefully evaluated during the pre-surgery physical examination, and subjected to cytological evaluation when enlarged (Sorenmo *et al*., 2011). The histological examination of regional lymph nodes is performed when inguinal nodes are included as part of the mastectomy specimen or when the cytology is positive for metastases. This justifies that even prospective cohorts included cases without histological examination of the regional lymph nodes (Santos *et al*., 2013; Peña *et al*., 2013). However, several authors confirmed that the histological evidence of metastases in regional lymph nodes (at the time of the diagnosis) is a significant prognostic factor (reviewed by Sorenmo *et al.*, 2011). Another important issue related to regional lymph node assessment in malignant CMT is the use of immunohistochemistry as an auxiliary method to detect occult metastases. The TNM staging system for human breast carcinomas indicates that the presence of isolated metastatic cells or clusters with less than 0.2 mm (or 200 cells) in a lymph node, should not be included in

the count of affected lymph nodes (Senkus *et al*., 2013). In female dogs, Matos *et al*. (2006a) used immunohistochemistry with antibodies against cytokeratins and detected micrometastasis in 9% of regional lymph nodes. The clinical relevance of this finding has never been completely appraised. However, it should be, at least, considered as a sign of aggressiveness of the tumor (Matos *et al*., 2006a), and consequently, alert the clinician for the need of more rigorous follow-up of those animals. Another important issue related to regional lymph node, corresponds to differences in lymph drainage in healthy and in neoplastic mammary gland (Pereira *et al*., 2003; Patsikas *et al*., 2006). Conventionally, it is assumed that the first two pairs drain to axillary nodes, the two last pairs drain to inguinal lymph nodes, while the lymph of the third pair could simultaneously be collected by both lymph stations (Pereira *et al*., 2003). However, it has been reported that new anastomoses between lymphatic vessels of the different mammary pairs can develop and other lymph nodes (as sternal, medial iliac or popliteal) can be involved when the mammary gland is affected by a neoplastic disease (Pereira *et al*., 2003; Patsikas *et al*., 2006). In this vein, all the evidence substantiates that the entire lymph nodes stations should be carefully evaluated during the physical examination and additional procedures should be performed if abnormalities are detected (Matos *et al*., 2006a; Sorenmo *et al*., 2011).

### 1.3 Histological grade in canine mammary tumors

Traditionally, the grading of malignant CMT has followed the human counterparts, as also occurred with the histological classification (Hampe and Misdorp, 1974). The comparative canine *versus* human approach was a goal when the classification of domestic tumors was designed under the auspice of the WHO (Beveridge and Sonin, 1974). In that first official classification, Hampe and Misdorp (1974) opened the window for using the level of tubular differentiation in the canine mammary malignancy grading system, as occurred in human pathology. Two years later, Misdorp and Hart (1976) proposed a grading method based on the existing human grading systems, *i.e.,* Patey and Scarff (1928) and Bloom and Richardson (1957) (as detailed in the section 1.4 of this chapter). It was proposed that all types of CMT should be graded according to the level of differentiation (tubule formation) and the degree of cellular anaplasia (which included irregular cell size, shape and staining of cells,

nuclei and number of mitoses). In that system, only the sarcomas were not included (Misdorp and Hart, 1976). Even if a higher grade of malignancy was associated with a worse prognosis, none of the individual grading parameters showed a statistical association with outcome (Misdorp and Hart, 1979). Interestingly, the authors stated that higher grade, higher level of differentiation and anaplasia were more common in simple carcinomas than in complex carcinomas. Regarding the mitotic counts, there was no significant difference between complex and simple carcinomas (Misdorp and Hart, 1979).

In 1983, Gilbertson *et al.* proposed a different staging and grading system for CMT, which was based on the stromal and vascular invasion, as well as in nuclear differentiation degree. The method also followed the human grading system developed by Black *et al.* (1955). It included the assessment of the degree of invasion (stromal and vascular / lymph node invasion), as well as the degree of nuclear differentiation (Table 1). Regarding this latter parameter, the numerical grade increased with the increasing degrees of differentiation (Gilbertson *et al*., 1983; Kurzman and Gilbertson, 1986). Significant differences were found between stages 0, I and II regarding disease-free interval (DFI); animals with stage II had a particularly high recurrence rate (Kurzman and Gilbertson, 1986). In contrast, dogs with nuclear grade 1 (atypical and poorly differentiated tumors) had more than fourfold increased risk of recurrence in the two years post-surgery and this effect was most apparent in the stage I of the disease (Kurzman and Gilbertson, 1986).

The Gilbertson system was adopted by other research groups (*e.g.*, Karayannopoulou *et al*., 2001), however, whilst in some studies only the degree of invasion was used (Papparella *et al*., 2002; Sarli *et al*., 2002), in others an emphasis was given to nuclear grade (Preziosi *et al*., 1995).

Methods similar to that used by Misdorp and Hart (1976) and, clearly influenced by the human Bloom and Richardson method (1957) have been adopted by French, Italian, Spanish and Portuguese research groups (*e.g.*, Lagadic and Estrada, 1990; Peleteiro, 1994; Restucci *et al*., 2000; de las Mulas *et al*., 2005: Sánchez-Céspedes *et al*., 2011). Peña *et al.* (1998) used a grading system based on human Bloom and Richardson method, in association with the vascular invasion included in the histological staging of Gilbertson *et al.* (1983).

In that study the presence of vascular invasion was used as an exclusive diagnostic feature of poorly differentiated tumors.

In the reference book “Tumors in Domestic Animals” edited by Meuten, Misdorp (2002) completely adopted the numerical human method of Bloom and Richardson (1957), which included three grading parameters: tubule formation, nuclear pleomorphism and mitoses / hyperchromatic nuclei per high-power field. Each of these was scored from 1 to 3, and the combined sum of the scores (from 3 to 9) defined the grade: scores 3 to 5, 6 to 7 and 8 to 9 defined grade I, II and III, respectively (Table 2).

**Table 1** – Classification system of the histological stage and grade according to Gilberston *et al*. (1983) criteria.

| **Score** | **Histological stage – degree of invasion** |
|---|---|
| **0** | No stromal invasion |
| **I** | Stromal invasion |
| **II** | Vascular / lymphatic invasion and/or metastases to lymph nodes |
| **III** | Systemic / distant metastases |
| **Score** | **Nuclear grade – degree of nuclear differentiation** |
| **1** | “Atypical” / poorly differentiated |
| **2** | “Intermediate” / moderately differentiated |
| **3** | “Typical” / well differentiated |

Despite some controversy still exists, the grading system most used over the last decade has been the standard method of the human breast cancer, the so-called Elston and Ellis method or Nottingham method (*e.g.*, Karayannopoulou *et al*., 2001; Nieto *et al*., 2003; Karayannopoulou *et al*., 2005; Clemente *et al*., 2010b; Carvalho *et al*., 2011; Santos *et al*., 2011; Kim *et al*., 2013). This human method was based on a large cohort of patients treated and followed at the Nottingham Hospital, United Kingdom (as detailed in the section 1.4 this chapter). The method presented high similarity with that of Bloom and Richardson, but the criteria defined for each parameter were more objective (Elston and Ellis, 1991). In brief, tubule formation became assessed semi-quantitatively (categorized in 1-3 according to the percentage of epithelium arranged in luminal structures). Nuclear pleomorphism score was established in

comparison with the normal surrounding parenchyma, and finally, the mitotic count was categorized according to the number of mitoses and the field diameter of the microscope (Elston and Ellis, 1991) (Table 3).

**Table 2** – Histological grading system according to Misdorp (2002) criteria.

| | | **Score** | |
|---|---|---|---|
| **Parameter** | **1** | **2** | **3** |
| Tubule formation | Well marked | Moderate | Few or no tubules |
| Nuclear pleomorphism | Mild pleomorphism and staining | Moderate pleomorphism and staining | Marked pleomorphism and staining |
| Mitotic figures / hyperchromatic nuclei (per high-power field) | Occasional | 2-3 | > 2-3 |
| Sum of scores | 3 - 5 | 6 - 7 | 8 - 9 |
| **Grade** | **I** | **II** | **III** |

**Table 3** – Nottingham histological grade method (Elston and Ellis, 1991).

| | | **Score** | |
|---|---|---|---|
| **Parameter** | **1** | **2** | **3** |
| Tubule formation | > 75% | 10-75% | <10% |
| Nuclear pleomorphism* | absent | moderate | marked |
| Mitotic count** | <9 | 9-17 | >17 |
| Sum of scores | 3 - 5 | 6 - 7 | 8 - 9 |
| **Grade** | **I** | **II** | **III** |

* compared to normal surrounding parenchyma; ** counted in 10 high-power fields; cut-offs depending on microscopic field diameter of the microscope; in this case, it was 0.55 mm.

It is noteworthy that the Nottingham method has been adopted by several research groups dedicated to the study of CMT and from different parts of the world (Table 4). However, it is nowadays recognized that the use of human grading methods requires adjustment efforts in the veterinary field (Goldschmidt *et al*., 2011; Matos *et al*., 2012; Mills *et al*., 2015). In literature, only one

retrospective study compared the Misdorp (adapted from the human Bloom and Richardson's method) and Nottingham method adapted to CMT (Rasotto *et al*., 2012). According to that study, the latter method was better for predicting the metastatic capacity of CMT, because a considerable proportion of tumors allocated to grade I (*i.e.*, well differentiated) by the Misdorp method presented vascular / lymph node invasion. The more rigorous mitotic count and the exclusion of hyperchromatic nuclei in the Nottingham method were described as main advantages (Rasotto *et al*., 2012).

Recently, Peña *et al.* (2013) proposed changes to the Nottingham method, including specific scores in particular subtypes of malignant CMT (*e.g.*, in malignant myoepithelioma tubule formation should be scored 2) and the assessment of nuclear pleomorphism and mitotic counts in all the malignant cell types (and not exclusively in the luminal epithelium). These topics are still a matter of debate, and four main drawbacks can be pointed to this new method. In first place, considering that myoepithelial cells are unable to be organized in tubular structures without luminal epithelial cells, the above mentioned recommendation is arguable. Additionally, the actual contribution of each malignant component for the assessment of nuclear pleomorphism is not clear in this new proposed grading method. Moreover, the authors did not suggest a grading method to carcinosarcomas and, lastly, they did not perform a comparative analysis between their modified method and the original Nottingham's method regarding their ability to predict survival in CMT. Such a comparative approach would be warranted for the sake of the validation of the proposed modifications.

Nowadays, the prognostic significance of the histological grade in malignant CMT is still under study (Peña *et al*., 1998; Nieto *et al*., 2000; Peña *et al*., 2013; Santos *et al*., 2013; Mainenti *et al*., 2014). Karayannopoulou *et al.* (2005) used the original Nottingham grading method for different types of carcinomas and concluded that the tumor grade was directly related with prognosis, with the single exception of simple carcinomas, where no differences were demonstrated between grades II and III. Furthermore, undifferentiated tumors (grade III) presented a 21-fold increased risk of death as compared to the other two grades combined (Karayannopoulou *et al.*, 2005). In that same year, de las

Mulas *et al.* (2005) confirmed the independent prognostic value of histological grade (performed using the human Bloom and Richardson's method as basis), regarding the DFI. However, this was observed when grade I plus grade II tumors were compared with grade III (de las Mulas *et al*., 2005). Additionaly, another study demonstrated a non-independent worse prognostic of grade III tumors, for tumor-related death and survival time, when compared with jointed grades I and II (Santos *et al.*, 2013). Such grouping of grades (I and II *versus* III) in the statistical analysis of prognosis raises the issue of the actual existence a three-tier grading system in CMT and adds doubts on the validity of the grading method in this species. Recently, two studies demonstrated that the Nottingham method with modifications proposed by Peña *et al.* (2013) provided independent prognostic information (Peña *et al.*, 2013; Mainenti *et al.*, 2014). Still, it has been stressed that in order to be universally accepted and adopted in CMT, any grading method should be validated in various prospective cohorts (Goldschmidt *et al.*, 2011; Matos *et al*., 2012). Until the present day, there have been relatively few efforts in that direction.

In human pathology, the criticism regarding histological grade resides mainly in the: 1) lack of reproducibility of grade and its parameters; 2) great proportion of tumors allocated to less discriminative grade II; and 3) problems associated with grading special types of carcinomas (Dalton *et al*., 1994; Volpi *et al*., 2004; Meyer *et al*., 2005; Rakha *et al*., 2008a). Regarding the first issue, the interobserver reproducibility is, to the best of our knowledge, completely unknown in CMT. In the majority of reports the number of grading observers was not provided (Table 4), while in others it was stated that two observers classified and graded the tumors but the statistic agreement values were not provided (Restucci *et al*., 2000; Karayannopoulou *et al.,* 2005; Rasotto *et al*., 2012). Notably, the overlooking of reproducibility of histological grade in CMT is contradictory, at some extent, with the most recent guidelines in veterinary oncology, which clearly state that the measurement of interobserver variability is critical to validate any prognostic marker, before it can be translated to a clinical setting (Webster *et al*., 2011). Moreover, the significance and distribution of the three parameters of the histological grade, as well as their individual influence on survival outcome has never been scrutinized in CMT. As far as we know,

only the classic studies by Misdorp and Hart (1976, 1979), performed an evaluation of the prognostic value of each grading parameter.

Concerning the distribution of tumors by the three grades, the disproportionate high number of cases graded II, as occurred in some human cohorts, seems not to be a major problem in CMT (Table 4). Considering the more than twenty studies published over the last two decades and the different grading methods (Table 4), grade II comprised 33% of cases, whereas grade I and III corresponded to 36% and 31%, respectively. Still, the proportion of each grade in the studies would depend on several factors, including intrinsic biological characteristics of the tumors, sampling schemes used to select the case series or pre-analytical parameters, such as tissue fixation and preparation (Rakha *et al*., 2010). It should be noted that in some CMT cohorts the distribution of cases by the three grades was not provided (*e.g.*, Clemente *et al*., 2010b; Mainenti *et al*., 2014), and the selection/inclusion criteria were not always completely described.

In their original publication, Elston and Ellis (1991) stressed that the assessment of histological grade is not restricted to ductal carcinomas and special types should also be graded. Nevertheless, the usefulness of histological grade in some subtypes of tumors such as lobular and medullary carcinomas is still controversial (Sinha *et al*., 2000; Adams *et al*., 2009; Rakha *et al*., 2010). Regarding lobular carcinomas, it has been argued that two of the three grading parameters present little variation (tubule formation is typically scored 3 and mitotic count scored 1) and thus, grading is mainly derived from differences in the nuclear pleomorphism, which is the least reproducible grading feature (Adams *et al*., 2009). In some studies there were no differences regarding survival between grade I and II lobular carcinomas and very few tumors were graded III (Sinha *et al*., 2000). More recently, the research group of the Nottingham University Hospital confirmed, in a large cohort (with more than 500 patients), that grade was an independent prognostic factor in lobular carcinoma and that a difference in survival was observed between all the three grades (Rakha *et al*., 2008a). Accordingly, the routine assessment of histological grade using the Nottingham criteria in lobular carcinomas is currently recommended (Rakha *et al*., 2008a; 2010). With medullary

carcinomas the scenario is quite different and this subtype of carcinoma might appear to be an exception regarding the importance of histological grade (Elston and Ellis, 1998; Rakha *et al*., 2010). This type of carcinoma is usually graded III, but their prognosis is more favorable than this grade would imply (Jacquemier *et al*., 2012). In this vein, some authors believe that this data warrants further confirmation (Rakha *et al*., 2010).

In CMT the Nottingham method has been performed in different subtypes of malignant tumors, including complex and mixed carcinomas with myoepithelial and mesenchymal elements. Those elements, including the resting or proliferative myoepithelium, typically present slightly or no atypia (Peña *et al*., 2014) and so, the grade is mainly based in the luminal epithelial cells characteristics (Mainenti *et al*., 2014). That approach is in accordance with previous evidence that the malignant luminal epithelium is the main responsible for the progression of disease to local recurrence or systemic metastases (Monlux *et al*., 1977; Gilbertson *et al*., 1983; Kurzman and Gilbertson, 1986; Moulton *et al*., 1999).

**Table 4** – Grading methods in CMT studies published over the last two decades.

| Study | Cases | Obs | Method | G I | G II | G III |
|---|---|---|---|---|---|---|
| Preziosi *et al*. (1995) | 48 | np | NG Gilbertson | 8 | 24 | 16 |
| Peña *et al*. (1998) | 39 | np | BR/Misdorp + VI | 6 | 10 | 13 |
| Karayannopoulou *et al*. (2001) | 16 | 1 | NG Gilbertson<br>Nottingham | 6<br>6 | 5<br>5 | 5<br>5 |
| Karayannopoulou *et al*. (2005) | 85 | 2 | Nottingham | 27 | 28 | 30 |
| de las Mulas *et al.* (2005) | 155 | 2 | BR/Misdorp | 77 | 49 | 29 |
| Matos *et al*. (2006b) | 77 | np | Nottingham | 16 | 34 | 27 |
| Restucci *et al.* (2007) | 25 | 2 | Misdorp | np | np | np |
| Gama *et al*. (2008) | 102 | np | Nottingham | 14 | 33 | 45 |
| de Oliveira *et al*. (2009) | 32 | 2 | Nottingham | 6 | 15 | 11 |
| Madrazo *et al*. (2009) | 63 | np | Misdorp | 39 | 20 | 4 |
| Clemente *et al*. (2010b)[1] | 41 | np | Nottingham | np | np | np |
| Gama *et al*. (2010) | 102 | np | Nottingham | 17 | 34 | 51 |
| Santos *et al*. (2010b) | 64 | 2 | Nottingham | 13 | 28 | 23 |
| Sánchez-Céspedes *et al*. (2011) | 63 | np | BR/Misdorp | 32 | 23 | 8 |
| Yoshimura *et al*. 2011 | 42 | np | Nottingham | 11 | 12 | 19 |
| Rasotto *et al*. (2012) | 245 | 2 | Misdorp<br>Nottingham | 78<br>30 | 90<br>100 | 77<br>115 |
| Manuali *et al*. (2012) | 50 | np | Nottingham | 33 | 14 | 3 |
| Kim *et al.* (2012) | 37 | np | Nottingham | 8 | 11 | 18 |
| Santos *et al*. (2011) | 94 | 2 | Nottingham | 21 | 43 | 30 |
| Peña *et al*. (2013) | 65 | np | Mod Nottingham | 29 | 19 | 17 |
| Kim *et al*. (2013) | 47 | np | Nottingham | 11 | 14 | 22 |
| Andreasen *et al*. (2014)[2] | 31 | np | Misdorp/Nottingham | 25 | 4 | 2 |
| Guil-Luna *et al.* (2014) | 46 | np | BR | 22 | 17 | 7 |
| Guimarães *et al*. (2014)[2] | 43 | np | Misdorp/Nottingham | 17 | 13 | 13 |
| Im *et al*. (2014) | 340 | np | Nottingham | 222 | 74 | 44 |
| Mainenti *et al*. (2014) | 79 | 2 | Mod Nottingham | 47 | 12 | 20 |
| Yoshimura *et al*. (2014)[3] | 72 | np | Mod Nottingham | 2 | 27 | 43 |

Legend: Obs – observers; G – grade; np – not provided; NG – nuclear grade; BR – Bloom and Richardson's method; Mod - modified; VI – vascular invasion.

[1]inflammatory carcinomas included.
[2i]not clear if the authors used the Misdorp or Nottingham criteria.
[4]only simple solid carcinomas.

## 1.4 Histological grade in human breast cancer – history of a constant renewed interest

Greenough is credited for applying grading systems to breast cancer for the first time (Greenough, 1925). In his pioneering work, six morphological features were considered (Table 5). This study showed that the classification of tumors in three grades of malignancy was important regarding prognosis. Notably, this author also introduced the notion of reproducibility of grading (Greenough, 1925).

Three years later, Patey and Scarff (1928) stated that the study of the relation between histological grade and prognosis could not be done without accounting for the clinical stage of the disease. Curiously, these authors used only three of the six morphological features of Greenough (Table 5). They confirmed the relation between the histological grade and clinical course in different stages of the disease (Patey and Scarff, 1928).

During the war years, Bloom and Richardson revitalized the grading method of Patey and Scarff (Bloom and Richardson, 1957). In their study of more than 1,500 patients, three grading parameters were considered but, for the first time, these were scored from 1 to 3, being the final combined score used to assign the overall tumor grade (Table 5). The grade computed by this method was correlated with 5, 10 and 15 years of survival. Despite this evidence, the authors advised that the histological grade should be used in association with other factors, like the axillary node status, for the guidance of prognosis (Bloom and Richardson, 1957).

In that same period, Black *et al.* (1955) developed a grading system based only in the nuclear features of cells. The regularity of nuclear limits, the chromatin pattern, the presence of nucleoli and mitotic figures were considered to allocate a tumor to grades 0 to 4 (Black *et al.*, 1955). In this study there was a clear linear relation between nuclear grade and survival (Black *et al*., 1955). The same research group provided evidence in subsequent studies that nuclear grade system had prognostic value (*e.g.*, Black *et al.*, 1975). Even so, other groups failed to observe a relation between the nuclear grade and the breast cancer survival (*e.g.*, Kister *et al.*, 1969). Later on, in 1968, the WHO adopted the Bloom and Richardson method (Roberti, 1997). Despite this, some authors

used elements of the two methods simultaneously (Fisher *et al.*, 1975). In the Bloom and Richardson method, the number of cases allocated to the less discriminative (grade II) category was very high, despite the good correlation between grade and survival.

Le Doussal *et al.* (1989) evaluated the prognostic value of each of the grading parameter of the Bloom and Richardson method and concluded that nuclear pleomorphism and mitotic counts were the highest predictors for all survival measures. Based on that evidence, these authors modified the grading system by defining 5 grading categories (according to the combined score of the nuclear pleomorphism and mitotic count). This approach allowed the stratification of cases firstly allocated to grade II in two groups with different risk of developing metastases (le Doussal *et al.*, 1989).

Three decades after the development of the Bloom and Richardson grading method, Parham *et al.* (1992) demonstrated that the mitotic count was the most significant grading parameter. Based on that evidence and in previous studies showing that a significant association between survival and necrosis existed (*e.g.*, Fisher *et al.*, 1978), the authors proposed a grading system incorporating necrosis and mitotic counts (Parham *et al.*, 1992). With this system the tumors were divided in 4 grades and, according to the authors, this allowed a better stratification of patients. Nevertheless, none of these previous methods had significant followers (Elston and Ellis, 1998).

Elston and Ellis are widely renowned because of their refinement of the criteria of the Bloom and Richardson grading method. These authors used a cohort of almost 2,000 patients treated in Nottingham (and because of that the revised grade system was later named Nottingham grade method) and answered to the criticism of the lower reproducibility of the previous Bloom and Richardson's grade. The novelty of the Nottingham method was that (Elston and Ellis, 1991; 1998):

1) tubule formation was assessed in a semi-quantitative fashion: score 1 was attributed to tumors with more than 75% of luminal structures, score 2 to tumors with 10 and 75% of tubular gland formation and score 3 to those with less than 10% of tubular structures. These cut-offs were not

arbitrary, but based on a previous study which showed that they allowed the best separation in survival curves.

2) for nuclear pleomorphism assessment the normal cells of the surrounding mammary tissue served as reference.
3) for mitotic counts, a score was given according to the field area of high-power lens used and only unequivocal mitotic figures were counted, excluding apoptotic and hyperchromatic nuclei.

Nottingham grading system became the international gold standard for grading breast cancer (Ellis *et al*., 2012) and its prognostic value was confirmed by several prospective cohorts around the world (Elston *et al*., 1999; Frkovis-Grazio and Bracko, 2002; Volpi *et al*., 2004; Rakha *et al*., 2008a; 2008b; Chen *et al*., 2011).

Nottingham histological grade has been included in a prognostic index, the so-called the Nottingham Prognostic Index, NPI (Blamey, 1996; Lee and Ellis, 2008). This index allows a stratification of breast cancer patients (Rampaul *et al.*, 2001) and incorporates three independent prognostic factors: tumoral size, lymph node stage and histological grade (Haybittle *et a.l*, 1982; Blamey, 1996). The NPI was built according to the coefficients (β values) obtained in Cox multivariable regression analysis (Haybittle *et al.*, 1982; Blamey, 1996):

NPI = [tumoral size (cm) x 0.2] + histological grade (1, 2 or 3) + lymph node stage (1, 2 or 3).

Regarding tumor size, it corresponded to the greatest diameter determined by the pathologist during the macroscopic examination of fresh specimens or during the microscopic examination in very small invasive carcinomas (Elston *et al.*, 1999). The histological grade was 1, 2 or 3, corresponding to grades I, II and III, respectively. As to the lymph node stage, it was also a three-tier system: in stage 1, no evidence of lymph node involvement existed, whilst in stage 2 up to 3 axillary nodes had metastases and, finally, in stage 3 more than 4 nodes were affected (or one extra-axillary node with metastatic carcinoma) (Lee and Ellis, 2008).

Even if the first histological grading method for human breast cancer was described over 90 years ago (Table 5), the interest of the scientific community for this topic remains renewed. Moreover, the prognostic significance of Nottingham histological grade has been constantly reappraised (*e.g.*, Rakha et al., 2008b). More recently, Thomas *et al*. (2009a) analyzed the prognostic significance of Elston and Ellis grade and its parameters and, based on their statistical results, they proposed a simplified binary scoring system. Accordingly, only two scores of each parameter would exist and their final combined score would range from 3 to 6, being score 3 allocated to grade I, score 4 and 5 to grade II and score 6 to grade III tumors (Thomas *et al*., 2009a). The authors claimed that their simplified system presented a prognostic value similar to the Nottingham method, with the great advantage that it would be more reproducible, because pathological assessments tend to vary less when variables are dichotomized (*i.e.,* high *versus* low) (Thomas *et al*., 2009a). In the last few years, Nottingham histological grade applicability was extended to needle core biopsies (Kwork *et al*., 2010; O'Shea *et al*., 2011). These biopsies are used for the preoperative diagnosis of a breast lesion but the mitotic count in these specimens tended to be underestimated compared to those obtained in the surgical excised specimens (Kwork *et al*., 2010; O'Shea *et al*., 2011). Modifications of the thresholds for scoring mitotic count and the use of immunohistochemical proliferative markers have been proposed as methods for improving accuracy of Nottingham histological grade in core biopsies (Kwork *et al*., 2010; O'Shea *et al*., 2011).

Meanwhile, the interest in the histological grade has been renewed by the advent of new techniques, such as tissue microarrays. Different morphological parameters have been tested in tissue microarray sections of human breast carcinomas aiming to develop a microarray grade highly correlated to the standard Nottingham method performed in the entire tumor (Dalton and Page, 2012). From the authors perspective the development of a grade suitable for very small samples of tumors, would represent a step forward in the investigation of human breast cancer (Dalton and Page, 2012).

For this revision of the literature a major conclusion can be draw: human pathologists are highly committed to grading breast cancer and improving their

performance when assigning a grade to a tumor, with the ultimate goal of providing reliable prognostic data for the better management of their patient.

**Table 5** – Historical milestones of human breast cancer grading systems.

| Year | Author | Number of parameters | | Comments |
|---|---|---|---|---|
| 1925 | Greenough | 6 | Tubule formation<br>Secretor activity<br>Cell and nuclear size<br>Variation in cell and nuclear size<br>Nuclear hyperchromatism<br>Mitotic figures | |
| 1928 | Patey & Scarff | 3 | Tubule formation<br>Variation in size and shape of nuclei<br>Hyperchromatism | Presence/absence of parameters defined low/moderate and/marked grades of malignancy |
| 1955 | Black *et al.* | 4 | Nuclear borders<br>Chromatin pattern<br>Nucleoli<br>Mitotic figures | Five nuclear grades, in which 0 was the most malignant, and 4 the less malignant |
| 1957 | Bloom & Richardson | 3 | Tubule formation<br>Nuclear pleomorphism<br>Mitotic count | Each parameter scored 1 to 3. Their sum defines grade I (3-5), II (6-7) and III (8-9) |
| 1989 | Doussal *et al.* | 2 | Nuclear pleomorphism<br>Mitotic count | Five grades. This modification included 2 parameters of Bloom & Richardson |
| 1991 | Elston & Ellis (Nottingham method) | 3 | Tubule formation (score 1:<10%; 2: 10-75%; 3: >75%)<br>Nuclear pleomorphism (comparing to normal cells)<br>Mitotic count (cut-offs defined by microscope field diameter) | Each parameter scored 1 to 3. Their sum defines grade, as in Bloom & Richardson |
| 1992 | Parham *et al*. | 2 | Mitotic count (from Bloom & Richardson)<br>Necrosis | Five grades |

### 1.5 Quantitative methods for objectivizing grade

The introduction of quantitative measurements of grading parameters, which are continuous variables, has the advantage of better reflecting the biological *continuum* of tumor progression and could obviate the need of a subjective categorization (Ladekarl, 1998). Nowadays, three modalities of quantitative methods are available for histopathologists: morphometry, image analysis and stereology. Morphometry corresponds to the analysis of microscopic images using a caliper micrometer or a digital tool to perform direct measurements (Marcos *et al*., 2012). Regarding image analysis, it includes the capture of digital images of tissues, and afterwards a *softwar*e analyzes the pixels of images, converting them into counts of tissue structures or cells (Marcos *et al*., 2012). In contrast, stereology is a science that samples objects (such are histological sections) and these samples are used to estimate tridimensional (3D) characteristics of objects (Gunderson *et al*., 2013). Geometrical probes are used, which consist of test systems composed by points, lines or boxes (test volumes). These are superimposed to the tissue sections in order to perform counts that, when included in a mathematic equation, allow the recovery of the 3D information of tissues (Gunderson *et al*., 1988).

Stereological methods are intimately associated with a correct sampling (*i.e.,* truly representative) of tissues and they are unique in providing objective and unbiased estimations of different morphological features, such as numbers or sizes of cells or nuclei (Marcos *et al*., 2012). In order to use these methods, tissue handling, processing and sectioning should be kept within reasonably standardized limits (Sørensen, 1992). Nevertheless, the possibility of bias related to tissue handling, when stereology is applied to routine diagnostic material, should not cloud the advantages of stereology over traditional two-dimensional (2D) techniques (Kamp *et al*., 2009). These latter are highly influenced by the shape, orientation and size of the particles being counted (Kamp *et al*., 2009).

In the 80's and 90's of the 20th century, studies focusing on measuring and counting morphological features of human breast cancer became more frequent. Haumeder is credited for confirming that the nuclear and nucleolar area of malignant breast cells were larger than that of the non-malignant tissue (reviewed by Meijer *et al.*, 1997). After this milestone, both morphometrical and

stereological approaches have been used for studying human breast carcinomas, producing different estimators direct or indirectly related to the grading parameters (Table 6).

**Table 6** – Two-dimensional and three-dimensional quantitative estimators used in human breast carcinomas (adapted from Ladekarl, 2004).

| **Feature** | **2D parameter** | **3D parameter** |
|---|---|---|
| Tubule formation / cellularity-related parameters | Nuclear profile numerical density<br>Percentage of neoplastic epithelium<br>Fraction of fields with malignant tubules<br>Cellularity index | Nuclear numerical density [$N_V$ (nuclei, tumor)]<br>Nuclear volume density [$V_V$ (nuclei, tumor)] |
| Nuclear size | Mean nuclear profile area | Number-weighted mean nuclear volume ($\overline{v}_N$)<br>Volume-weighted mean nuclear volume ($\overline{v}_V$) |
| Mitotic activity | Mitotic profile density<br>Mitotic profile frequency | Mitotic density [$N_V$ (mitoses, tumor)]<br>Mitotic frequency [$N_N$ (mitoses, nuclei)]<br>Volume corrected mitotic index (M/V index) |

The percentage of malignant cells within breast carcinomas was investigated by Parham *et al.* (1988). Using routine slides, which incorporated the center and the periphery of tumors the percentage of neoplastic epithelium was determined in 30 consecutive fields by using a grid of points. The percentage of tumor cells showed a wide variation between cases, and those with higher proportion of neoplastic cells in relation to stroma had a better prognosis (Parham *et al*., 1988). However, no association between the percentage of tumor area and histological grade was observed (Parham *et al*., 1988).

A cellularity index was computed by van der Linden *et al.* (1986), this time using as few as five fields and counting only epithelial cells that did not contact with

the lines of a test system composed by squares (each with a defined area). This index tended to be lower in tumors that recurred (van der Linden *et al*., 1986).

Tubular formation is one of the grading parameters of breast cancer that has been addressed quantitatively, but using a different strategy. The tumor was screened at the medium magnification and all the fields showing at least one unambiguous tubular malignant structure were considered positive (Kronqvist *et al*., 1999). The ratio between number of positive fields and the total fields in the tumor was called fraction of fields with malignant tubules and used to measure tubule formation. Additionally, cut-offs for these quantitative features were defined in order to score tumors (Kronqvist *et al*., 1999; 2000; 2002).

In human breast cancer, two cellularity-related parameters were established using stereological methodology (Ladekarl and Sørensen, 1993a; 1993b; Artacha-Pérula and Roldán-Villalobos, 1997; Ladekarl *et al*., 1997): 1) the nuclear numerical density [$N_V$ (nuclei, tumor)], which means the number of neoplastic nuclei per volume of tumor; 2) the nuclear volume density [$V_V$ (nuclei, tumor)], also known as the volume fraction of nuclei, that is the fraction of the total volume occupied by neoplastic nuclei. For estimating the first parameter thick sections are used and scanned, following the optical disector methodology (Fig. 1A). A nuclear volume density is estimated in thin sections by the point counting method, in which points hitting nuclear profiles and the reference space are counted (this latter corresponding to the tumor present in sections) (Howard and Reed, 2005).

Probably, the mitotic activity has been the grading parameter with more quantification studies. In order to cope with the variation of cellularity between tumors, mitotic counts have been corrected to the percentage of neoplastic epithelium present in each analyzed field. This methodology, which was introduced in human pathology by Haapasalo *et al.* (1989), included the simultaneous counting of mitotic figures and estimation of the percentage of the volume of neoplastic tissue by a point counting procedure in each field; this generated the so-called volume corrected mitotic index (M/V index) (Haapasalo *et al*., 1989). The M/V index has been reported to be a powerful factor for predicting survival in human breast carcinomas (Lipponen *et al*., 1991; Aaltomaa *et al*., 1991; 1992; Jannink *et al*., 1995; Kronqvist *et al*., 1998). Other

morphometric approaches have been used to count mitoses in human breast cancer, namely the mitotic profile density (*i.e.,* number of mitotic profiles per tissue area) and frequency (*i.e.,* number of mitotic profiles per 1000 nuclear profiles). For these 2D estimators, and for increasing the efficiency of the procedure, counting frames with different areas were superimposed to the fields of vision (Ladekarl and Sørensen, 1993a; 1993b).

In stereology, the mitotic density (*i.e.,* the number of mitotic figures per unit of volume) was already estimated in thick histological sections of human breast cancer by the optical disector (Ladekarl *et al*., 1997). By that procedure, the 3D mitotic frequency was computed (2.8 mitotic figures per 1000 nuclei in ductal carcinomas, with higher in lymph node positive cases). This figure was quite similar to that estimated by a simpler 2D morphometrical method in thin sections (mitotic profile frequency of 2.1 per 1000 nuclei), and thus, it remains questionable whether the use of a more technical demanding technique, such as the optical disector, does have a real clinical utility (Ladekarl, 1995; 2004).

Mitotic counts included in grading systems of CMT have been expressed as number of mitotic figures per each high-power field or per 10 high-power fields selected in the most proliferative area of a lesion (Misdorp, 2002; Karayannopoulou *et al*., 2005; Goldschmidt *et al*., 2011). Still, a research group has used a different approach: by using image analysis, Sarli *et al.* (1999; 2002) estimated the number of mitotic figures per 1000 neoplastic cells. Briefly, the total area of the nuclei of the neoplastic cells in 10 high-power fields was estimated and divided by the mean nuclear area (determined in 10 nuclei) in order to estimate the number of nuclear profiles. Despite the interesting approach, this mitotic index seemed not useful for prognostic purposes (Sarli *et al*., 1999; 2002).

As to mitotic counts, nuclear morphologic features of human breast cancer have been evaluated by morphometrical and stereological methods. In 1993, Ladekarl and Sørensen published a study focusing on the assessment of various quantitative nuclear parameters in *in situ* and invasive ductal and lobular breast carcinomas, in which morphometrical and stereological methods were applied. Regarding the former, the mean nuclear profile area and the nuclear profile numerical density (*i.e.,* number of nuclear profiles per tissue

area) were estimated (Ladekarl and Sørensen, 1993a). As previously mentioned, for these 2D estimates, counting frames with defined areas were used and the procedure fairly corresponded to that recommended in the stereological unbiased counting rule (Gundersen, 1977). Regarding to the stereological parameters estimated by Ladekarl and Sørensen (1993a) they comprised the volume-weighted mean nuclear volume ($\bar{v}_V$). The $\bar{v}_V$ was estimated by the point sampled intercepts (PSI) method, which uses a test grid made of parallel lines bearing a systematic pattern of points (Sørensen, 1992; Ladekarl and Sørensen, 1993a; Gunderson *et al*., 1988). This parameter increases as the nuclei enlarges, being further augmented when a substantial variation in their size exists (Fig. 1B). It is mostly used in histopathology, since it grades quantitatively the nuclear size pleomorphism, correlating with the prognosis of various neoplasias (Sørensen, 1992; Ladekarl, 1998).

The prognostic value of morphometrical and stereological estimators in human breast carcinomas was already evaluated using a multivariable analysis (Ladekarl and Sørensen, 1993b; Ladekarl, 1995). The $\bar{v}_{V,}$ the nuclear profile density number and the mitotic profile frequency showed independent prognostic value. A prognostic index that included the quantitative variables and the clinical stage was proposed (Ladekarl and Sørensen, 1993b). The authors discussed the limitations of the 2D morphometrical parameters, such as the nuclear profile numerical density and the mitotic profile frequency, and emphasized the value of the stereological $\bar{v}_{V,}$ not only because of the simplicity of the estimation method, but also because of its applicability to routine tumor sections (Ladekarl and Sørensen, 1993b; Ladekarl, 1995).

By the same time, a Spanish group reached to similar conclusions (Roldán-Villalobos *et al*., 1996; Artacha-Pérula and Roldán-Villalobos, 1997). By studying the $\bar{v}_V$ in human ductal carcinomas, these authors defined a cut-off of 425 $\mu m^3$ that was significantly associated with short survival (Roldán-Villalobos *et al*., 1996). They also estimated other stereological parameters in breast tumors of the 3 histological grades, such as nuclear numerical density [$N_V$ (nuclei, tumor)], the nuclear volume fraction, and the number-weighted mean nuclear volume ($\bar{v}_N$). The latter corresponds to the mean volume of nuclei, being determined using the nucleator technique (Fig. 1C) (Gundersen *et al*.,

1988). The $\overline{v}_N$ does not provide direct information regarding the variability of the nuclear volume, and this is the main difference for the $\overline{v}_V$ (Sørensen, 1992).

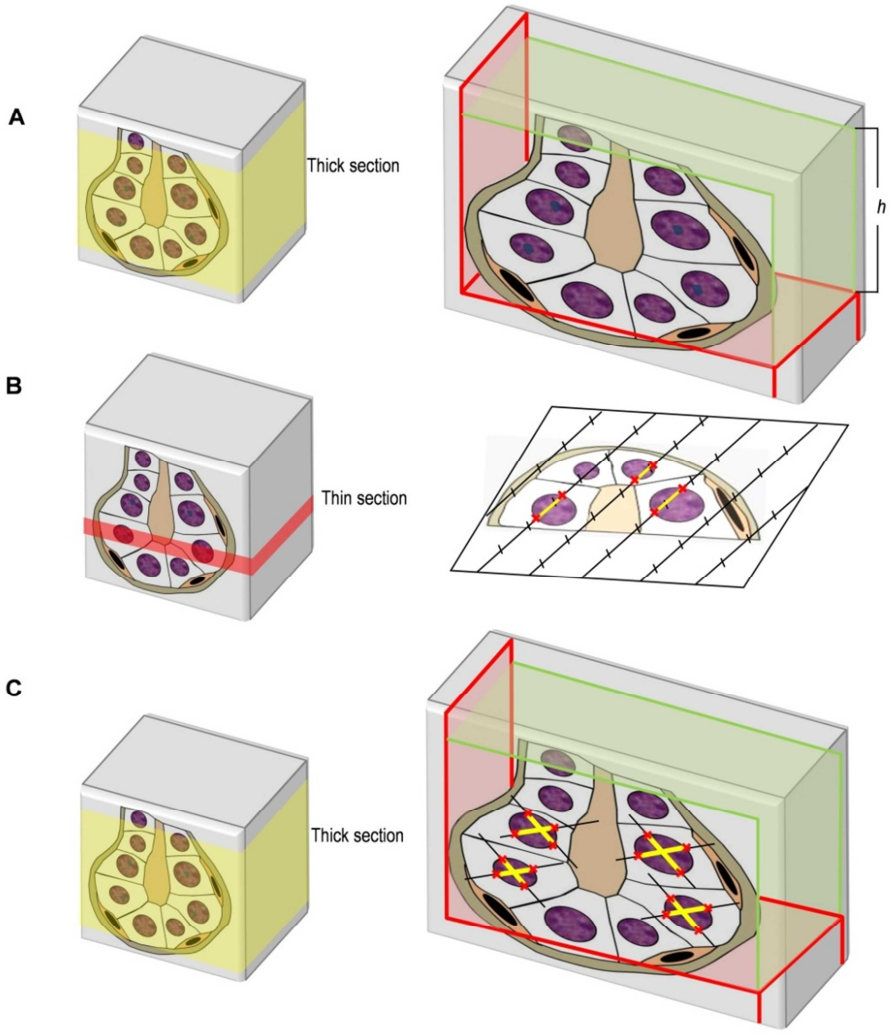


**Fig. 1** – Overview of the estimation of the nuclear mean volumes and numerical density. (A) Estimation of nuclei numerical density using the optical disector in thick sections. Nuclei are counted as they appear in focus within the disector height (*h*) and within or touching inclusion lines (green) but not the exclusion ones (red). In this example, 8 nuclei would be counted. (B) Estimation of the volume-weighted mean nuclear volume ($\overline{v}_V$) according to the point sampled intercepts method. The nuclei are sampled according to their volume, by overlaying a grid of points at random and, for each nucleus hit by a point, the intersection between the line and nuclei is marked (red crosses) and distance between both ends of the nuclei measured (yellow line), which is used to estimate the $\overline{v}_V$. This procedure uses thin sections. (C) Nucleator method for estimating number-weighted mean cell volume ($\overline{v}_N$). After applying the disector to sample cells the stereological system generates two lines passing through the nucleolus; intersections between the lines and nuclear borders (red crosses) is marker and the average distance (yellow lines) used to estimate the $\overline{v}_{N.}$ In this case thick sections are needed.

According to the results of that group, the $N_V$ (nuclei, tumor) tended to be lower in poorly differentiated tumors, but no statistical difference was observed between tumors of the grade I, II and III. In contrast, the nuclear volume fraction increased with the grade, and both the $\overline{v}_V$ and the $\overline{v}_N$ were significantly higher in grade III tumors compared with grade I and grade II tumors. These two nuclear volume estimates were positively correlated with each other, were significantly associated with survival outcome, and therefore appeared as quantitative and useful prognostic factors (Artacha-Pérula and Roldán-Vilalobos, 1997).

Various tumor parameters can be assessed by quantitative methods, but there is a general agreement that variables related to nuclear size and mitotic counts have an increased prognostic importance in human breast cancer (Ladekarl, 1998; Baak *et al*., 2005; Skaland *et al*., 2008; Baak *et al*., 2009). In most cases, the 2D and 3D estimators showed significant correlations, which were better for the mitotic activity estimators (Ladekarl, 2004). In human breast cancer, unbiased 3D quantification techniques have been recommended for estimating nuclear size of neoplastic cells, whereas for mitotic activity the 2D techniques can be sufficiently accurate for grading purposes (Ladekarl, 2004).

The interest in quantitative variables in human breast cancer extends to the present days. In a recent report nuclear parameters in *in situ* carcinomas and in ductal hyperplasia were studied in digitalized routine sections by computer image processing and analysis (Dong *et al*., 2014). This study demonstrated that nuclear quantitative features allowed a differential classification of *in situ* carcinomas and hyperplasia, and, in case of carcinomas, the distinction between high and low nuclear grades was possible (Dong *et al*., 2014). The use of quantitative features of cells for the diagnosis and grading of malignancy, could act as a second-reader in borderline and dubious cases, being a real-time support for the decision-making of pathologists (Dong *et al*., 2014).

Despite this promising data based on 2D and 3D quantification in human breast cancer, a scarce interest has been devoted to quantitative analysis of cellular features in CMT. As far as we know, a 3D quantification (*i.e.*, based on stereological methods) has never been performed in CMT. Computer-assisted image analysis has been used to estimate 2D/morphometrical nuclear characteristics in CMT (Simeonov and Simeonova, 2006; 2007; de Vico *et al*.,

2007). Two of those studies were performed in cytological preparations and did not include clinicopathological data, other than the histological diagnosis (Simeonov and Simeonova, 2006; 2007). Interestingly, the authors reported that the mean nuclear diameter, perimeter, and roundness (also known as nuclear form factor) were significantly higher in malignant CMT compared to benign ones. In a series of CMT, de Vico *et al.* (2007) estimated the mean nuclear area, the mean nuclear form factor and their respective standard deviations. The estimates of mean nuclear area were significantly higher in lymph node positive cases. Therefore, this parameter appeared as a good morphometrical discriminator of metastatic tumors (de Vico *et al*., 2007). Yet, the utility of quantitative and unbiased estimators for improving the reproducibility, accuracy and prognostic value of the histological grade in CMT remains to be determined.

### 1.6 Genomic studies: a tool with clinical implications

Cancer is rooted in inherited or acquired genetic and epigenetic alterations (Wang, 2013). The successive accumulation of alterations in the genome contributes to the acquisition of several functional and morphological characteristics by neoplastic cells, such as sustained proliferative activity or invasion (Hanahan and Weinberg, 2011). In the perspective of cancer development, the most relevant genetic changes (numeric or structural) or epigenetic alterations (such as DNA methylation and histone modifications) are those affecting oncogenes, tumor suppressor genes, or the DNA repair system, also named as caretaker of the genome (Hanahan and Weinberg, 2011).

Significant efforts have been focused in the comprehensive characterization of genetic underpinnings of the major human tumor types (Wang, 2013). In human breast cancer the knowledge of chromosome changes associated with the carcinogenesis have significantly improved over the last 30 years (Steinarsdottir *et al*., 2011). The study of DNA changes progressed along with the development of techniques (Costa *et al*., 2008). Early studies used flow cytometry to detect genomic changes, without chromosome specificity and karyotyping methodology, which required living cells (Dressler *et al*., 1988; Heim *et al*., 1997; Teixeira *et al*., 2002; Costa *et al*., 2008). The standard chromosome banding analysis is a good method for an initial screening for

karyotype abnormalities (Teixeira, 2002). However, major drawbacks exist, namely: its low resolution, which limits the detection of highly complex karyotypes, and its requirement of living cells, either from fresh tumoral specimens or obtained from short-term culture of tumor cells (Teixeira *et al*., 2001; Tan and Reis-Filho, 2008).

The dependence of short-term culture was considered a potential pitfall for the use of conventional cytogenetic analyses in the study of solid tumors, including breast cancer (Teixeira *et al*., 2001). The next logical methodological step was to use a technique independent on the availability of metaphases from the neoplastic cells. This was accomplished by the use of comparative genomic hybridization (CGH) (Teixeira *et al*., 2002). This method allows the detection of changes in the copy number of chromosomes, providing a global overview of chromosomal gains and losses throughout the whole genome of the tumor (Weiss *et al*., 2003). CGH uses differentially labeled total genomic tumor DNA and normal reference DNA (obtained from unrelated healthy individuals) that are cohybridized onto normal metaphase chromosome spreads on a glass slide (Kallioniemi *et al*., 1993; Teixeira *et al*., 2002; van Beers and Nederlof, 2006; Costa *et al*., 2008). The ratio of fluorescence intensities of tumor and normal DNA is measured along the chromosome axis, which allows the mapping of chromosomal gains and losses (Teixeira *et al*., 2002; Costa *et al*., 2008).

More recently, the resolution of the CGH method was improved by the development of array-based CGH technologies (aCGH) (Costa *et al*., 2008). In aCGH, the metaphase was replaced by DNA fragments arrayed on a microscope slide, for which the exact chromosomal location is known from the genome sequence (Costa *et al*., 2008). These aCGH platforms could be constructed using bacterial artificial chromosome (BAC) clones which provide a resolution of about 1 Megabase (Mb), or short 130-600 base pair (bp) single-stranded molecules (oligonucleotides) with a theoretical resolution of up to 2 kilobase (kb) (Climent *et al*., 2007; Tan and Reis-Filho, 2008). Currently, aCGH is the method of choice for screening and characterizing copy number variations of human solid tumors, allowing, for the first time, the full classification of complex karyotypes (Climent *et al*., 2007; van Beers and Nederlof, 2006). It should be stressed that despite its high resolution, aCGH cannot detect ploidy

variations and balanced reciprocal aberrations and that aCGH is a relatively expensive technique that requires specific equipment and specialized interpretation skills (van Beers and Nederlof, 2006).

In breast cancer, CGH technologies have been applied for: 1) searching for genomic regions harboring candidate genes involved in carcinogenesis; 2) studying intratumoral heterogeneity; 3) establishing the clonal relation between multiple tumors as well as between the tumor and their metastases; and 4) identifying DNA copy number changes that could be prognostic or predictive markers (Reis-Filho *et al*., 2005; Climent *et al*., 2007; Andre *et al*., 2009).

CGH studies already revealed that the most common forms of invasive breast carcinomas are associated with recurrent unbalanced chromosomes changes, namely gains in 1q, 8q, 11q, 16p, 17q and losses of 1p, 13q, 16p and 17p (Reis-Filho *et al*., 2005). Some of those aberrations were associated with poor prognosis, such as gains of 3q or 8q, whereas the loss of 16p correlated with good prognosis (Reis-Filho *et al*., 2005; Horlings *et al*., 2010). The profiling of DNA copy number changes was associated with different clinicopathological features, including grade (Bergamaschi *et al*., 2006), estrogen receptor status (Loo *et al*., 2004), gene-expression subtype (Andre *et al*., 2009), and survival (reviewed by Climent *et al*., 2007).

CGH methodology also confirmed that some human breast carcinomas are composed by different cells, clonally unrelated, thus supporting the existence of polyclonality, previously suggested by conventional chromosome banding techniques (Teixeira *et al*., 1994; Heim *et al*., 1997; Teixeira *et al*., 2001; Torres *et al*., 2007). This evidence contradicted the almost unanimous conviction that carcinomas were monoclonal in their origin (Heim *et al*., 2001). Moreover, by CGH and a robust statistical analysis, it was reported that significant divergent clonal evolution occurred in primary breast cancer and their lymph node metastases and that, apparently, the metastases developed during the early stages of tumorigenesis (Torres *et al*., 2007).

Another important issue of breast cancer was explored by classic cytogenetic analysis and CGH. Both techniques were used to disclose the clonal association between synchronous breast carcinomas (Teixeira *et al*., 1996; 1997; 2004). In this regard, the existing evidence supports that ipsilateral

lesions are clonally related, and could represent a process of intra-mammary spreading of a single breast cancer, while most synchronous bilateral breast cancer cases seemed to correspond to independent tumors rather than metastatic events (Agelopoulous *et al*., 2003; Teixeira *et al*., 2004; Hwang *et al*., 2004). Accordingly, CGH offered the unprecedented possibility to confirm if multiple breast carcinomas really represented multiple primary entities, which certainly would have an obvious clinical applicability, being helpful for delineating appropriate therapies (Teixeira *et al*., 2004; Bendifallah *et al*., 2010).

In veterinary pathology, the issue of clinical and morphological heterogeneity, as well as multiplicity of CMT, is much more important than in humans. However, unlike breast cancer, CMT are poorly characterized regarding cancer-associated genomic aberrations. Understanding the fundamental biology of CMT is crucial, not only for improving the management of the disease, but also to further support the dog as a potential model for studying human breast cancer (Rowell *et al*., 2011). Classic chromosome banding studies of CMT are rare (Mellink *et al*., 1989; Mayr *et al*., 1990; 1993). The presence of bi-armed chromosomes (putatively due to isochromosome formation) and the loss of X were already reported. In one study, the fluorescence *in situ* hybridization (FISH) technique was performed in cells lines derived from malignant CMT and reported abnormalities comprised the chromosomes 8 or 11, 13 or 15, 37, 38 and X; the changes included the loss and formation of isochromosomes, as well as centric fusion (Tap *et al*., 1998).

It should be noted that conventional cytogenetic analyses in dogs are technically demanding, because the normal diploid number of dogs is very large (39 pairs of chromosomes, as opposed to the 23 pairs of humans) and the canine chromosomes (except the X and Y chromosomes) are acrocentric, similar in size, shape and banding pattern (Reimann-Berg *et al*., 2012). Therefore, the genomic studies of canine cancers have greatly benefited from the introduction and development of metaphase and aCGH (Thomas *et al*., 2007; 2008). Genome integrated canine BAC arrays for CGH analyses were developed with clones spaced at 10Mb and 1Mb (Thomas *et al*., 2007; 2008). These authors developed a comprehensive genome-wide set of single *locus* probe FISH reagent for the canine genome (Thomas *et al*., 2008). In

combination, these tools already begun to make an huge impact on the understanding of cancer-associated genomic changes in a variety of cancers, including hematopoietic malignancies (Breen and Modiano, 2008; Thomas *et al.*, 2011), intracranial malignancies (Thomas *et al*., 2009b) and sarcomas (Thomas *et al*., 2009c; Angstadt *et al*., 2011; Hedan *et al*., 2011). More recently, the resolution for canine aCGH has been extended using an oligonucleotide platform comprising 180,000 60-mer features, which are currently under use (Breen and Thomas, 2012). These genetic resources offer new opportunities to investigate the type and nature of recurrent chromosomal abnormalities of CMT. These tools may also contribute to further understand the genetic process involved in CMT, by identifying regions of canine genome with carcinogenesis associated genes, as has been demonstrated in other tumors of dogs (Breen and Thomas, 2012; Thomas *et al*., 2014). Interestingly, in some of those studies, interspecies genetic overlap between canine and human tumors were reported (Rowell *et al*., 2011; Breen and Thomas, 2012).

Until the present, only two studies reported genomic alterations in malignant CMT using modern genomic-wide methodology (Beck *et al*., 2013; Liu *et al*., 2014). In those studies a total of 17 animals with CMT were included — a figure that drastically differs from the number of breast cancer cases studied so far. In the work by Beck *et al.* (2013) five CMT of different subtypes (simple carcinomas, complex carcinomas and carcinosarcoma) were submitted to whole-genome sequencing, aiming to detect copy number and structural aberrations. In all but one case, the authors described genomic aberrations, affecting several known cancer associated genes, such as *c-MYC* and KIT (Beck *et al*., 2013). A recurrent deletion of chromosome 27 was observed in the analyzed series. Moreover, similarities to human breast cancer and specific canine alterations were also reported (Beck *et al*., 2013). Last year, Liu *et al.* (2014) performed aCGH and whole-genome sequencing in 12 cases of CMT. According to the authors, simple carcinomas were associated with extensive genetic aberrations while complex carcinomas presented no major copy number alterations, but were associated with several histone modifications. Based on their findings, the authors hypothesized that complex carcinomas could arise from epigenetic changes, rather than genomic aberrations (Liu *et al*., 2014).

A generalized optimism exists regarding the clinical applicability of the genetic characterization of human cancer, even if the translation of the data from the bench to the clinical setting will take several years (Caldas, 2011). In veterinary oncology, and specifically in CMT, there is a long road ahead. However efforts should be pursued to increase the knowledge on genetic changes and mechanisms involved in CMT development. There are a couple of issues with direct clinical implications that deserve further studies. Firstly, the genetic characterization of the malignant CMT will be crucial to support the stratification of cases with different prognosis. Secondly, understanding the genetic connection between the different neoplastic mammary nodules will provide evidence-based options regarding the surgical and eventual adjuvant therapy in the setting of multiple CMT.

## 2. Aims

Pathologists and clinicians are still challenged for an accurate prognosis definition in each case of malignant CMT. From the pathologist's perspective, a reliable and reproducible grading method is essential. Considering this, we aimed to:

- perform a comprehensive evaluation of the prognostic value of histological grade in malignant CMT, considering the individual role of each grading parameter (tubule formation, nuclear pleomorphism and mitotic count) and their association with survival;
- test quantification strategies, using stereology and morphometrical approaches that could contribute to a more objective assessment of the grading parameters;
- study the level of interobserver reproducibility of the histological grade and their parameters in a series of malignant CMT;
- evaluate the feasibility of a prognostic formula for malignant CMT, namely by adapted human Nottingham Prognostic Index, which incorporates the histological grade.

Keeping in mind that the grading method for malignant CMT has been adapted from human breast cancer grading systems, we also intended to:

- investigate potentially new grading parameters, easily assessed during the histopathological examination of malignant CMT in order to contribute to the future development of a canine-specific histological grading method.

Both clinicians and pathologists agree that the genetic basis of CMT remains poorly understood; this clearly contrasts with the human breast cancer. Particularly, in the case of multiple tumors, it is unknown if they share the genetic background or represent truly independent tumors. Considering this, we aimed to:

- study genetic alterations in malignant CMT and assess the clonal relation between different synchronous tumors using array comparative genomic hybridization.

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# CHAPTER 2

## NUCLEAR PLEOMORPHISM IN CANINE MAMMARY TUMORS: A STEREOLOGICAL APPROACH

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**Summary**

Canine mammary neoplasia is highly heterogeneous in morphological appearance and in biological behavior. The successful clinical management of this disease should rely on robust prognostic factors. Histological grade, determined by the human Nottingham histological grade method (NHG), has been considered one of such factors. Despite the adoption of this method, it is unknown if interobserver agreement exists regarding the assessment of its parameters in canine mammary carcinomas. In this study, the agreement between two observers regarding the NHG nuclear pleomorphism scoring was evaluated in 89 canine mammary carcinomas. In each case, the histological evidence of vascular invasion and/or regional lymph node metastases, regarded as early signs of tumor aggressiveness, was recorded. In 48 animals two years follow-up data was available. Nuclear pleomorphism was also quantitatively assessed by a stereological method, which allowed for an unbiased estimation of nuclear size and its variability, by determining the volume-weighted mean nuclear volume ($\overline{v}_V$). Differences in the $\overline{v}_V$ estimations of the normal parenchyma, benign and malignant tumors were comparatively evaluated. In malignant tumors the stereological estimations of nuclear pleomorphism were compared with the histological score of nuclear pleomorphism included in the NHG. Additionally, the prognostic significance of clinicopathological features in carcinomas included the nuclear score and the $\overline{v}_V$ was evaluated. A modest agreement between the two observers in the histological score of nuclear pleomorphism was obtained. The value of $\overline{v}_V$ significantly increased from normal parenchyma, to benign and to malignant tumors. Carcinomas scored as 1 and 2 presented similar $\overline{v}_V$ and only tumors scored 3 presented significantly higher estimates. The $\overline{v}_V$ was not associated with vascular invasion and/or lymph node metastases, but was higher in tumors that progressed during follow-up. In multivariable analysis, only tumor size stood up as an independent factor regarding evidence of aggressiveness and an optimal cut-off of 2.9 cm was defined. According to these results, we propose that the $\overline{v}_V$ could be included in the toolbox for assisting in the diagnosis and grading of canine mammary tumors.

## 2.1 Introduction

Mammary gland tumors are the most frequent neoplasia in female dogs, particularly in countries where spaying is not performed routinely early in life (Sorenmo, 2003). Approximately half of the affected dogs have malignant disease, based on the histopathological examination (Lana *et al.*, 2007). Metastases to distant organs are the most common cause of morbidity and mortality associated with malignant canine mammary tumors (CMT) (Clemente *et al.*, 2010; Lana *et al.*, 2007). It is now clear that CMT is a complex disease characterized by a heterogeneous clinical and biological behavior. This probably contributes to the limited therapeutic options that are currently available (Goldschmidt *et al.*, 2011; Klopfleisch *et al.*, 2011, Sorenmo *et al.*, 2011). Searching for new prognostic parameters is of utmost importance and this has been addressed by several studies (*e.g.*, Philibert *et al.*, 2003; Chang *et al.*, 2005; Santos *et al.*, 2013).

Histological grade is well established as an important prognostic factor in human breast cancer (Rakha *et al.*, 2010). In veterinary pathology, different grading systems have been used and claimed to have prognostic value, but such variety of methods represents a drawback for establishing grade as prognostic factor (Matos *et al.*, 2012). One of the most frequently used methods for histological grading of malignant CMT is the NHG, originally developed for human breast cancer (Elston and Ellis, 1991). Karayannopoulou *et al.* (2005) provided some evidence that this method had advantages when compared to previous methods for prognostic purposes in dogs. The NHG is based on the semi-quantitative assessment of three morphological features: tubule formation, mitotic counts and nuclear pleomorphism. The latter should mainly be evaluated by comparing the nuclear size and its variation with normal mammary epithelial cells (Elston and Ellis, 1998). Nuclear pleomorphism is considered a hallmark of malignant transformation, increasing in parallel to the absence of cell differentiation (Sørensen, 1992; Ladekarl, 2004). However, it has been claimed that karyomegaly often leads to overdiagnosis of malignancy in CMT (Matos *et al.*, 2012; Peña *et al.*, 2013). Still, objective and unbiased estimations of nuclear size and nuclear pleomorphism were never compared in benign and malignant CMT, and their role in prognosis is largely unknown.

Nowadays, it is recognized that the histological grading system of malignant CMT deserves further investigation (Goldschmidt *et al.*, 2011; Matos *et al.*, 2012). Irrespective of the method, grading is usually based on a subjective, experience-dependent judgment by the pathologist and on qualitative or semi-quantitative evaluations of morphologic and cytological features (Sørensen, 1992; Artacha-Pérula and Roldán-Vilalobos, 1997). Not only the observer, but also the selection of the tumor areas to assess grade can significantly influence the scoring of parameters. This is especially relevant for CMT, which are intrinsically very heterogeneous (Klopfleisch *et al.*, 2011). The subjective nature of morphological evaluation is associated with a high risk of inter- and intraobserver variations that can hamper the reproducibility and accuracy of the biological information, jeopardizing a comparative analysis between studies (Artacha-Pérula and Roldán-Vilalobos, 1997). A simple way to overcome such subjectivity is to use unbiased quantitative parameters for scoring neoplasms, namely by applying appropriate stereological methods (Ladekarl, 1995). This has been performed in various human tumors, including breast cancer (Sørensen, 1992; Ladekarl and Sørensen, 1993; Ladekarl, 1995; Steiner *et al.*, 1994; Artacha-Pérula and Roldán-Vilalobos, 1997; Ladekarl, 1998; Yörükoglu *et al.*, 1998; Soda *et al.*, 1999; Fujikawa *et al.*, 2000). The design-based stereological estimates are precise, shape-independent, and allow an objective evaluation of several cytological features, including nuclear pleomorphism (Ladekarl, 1998). However, to the best of our knowledge, stereological methods were never applied for studying CMT.

The point sampled intercepts (PSI) method is considered the easiest way to objectively quantify the nuclear size and its pleomorphism (Gundersen and Jensen, 1985; Ladekarl, 1998). The PSI generates estimations of the volume-weighted mean nuclear volume, $\bar{v}_V$ (Sørensen, 1992). The $\bar{v}_V$ is not an intuitive parameter: it involves sampling the nuclei in proportion to their individual volumes; cells with bigger nucleus are more likely to be sampled and, thus contributing more to the estimate (Gundersen *et al*., 2013). The $\bar{v}_V$ increases as the nucleus enlarges, being further augmented when a substantial variation in its size exists. Therefore this parameter combines information on nuclear size and its variability (Sørensen, 1992; Ladekarl, 2004). This parameter is mostly

used in histopathology and it has been emphasized that the $\overline{v}_V$ estimates provide relevant information for diagnosis and grading of human breast tumors (Ladekarl and Sørensen, 1993; Ladekarl, 1995; Roldán-Vilalobos *et al*., 1996; Artacha-Pérula and Roldán-Vilalobos, 1997).

The aims of this study were to: 1) assess the nuclear pleomorphism of CMT and normal mammary parenchyma using stereological tools, namely the PSI method; 2) compare the stereological estimation of nuclear pleomorphism in normal parenchyma, benign and malignant CMT; 3) compare the stereological estimations with the histological score of nuclear pleomorphism in malignant tumors; 4) verify the prognostic significance of the nuclear pleomorphism estimations, by using univariable and multivariable analyses.

## 2.2 Material and methods

### *2.2.1 Clinical cases and histological analysis*

Pathology archives from ICBAS, University of Porto were accessed to select retrospectively 122 spontaneous CMT (33 benign and 89 malignant) that had been surgically removed. The selection of cases was blinded to clinical data. Six normal mammary gland cases (one case per each estrous phase, *i.e.,* proestrous, estrous, early diestrous, late diestrous, early anestrous and late anestrous), previously included in a study (Santos *et al.*, 2010) were also selected. For animals bearing mammary tumors, owners gave informed consent for the surgery with curative intents, after declining postoperative adjuvant therapy. Histological evaluations were performed in all the slides containing the largest cross section of the tumor. The histological diagnosis was performed by two observers (MS and PDP) using the criteria of the World Health Organization classification (Misdorp *et al.*, 1999). In malignant CMT the following clinicopathological parameters were recorded: the largest diameter accurately measured with a caliper, before surgery by the clinician (AdM or AS); the presence of peritumoral vascular invasion (defined as the presence of tumor emboli within endothelial-lining spaces without distinguishing between lymphatic and blood vessels) and regional lymph node metastases [lymph nodes were evaluated in routine slides and after immunolabelling with pancytokeratin AE1/AE3 and cytokeratin 14, as previously described by Matos *et al.* (2006)];

the histological scoring of nuclear pleomorphism according to the NHG criteria (Elston and Ellis, 1991; Karayannopoulou *et al.*, 2005), considering only luminal epithelial cells. Two observers (MS and PDP) assigned the nuclear grading score independently in order to determine the interobserver agreement. Subsequently, in cases with discrepant scores, a consensus was obtained by reviewing the slides using a multi-head microscope. Briefly, nuclei were scored as 1 if no visible increase in size and shape variability was detected, comparing to normal surrounding mammary epithelial cells; scored as 2 when moderate variation in size and shape existed (nuclei were generally larger than the normal ones); scored as 3 when a marked increased variation in size and shape was present, with very large and bizarre forms observed in at least one quarter of the tumor area (Fig. 1) (Elston and Ellis, 1991).

*2.2.2 Stereological analysis*

A systematic random sampling approach for the selection of the fields was used in each slide, meaning that the first field of sampling was randomly selected and thereafter fields were sampled systematically by adjusting the distance between individual fields of vision roughly proportional to the overall area of the tissue of interest; all the parenchyma (in normal mammary glands) and all the tumor area (in CMT cases) present in the slides was considered. The stereological analysis was performed with a workstation comprising a microscope (BX-50 Olympus, Japan) equipped with a 100x oil immersion objective (Olympus Uplan), a CCD video camera (Sony, Japan) connected to a PC monitor, and a motorized stage (Prior, United Kingdom) for stepwise displacements in x-y directions; the workstation was controlled by the software CAST-Grid (Version 1.5, Olympus, Denmark). The $\bar{v}_V$ was estimated by the PSI method (Gundersen and Jensen, 1985). This parameter quantifies the nuclear size and pleomorphism, estimated with a test-system made of parallel lines bearing a systematic pattern of points (Fig. 2). Only the nuclear profiles of epithelial cells hit by one of these points were sampled. On these profiles, the line segments overlying the nucleus were measured (from boundary to boundary) (Fig. 2); this resulted in a length (*l*) that was used to estimate the $\bar{v}_V$ as follows (Gundersen and Jensen, 1985):

$$\bar{v}_v(nucleus) = (\pi/3) \cdot \bar{l}_0^3$$

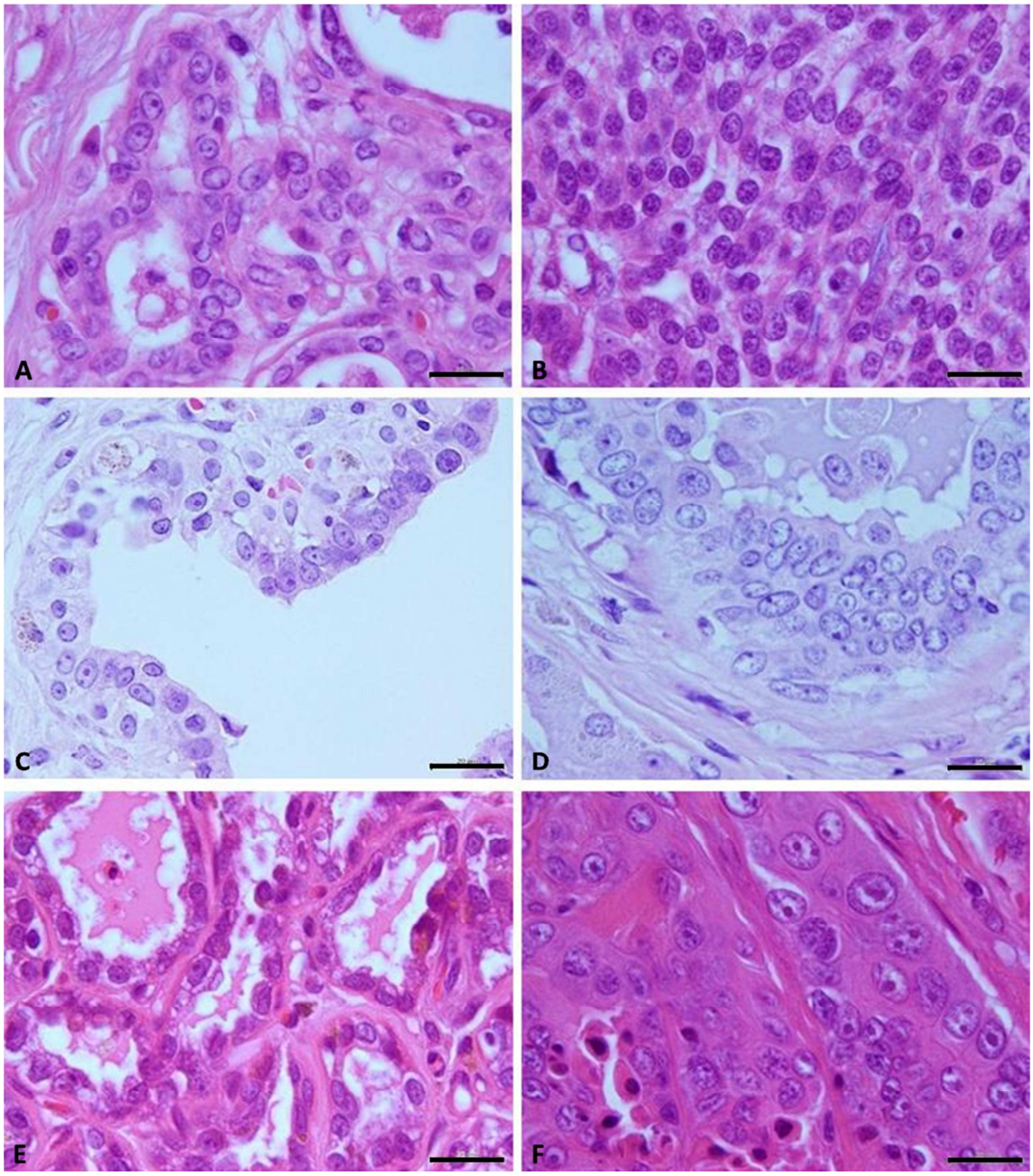


**Fig. 1** – Histological normal surrounding mammary tissue (A, C, E) and tumor cells (B, D, F) in three cases of canine mammary carcinomas. Nuclear pleomorphism scoring followed the criteria of the Nottingham histological grade method: score 1 (image B) presents no increased size and shape variability comparing to normal epithelial cells (image A); score 2 (image D) has moderate variation and larger nuclei than normal ones (image C); whereas score 3 (image F) shows marked increased variation in size and shape, with very large nuclei and prominent nucleoli comparing with normal adjacent parenchyma (image E). Hematoxylin-eosin, Bar 20 µm.

Estimates of the $\overline{v}_V$ are volume-weighted, because the nuclear profiles are point sampled with a chance proportional to their volume. It is assumed herein that, globally, the nuclei do not display a preferred three-dimensional orientation, *i.e.,*

that the nuclei are isotropically oriented; our qualitative observations do support the presumption. For estimating the $\overline{v}_V$, a total nuclear profiles of 92 (39, SD, standard deviation), 115 (43) and 166 (69) was measured, on average, in each slide of normal mammary parenchyma, benign and malignant tumor cases, respectively. The coefficient of variation (CV = SD/mean) was also computed, as well as the coefficient of error (CE), estimated according to the formula:

$$CE = 100 \cdot CV \div \sqrt{n}$$

In which, n stands for the cases included in each group.

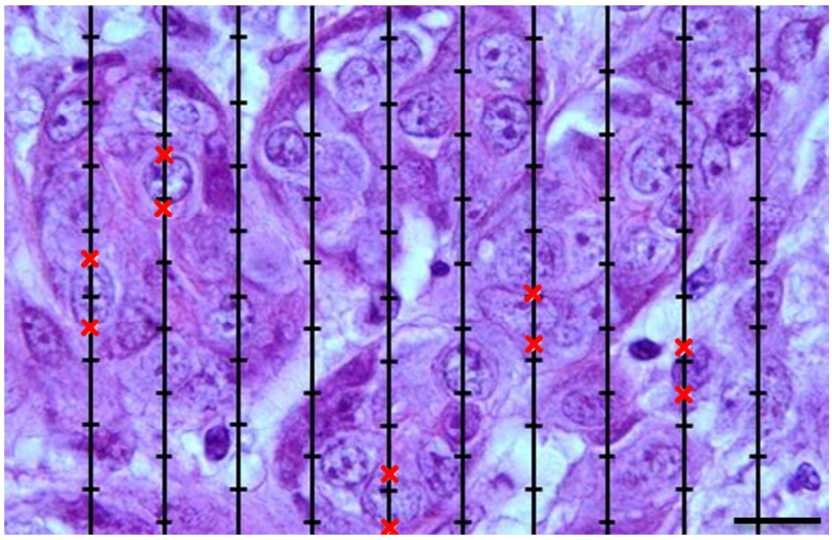

**Fig. 2** – Estimation of the volume-weighted mean nuclear volume ($\overline{v}_V$) with the point sampled intercepts method. A test system made of parallel lines bearing a systematic pattern of points is generated by the software. Nuclei in focus that are hit by one of the points are selected. In these nuclei, the distance between the intersections of the nuclear boundaries with the lines (marked with red crosses) was measured. Hematoxylin-eosin, Bar 13 µm.

*2.2.3 Follow-up and survival study*

Follow-up data were collected over two years following the protocol detailed in Santos *et al.* (2011). Briefly, each female dog was evaluated prior to surgery, 3 weeks after the procedure and every 3 months thereafter. Each evaluation included a complete physical examination, thoracic radiographs (3 views) and an abdominal ultrasound. Any clinical signs or lesions that could be related to tumor progression, either in the scheduled evaluations or in between them was thoroughly investigated (*e.g.*, fine-needle aspiration, ultrasound-guided biopsy, and skeletal radiography). A complete necropsy was performed when the

animals died spontaneously or were euthanized in search for evidences of subclinical local recurrence or metastatic disease. Overall survival (OS) was calculated from the date of surgery to the date of animal death/euthanasia due to tumor metastasis. Disease-free interval (DFI) was calculated from the date of surgery to the date of detection of tumor progression, *i.e.,* confirmed local recurrence and/or metastases. In the survival study, cases were censored if: 1) animals died due to causes unrelated to tumor; 2) were lost to follow-up; 3) were alive and free of distant metastases 24 months after surgery.

### *2.2.4 Statistical analysis*

Cohen's Kappa statistic was used to assess the interobserver agreement for the histological scoring of nuclear pleomorphism. Additionally, an asymptotic marginal homogeneity test was used, to evaluate if statistical differences between the observers existed.

One-way ANOVA followed by Tukey multicomparison test were used to check for differences in $\bar{v}_V$ of normal mammary parenchyma, benign and malignant tumors. The same approach was performed for investigating differences in the stereological estimation in each nuclear pleomorphism consensual score. ROC curves analyses were used to determine if tumor size and $\bar{v}_V$ values could discriminate malignant tumors with or without evidence of vascular invasion and/or lymph node metastases. In malignant tumors, the association between clinico-pathological parameters (tumor size, histological type, nuclear pleomorphism consensual score, and $\bar{v}_V$) and the evidence of vascular invasion and/or lymph node metastases was assessed by logistic regression models. Pearson Chi-square or Fisher test (when the assumptions for the Pearson Chi-square were not fulfilled) were used to assess which categorical variables should enter in the multivariable logistic model, while independent t-test was used for continuous variables. It is opportune to mention that in addition to the histological type, malignant tumors were grouped for statistical purposes, into two categories: 1) solid and anaplastic types; 2) tubulopapillary and complex types (one case of squamous cell carcinoma was excluded). This was performed according to previous evidence that solid and anaplastic tumors show increased metastatic activity (Misdorp *et al.*, 1999; Rasotto *et al.*, 2012;

Peña *et al.*, 2013). As different categories of tumor size have been described in the literature, this parameter was analyzed in three different ways: 1) as a continuous variable; 2) grouped according to the WHO criteria (< 3cm; 3-5cm; > 5cm); 3) categorized as suggested by Santos *et al.* (2011) (< 3cm; ≥ 3cm).

A survival analysis of DFI and OS was performed to determine if differences existed between the group with and without evidence of vascular invasion/lymph node metastases. The Kaplan-Meier plots were used to show differences between these groups. Finally, a log-rank test (Mantel-Cox) was applied to analyze the significance of the differences between groups. The same approach was used for the results of the ROC analysis if significant. The association of nuclear pleomorphism consensual score and $\overline{v}_V$ with survival data was also evaluated by univariable analyses. The counts of malignant tumors with progression (recurrence and/or metastases and death) and malignant tumors without events during the entire follow-up period were used for those univariable analyses. In all the tests a *P* value lower than 0.05 was considered significant. The statistical analyses were performed in the program IBM SPSS Statistics, version 22 (IBM, New York, USA).

### 2.3 Results

The 122 CMT analyzed included 17 complex adenomas, 10 simple adenomas and 6 benign mixed tumors, 42 complex carcinomas, 23 solid carcinomas, 21 tubulopapillary carcinomas and 3 other carcinoma types (one squamous cell and two anaplastic carcinomas). In 26% of the malignant tumors there was histological evidence of vascular invasion and/or lymph node metastases at the time of the diagnosis. For survival analysis only cases presented as one malignant tumor per animal (*n* of animals = 40) and cases of multiple malignant tumors per animal with no evidence of disease progression (recurrence and/or metastases) during the follow-up period *(n* of animals = 8; *n* of tumors = 18) were considered. During the follow-up period 10 animals progressed (recurred and/or metastasized), 26 were disease-free at the end of the follow-up period, and the remaining cases were censored.

When scoring the nuclear pleomorphism in malignant tumors, the two observers disagreed in 30/89 cases (34%), with higher agreement in the score 3

comparing to scores 1 and 2 (Table 1). The Kappa value was 0.46 ($P < 0.001$), thus corresponding a low-moderate agreement (Vieira and Garrett, 2005). Asymptotic marginal homogeneity test was performed to check if there was a significant difference between the observers and none gave systematically lower or higher values than the other ($P = 0.16$).

In each subtype of carcinomas (except for those named “other carcinomas”) score 2 was given to 50-60% of the cases. In solid and tubulopapillary carcinomas, score 3 represented 30-40% of the cases, while in complex carcinomas only 19% of cases were score 3. In the latter, 29% had score 1 in nuclear pleomorphism (Table 2). It is noteworthy that no association was detected between nuclear pleomorphism scores and histological subtypes ($P = 0.26$).

Regarding the histological subtype, no significant association with evidence of vascular invasion and/or lymph node metastases was detected. However, when the tumors where grouped into two histological type categories (solid plus anaplastic and tubulopapillary plus complex), it was noticed that the latter presented a low risk of vascular invasion and/or lymph node metastases comparing to the other category ($P = 0.01$) (Table 3).

**Table 1** – Results and concordance of the nuclear scoring in 89 canine mammary carcinomas by two observers.

| | | **Nuclear grade observer A** | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | Total |
| **Nuclear grade observer B** | 1 | 8 | 4 | 0 | 12 |
| | 2 | 10 | 29 | 9 | 48 |
| | 3 | 1 | 6 | 22 | 29 |
| | Total | 19 | 39 | 31 | 89 |

**Table 2** – Nuclear grading scores, mean (standard deviation) of volume-weighted mean nuclear volume ($\overline{v}_V$) and frequency of vascular invasion and/or lymph node metastases in each carcinoma subtype ('others' include 1 squamous cell and 2 anaplastic carcinomas).

| | **Histological subtype** | | | |
|---|---|---|---|---|
| **Nuclear score** | Solid (*n*=23) | Tubulopapillary (*n*=21) | Complex (*n*=42) | Others (*n*=3) |
| 1 | 2 | 2 | 12 | 0 |
| 2 | 12 | 12 | 22 | 2 |
| 3 | 9 | 7 | 8 | 1 |
| $\overline{v}_V$ ($\mu m^3$) | 311(98) | 295(82) | 282(49) | 248(116) |
| **Invasion / metastases** | 11 | 5 | 7 | 0 |

In general, the stereological analysis was simple: the PSI method lasted 10 to 15 minutes per slide. The mean (SD, standard deviation) $\overline{v}_V$ was 128 $\mu m^3$ (64 $\mu m^3$) in normal mammary gland, 179 $\mu m^3$ (31 $\mu m^3$) in benign tumors and 291$\mu m^3$ (75 $\mu m^3$) in malignant tumors (Fig. 3). In the latter group, the mean (SD) $\overline{v}_V$ was 267 $\mu m^3$ (50 $\mu m^3$), 265 $\mu m^3$ (50 $\mu m^3$) and 357 $\mu m^3$ (92 $\mu m^3$) in tumors with nuclear scores 1, 2 and 3, respectively. Regarding normal parenchyma, a wide range of values of $\overline{v}_V$ was obtained (CV = 0.50); the higher value was estimated in early diestrous and the lower one in proestrous. The mean values of the stereological estimations were significantly different in normal parenchyma, benign and malignant tumors (Tukey test, $P < 0.001$ in all comparisons).

The CE of the estimates was 19% for normal parenchyma, 3% for the benign tumors and 3% for the group of malignant tumors. When the latter ones were grouped by the nuclear pleomorphism score, the CE was 5%, 7% and 5% for score 1, 2 and 3, respectively.

**Table 3** – Results of the univariable models and the final multivariable model using vascular invasion and/or lymph node metastases as the dependent variable in 89 canine mammary carcinomas.

| | Univariable analysis | | | Multivariable analysis | | |
|---|---|---|---|---|---|---|
| ***Independent variables*** | **OR** | **95% CI** | ***P*** | **OR** | **95% CI** | ***P*** |
| **Histological subtype** | | | | | | |
| *All categories:* | | | | | | |
| *TP* | Ref | | — | | | |
| *Solid* | 2.94 | 0.80 - 10.74 | 0.103 | | | |
| *Complex* | 0.64 | 0.17 - 2.32 | 0.498 | | | |
| *Others* | 0 | 0 - >100 | 0.991 | | | |
| *Two categories:* | | | | | | |
| *Solid and anaplastic* | Ref | | — | | | |
| *TP and complex* | 0.28 | 0.10 - 0.77 | 0.014 | 0.31 | 0.09 - 1.07 | 0.065 |
| **Tumor size** | | | | | | |
| *Continuum* (cm)* | 1.50 | 1.21 - 1.87 | <0.001 | 1.63 | 1.26 - 2.10 | <0.001 |
| *WHO categories* | | | | | | |
| < 3 cm | Ref | | — | | | |
| 3 - 5 cm | 8.67 | 2.24 - 33.53 | 0.002 | | | |
| > 5 cm | 12.43 | 3.47 - 44.43 | <0.001 | | | |
| *Santos et al., 2011 categories* | | | | | | |
| < 3 cm | Ref | | — | | | |
| ≥ 3 cm | 9.58 | 3.20 - 28.72 | <0.001 | | | |
| **Nuclear pleomorphism** | | | | | | |
| *Score 3* | Ref | | — | | | |
| *Score 2* | 0.42 | 0.15 - 1.20 | 0.101 | | | |
| *Score 1* | 0 | >0.001 - 0 | 0.991 | | | |
| *Two categories* | | | | | | |
| *Score 1 and 2* | Ref | | — | | | |
| *Score 3* | 3.42 | 1.26 - 9.29 | 0.017 | 2.57 | 0.76 - 8.63 | 0.126 |
| **Nuclear** $\overline{v}_V$ (µm3) | 1.004 | 0.99 - 1.01 | 0.248 | | | |

Legend: OR – odds ratio; CI – confidence interval; TP – tubulopapillary; $\overline{v}_V$ – volume-weighted mean nuclear volume; Ref – referent.

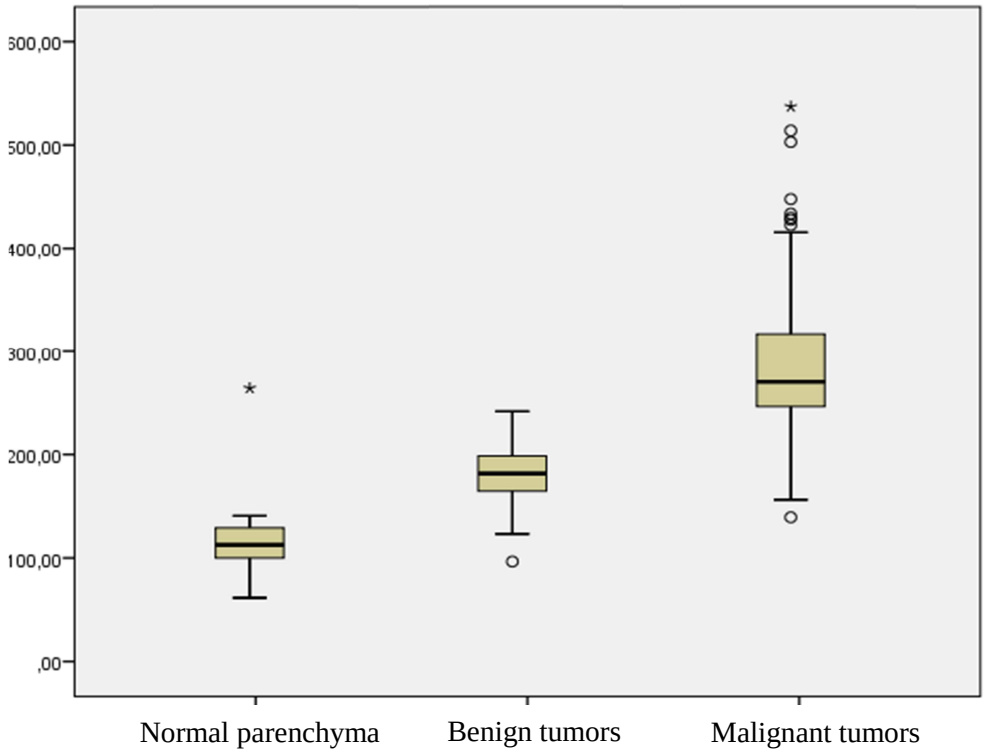


**Fig. 3** – Box plot with median, first and third quartile values and the distribution of the volume-weighted mean nuclear volume ($\overline{v}_V$) values in normal mammary parenchyma, benign and malignant canine mammary tumors. Only slight overlaps existed between $\overline{v}_V$ values of each group. Outliers are indicated by the different marks.

The ANOVA comparison between nuclear pleomorphism scores and $\overline{v}_V$ revealed significant differences between score 1 *versus* 3 ($P < 0.001$) and 2 *versus* 3 ($P < 0.001$). Tumors scored as 1 and 2 did not differ significantly regarding their $\overline{v}_V$. The histogram of $\overline{v}_V$ values in malignant tumors showed a larger proportion of values around 300 $\mu m^3$, mainly including tumors with nuclear scores 1 and 2; a much smaller peak appeared to exist after 400 $\mu m^3$, corresponding to tumors that scored 3 in nuclear pleomorphism (Fig. 4).

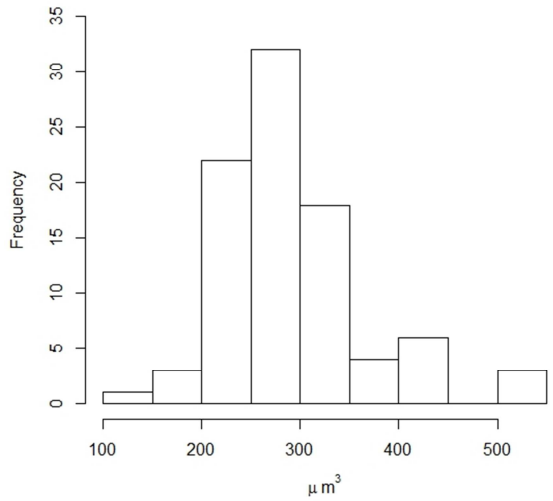


**Fig. 4** – Histogram of the volume-weighted mean nuclear volume ($\bar{v}_V$) values of the 89 canine mammary carcinomas. The higher peak of the values is around 300 µm$^3$.

In univariable analysis, the $\bar{v}_V$ was not associated with histological evidence of vascular invasion and/or lymph node metastases. Therefore, in descriptive ROC analyses the discriminating power of the $\bar{v}_V$ estimations between malignant tumors with and without histological evidence of vascular invasion and/or lymph node metastases was very low (AUC statistics 0.55, 95% confidence interval 0.41–0.70). According to the ROC findings, no optimal cut-off value of nuclear $\bar{v}_V$ could be defined for differentiating tumors with and without vascular invasion and/or lymph node metastases. The $\bar{v}_V$ cut-off of 420 µm$^3$ provided 96% specificity but only 22% sensitivity. However, when the $\bar{v}_V$ values of the tumors that progressed (recurred and/or metastasized) were compared to those that did not progress during the follow-up, it was noticed that the mean $\bar{v}_V$ in the former was significantly higher [393 µm$^3$ (74 µm$^3$) *versus* 270 µm$^3$ (68 µm$^3$); $P < 0.001$].

The nuclear pleomorphism score categories (score 1 plus score 2 *versus* score 3) was associated with evidence of vascular invasion and/or lymph node metastases ($P = 0.02$) and with tumor progression during the follow-up ($P < 0.001$). In fact, only tumors scored as 2 or 3 showed histological evidence of vascular invasion and/or lymph node metastases and progression (in the latter, 9 out 10 were scored 3, with only one case being nuclear score 2). In multivariable analysis, histological type categories and nuclear pleomorphism score failed to retain their association with evidence of vascular invasion and/or

lymph node metastases. Tumor size was the only factor with statistical significance as independent predictor of vascular invasion and/or lymph node metastases (Table 3). In the descriptive ROC analysis, the discriminative power of tumor size was high (AUC statistics 0.80, range 0.70-0.91) (Fig. 5). According to the ROC findings, the optimal cut-off tumor size value that would distinguish tumors with and without vascular invasion and/or lymph node metastases at the time of the diagnosis was 2.9 cm (sensitivity of 74% and specificity of 77%).

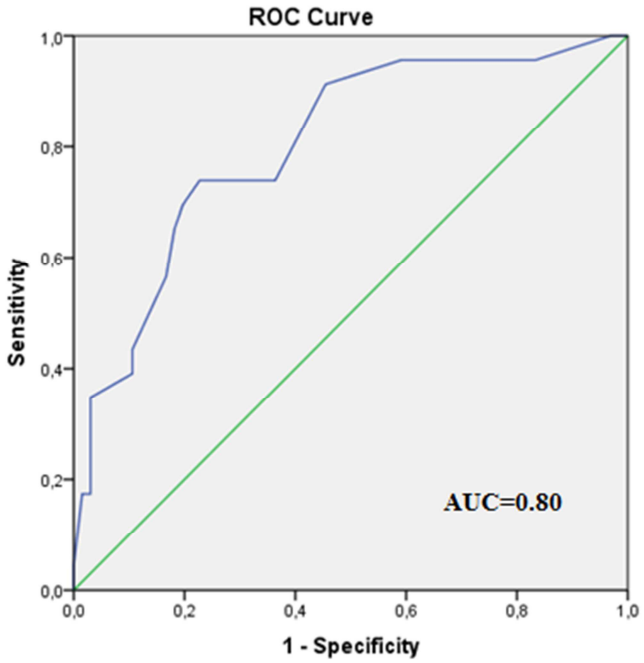


**Fig. 5** – Receiver operating curve (ROC) curve for tumor size values in 89 canine mammary carcinomas. The area under the curve (AUC) is 0.80 which indicates that tumor size has high discriminatory power between tumors with and without evidence of vascular invasion and/or lymph node metastases at the time of the diagnosis. An optimal cut-off of 2.9 cm with 74% sensitivity and 77% specificity was defined.

Regarding the survival study, the mean DFI (14.2 ± 2.7 months) and OS (15.2 ± 2.6 months) of the dogs bearing tumors with histological evidence of vascular invasion and/or lymph node metastases were significantly shorter than the DFI (22.4 ± 0.9 months) and OS (23.2 ± 0.5 months) of the cases without such evidence (Fig. 6). The tumors were also grouped by the tumor size cut-off value previously determined by the ROC curve in order to perform a survival analysis (Fig. 4). Tumors ≥ 2.9 cm in their maximum diameter were associated with poorer survival times ($P$ = 0.04 for DFI and $P$ = 0.02 for OS) (Fig. 7).

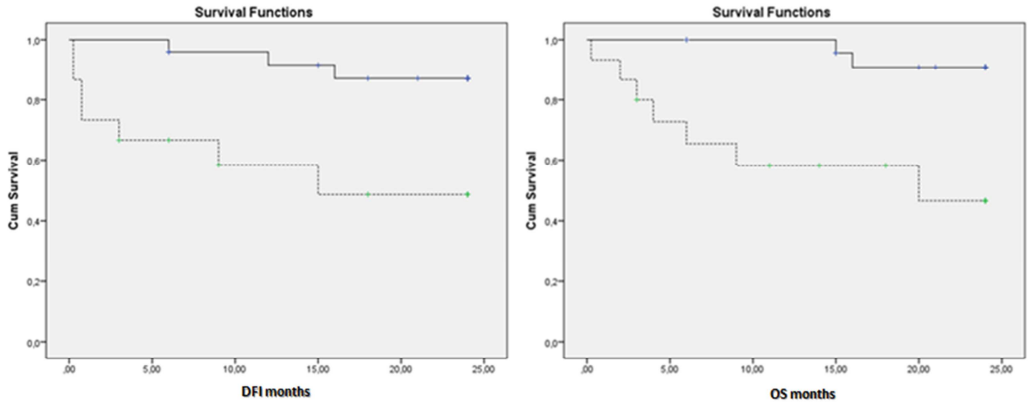


**Fig. 6** – Kaplan-Meier disease-free interval (DFI) and overall survival (OS) curves comparing cases with histological evidence of vascular invasion and/or lymph node metastases (dashed line) and cases without that evidence (continuous line) in 40 female dogs presenting a single mammary carcinoma. Vertical marks represent censored cases in each group. Histological evidence of vascular invasion and/or lymph node metastases was significantly associated with lower DFI and OS ($P$ = 0.004 and $P$ = 0.001, respectively).

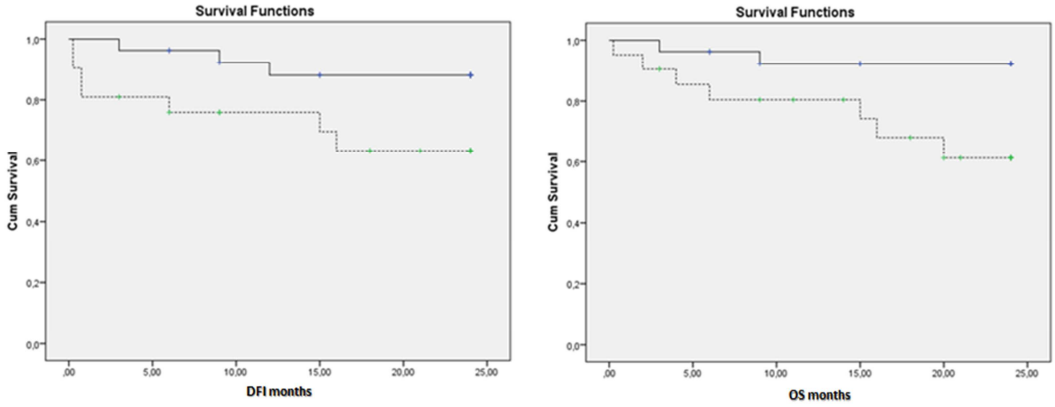


**Fig. 7** – Kaplan-Meier disease-free interval (DFI) and overall survival (OS) plots. Tumors were grouped on the basis of optimal cut-off size value determined by ROC curve analysis: size < 2.9 cm (continuous line) and size ≥ 2.9 cm (dashed line). Vertical marks represent censored cases in each group. Size ≥ 2.9 cm was associated with shorter DFI ($P$ = 0.04) and OS ($P$ = 0.02).

## 2.4 Discussion

In this study a comprehensive evaluation of the nuclear pleomorphism of CMT and its prognostic value was performed. The malignant tumors were divided according to the presence or absence of vascular invasion and/or lymph node metastases at the time of histological diagnosis, as previously described by

Rasotto *et al.* (2012). This could be viewed as one of the earliest morphological signs of tumor aggressive behavior and we propose that its recognition is essential for the development of effective post-operative metastatic disease prevention strategies, like chemo- or radiotherapy. This assumption was supported by the survival analysis of this series, where tumors with histological evidence of vascular invasion and/or lymph node metastases had poorer survival after surgery, thus corroborating previous studies (Hellmén *et al.*, 1993; Sarli *et al.*, 2002; Philibert *et al.*, 2003; Chang *et al.*, 2005).

The interobserver variability in scoring nuclear pleomorphism using the NHG criteria had not, to the best of our knowledge, been previously studied in CMT. In some of the most recent reports focusing on the use of the NHG method for mammary tumors of dogs, the concordance between the observers was not reported (Karayannopoulou *et al.*, 2005; Clemente *et al.*, 2010; Peña *et al.*, 2013). In this study, there was a modest agreement between the two observers regarding nuclear pleomorphism scoring. At a first glance, this fact could be a reason for concern since it anticipates a probable low reproducibility of histological grading in CMT. As already mentioned, malignant CMT are highly heterogeneous tumors and an inherent subjectivity associated with the selection of the highly malignant areas is likely to occur (Ladekarl, 1998; Misdorp, 2002; Kamaki *et al.*, 2006; Rakha *et al.*, 2010). Moreover, according to our stereological findings, tumors with nuclear scores 1 and 2 had objectively comparable $\overline{v}_V$. Therefore, a lack of concordance between observers in these nuclear scores, as observed in this study, is more prone to occur. It is noteworthy in human breast cancer, one study concluded that the $\overline{v}_V$ estimations were not correlated with the nuclear scores of histological grade (Ladekarl, 1995), and other study established that the estimations in grade I and grade II were similar (Artacha-Pérula and Roldán-Vilalobos, 1997).

The stereological assessment of nuclear pleomorphism by the PSI method uses a systematic random sampling, which is not only highly efficient but also obviates the subjectivity associated with the selection of the apparently more aggressive tumor areas (Ladekarl, 1998). To the best of our knowledge, nuclear pleomorphism in CMT has never been quantified by unbiased stereological

techniques. According to this statistical analysis, malignant tumors presented a significant higher nuclear pleomorphism compared to benign tumors.

The unbiased assessment of nuclear pleomorphism seems not to be related to the histological evidence of vascular invasion and/or lymph node metastases in malignant CMT. However, $\overline{v}_V$ was significantly higher in tumors that recurred and/or metastasized during the two years follow-up period. This opens the possibility that this parameter may be regarded as a survival predictor, in accordance with the human scenario, in which it has been correlated with outcome of patients affected by breast carcinoma (Artacha-Pérula and Roldán-Vilalobos, 1997; Laderkarl, 1998).

It is consensual that canine mammary neoplasms are morphologically distinct from those of women. Yet, the NHG was straightforwardly adapted to the dog, taking for granted that the evaluation of the parameters should be the same as used in human pathology (Matos *et al.*, 2012), and the three scores of nuclear pleomorphism were considered, without any validation studies. It is worth mentioning that the present study showed that tumors scored 1 or 2 in nuclear pleomorphism presented similar mean nuclear volumes and that there was a low interobserver concordance in nuclear score 1 and 2. Moreover, nuclear score 3 was associated with a higher probability of vascular invasion and/or lymph node metastases, and poor survival. Therefore, only two nuclear grading scores appear to exist in malignant CMTs: low grade and high grade, corresponding mostly to NHG nuclear scores 1 and 2 and to nuclear score 3, respectively.

There is a general agreement that the tumor size of canine malignant mammary tumors has a prognostic significance (Philibert *et al.*, 2003; Chang *et al.*, 2005; Sorenmo *et al.*, 2011). Our multivariable analysis results confirmed that tumor size represents an independent prognostic factor regarding the capacity of tumor vascular invasion and metastasize. Keeping in mind that different tumor size categories have been proposed and used (Misdorp, 2002; Santos *et al.,* 2011; Peña *et al.,* 2013), a ROC curve analysis was performed in order to define the tumor size threshold value with the highest sensitivity and specificity to predict aggressive tumor behavior. Our results highlighted that tumors with or bigger than 2.9 cm in their largest diameter were more associated with vascular

system invasion and/or metastases to the regional lymph nodes. The established cut-off of 2.9 cm supports that the tumor size categories (under and over 3 cm), defined by Sorenmo (2003) and used by Santos *et al.* (2011), are suitable for CMT and should be routinely applied, for better reproducibility of future studies. Moreover, it indirectly supports the model proposed by Sorenmo *et al.* (2009) that correlates the development of a malignant phenotype with increased tumor size. According to our findings, tumor size does matters and clinicians should consider more radical surgical procedures and meticulous periodical follow-up evaluations of animals affected by malignant tumors larger than 3 cm, with a special regard to the surgical removal of regional lymph nodes.

Unlike tumor size, nuclear pleomorphism was unable to show independent prognostic value when included in the multivariable model. This analysis obviates possible confounding factors when assessing prognostic factors, justifying the fact that some parameters, although related to prognosis in univariable analysis, lose their value in multivariable models (Peña *et al.*, 2013; Santos *et al.*, 2013). We think that the role of nuclear pleomorphism as a prognostic factor deserves to be studied in larger series by multivariable survival analysis.

To the best of our knowledge, this was the first time that a stereological tool was used to compare the nuclear pleomorphism in benign and malignant CMT. The results pointed to significant differences in the $\bar{v}_V$ from either tumor. This finding corroborated previous morphometric studies performed in cytological and histological preparations, which demonstrated that the nuclear area and perimeter were significantly higher in malignant CMT (Simeonov and Simeonova, 2006; 2007). We consider that the estimates of $\bar{v}_V$ could assist in the differential diagnosis of benign and malignant tumors, being particularly important for tumors presenting borderline malignancy. As to the normal parenchyma, the mean value of $\bar{v}_V$ was significantly lower that those from tumors, however a high variation during the estrous cycle seemed to occur. The value of $\bar{v}_V$ of the normal parenchyma during early diestrous was within the range of values seen in maligant tumors. Early diestrous mammary parenchyma is characterized by profilerative cells, presenting euchromatic and large nuclei,

with moderate size variability — this could justify the high values of the $\overline{v}_V$ estimated in this phase (Santos *et al.*, 2010). Thus, the variation of the $\overline{v}_V$ of normal parenchyma during the estrous cycle, as an objective estimation of nuclear volume variability, deserves attention from future studies. In malignant CMT, the seterological estimation of nuclear pleomorphism did not clearly discriminated tumors of the 3 different NHG nuclear scores. Some evidence that higher tumoral $\overline{v}_V$ could predict a poor animal outcome was also provided by the present study. However, the analyzed sample with follow-up data, particularly the number of cases with tumor related events, was relatively small, hampering further statistical analysis (*e.g.*, a ROC curve analysis of the $\overline{v}_V$ values in tumors that progressed and tumors that did not progressed during the follow-up period). In addition, the role of this stereological evaluation of nuclear volume in other subtypes of canine mammary malignant tumors, like carcinosarcomas, remains to be determined.

In this study, evidence that the nuclear pleomorphism evaluation in CMT by the NHG criteria is highly prone to interobserver variability was provided. As an immediate recommendation, the authors propose that this parameter should be evaluated simultaneously by, at least, two observers, that should be able to produce a consensus nuclear grading score. Meanwhile, prospective studies of larger series and rigorous follow-up data are needed to validate the possibility that only two nuclear pleomorphism scores should be considered in malignant CMT. We hypothesize that the $\overline{v}_V$, as an unbiased stereological that elegantly combines the estimation of nuclear size and its variability, can provide an objective threshold, allowing the differential diagnosis between benign and malignant CMT, as well as the distinction between low and high nuclear grade malignant tumors, based on reliable survival information.

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# CHAPTER 3

## INTEROBSERVER REPRODUCIBILITY OF HISTOLOGICAL GRADE IN CANINE MAMMARY CARCINOMAS

*[The contents of this chapter are in press in Journal of Comparative Pathology ]*

**Summary**

Histological grading of canine mammary carcinomas (CMC) has been performed using the human Nottingham histological grade method as basis. Evidence exists that the histological grade could be a prognostic factor in CMC; however, no data are available concerning interobserver variability in grading. In this study we analyzed the interobserver reproducibility between three observers when assigning individual parameter scores and grade to 46 CMC. The influence of tumor size and vascular invasion and/or lymph node metastases on the odds of grading disagreement was also evaluated. The mean kappa values were 0.71, 0.51, 0.69 and 0.70 for tubule formation, nuclear pleomorphism, mitotic counts and grade, respectively. There was moderate to good agreement in scoring parameters and tumor grading, with nuclear pleomorphism being the least reproducible. These findings are similar to those of human studies. The odds of grading disagreement increased with tumor size, but decreased with the presence of vascular invasion and/or lymph node metastases. Individual scoring differences were attenuated by reaching a consensus between two observers.

## 3.1 Introduction

The Nottingham histological grade (NHG), the standard method in human breast pathology, has been apllied (with or without modification) for grading canine mammary carcinomas (CMC) (Karayannopoulou *et al*., 2005; Peña *et al*., 2013). The NHG is composed of the sum of scores assigned to three morphological features (tubule formation, nuclear pleomorphism and mitotic count), each taking a value of one to three points (Elston and Ellis, 1991). A total score ≤ 5 points, 6-7 points, and 8-9 points denotes grade I, II, III carcinomas, respectively (Elston and Ellis, 1991).

Nowadays, the histological grade is widely used in CMC, but there are no studies focusing on the reproducibility of grading. This scenario contrasts markedly with human pathology, where the reproducibility of grading methods, including the NHG, has been debated for years (Stenkvist *et al.*, 1979; Frierson *et al.*, 1995; Robbins *et al.*, 1995; Dalton *et al.*, 2000; Meyer *et al.*, 2005). Furthermore, the measurement of interobserver variability in veterinary oncology is considered critical to validate prognostic markers (Webster *et al*., 2011).

In human studies, the agreement between observers has been estimated in three different ways: percentage of equal judgments, Cohen's kappa ($\kappa$) statistics and Spearman correlation coefficient (Stenkvist *et al.*, 1979; Longacre *et al.*, 2006). Each of these statistical methods has limitations and pitfalls; reporting all three may provide a better reproducibility assessment (Stenkvist *et al.*, 1979). The agreement percentage is intrinsically dependent on the number and frequency of the classifying categories (Stenkvist *et al.*, 1979). The $\kappa$ statistic implies the assumption that categories have the same width and the so-called $\kappa$ paradox may occur, namely when the frequencies of categories are clearly unbalanced (Sim and Wright, 2005). In those cases, the proportion of agreement may be high but the $\kappa$ value could be low and an interpretation based solely on the $\kappa$ value would lead to erroneous conclusions (Sim and Wright, 2005). Still, some controversy exists regarding the best $\kappa$ value (weighted or unweighted) to be used in breast cancer grade reproducibility studies (Chowdhury *et al.*, 2007). The unweighted $\kappa$ value ($\kappa_u$) considers all types of disagreements as equal, independently of their magnitude (Sim and

Wright, 2005). In contrast, the weighted κ value ($\kappa_w$) emphasizes large differences between ratings in ordinal scales (Sim and Wright, 2005). It should be noted that recent guidelines in veterinary oncology recommend the use of $\kappa_w$ statistics (Webster *et al*., 2011).

The aim of this study was to determine the interobserver agreement in grading simple CMC and in scoring each grading parameter, using the NHG. Additionally, the influence of clinicopathological parameters (tumor size, vascular invasion and/or lymph node and tumor progression) on the odds of grading disagreement was estimated.

## 3.2 Material and methods

### *3.2.1 Cases and histological analysis*

Pathology archives from the ICBAS, University of Porto were accessed to retrospectively select 46 spontaneous simple CMC that had been surgically removed. The selection of cases and their histological study were blinded to clinical data. For 30 cases follow-up data were collected over two years following the protocol detailed in Santos *et al.* (2013). Owners gave informed consent for both surgery and follow-up.

The histological diagnosis was reviewed by two observers to confirm that all cases fulfilled the criteria for simple carcinomas (composed of luminal epithelial cells) (Misdorp *et al*., 1999). For each case, tumor size (*i.e.,* largest diameter) and histological evidence of vascular invasion (defined as the presence of tumor emboli within endothelial-lined spaces and without distinguishing bteween lymphatic and blood vessels) and/or regional lymph node metastases were recorded. All the slides resulting from the largest cross section were used for grading. Three observers from the same institution (MS, a veterinary pathologist with 10 years of experience; PDP, a veterinary pathologist with 15 years of experience, both with special interest in canine mammary pathology; and CL, a medical pathologist and Professor of human pathology with more than 40 years of experience) graded all the tumors independently, using the NHG (Elston and Ellis, 1991; Karayannopoulou *et al*., 2005). Briefly, tubule formation was scored as 1, 2 or 3 when more than 75%, 10-75% or less than 10% of neoplastic cells, respectively, were arranged in structures exhibiting an obvious lumen. Nuclear

pleomorphism was scored as follows: score 1 denoted a slight increase in variability of nuclear size and shape, compared to normal surrounding epithelial cells; score 2 denoted moderate variation in nuclear size and shape; score 3 denoted marked variation in nuclear size and shape, with very large and bizarre forms. Mitotic figures were counted in 10 high-power fields and scored using the cut-offs defined by the field diameter of the microscope (field diameter of 0.55mm; field area of 0.238 $mm^2$; score 1 ≤ 8 mitotic figures, score 2 = 9-17 mitotic figures; score 3 ≥ 18 mitotic figures), thus assuring equivalence with assessments made by Elston and Ellis (Elston and Ellis, 1998; Karayannopoulou *et al*., 2005). The selection of the high-power field for mitotic counts was performed independently by each observer in the most mitotically active parts of the tumor (Elston and Ellis, 1991).

Cases with scoring discrepancies between the veterinary pathologists were reviewed using a multi-head microscope, in order to obtain a consensus. The consensus grade (observer 1 and 2) and its components were also compared with the grade assigned by the medical pathologist (observer 3).

*3.2.2 Statistical analysis*

The interobserver variability was measured by estimating the percentage of equal assessments. The $\kappa_u$ and $\kappa_w$ statistics were used to assess the paired interobserver agreement for histological grading and for parameter scoring. A value of $\kappa$ greater than 0.8 is considered to indicate almost perfect agreement, whereas $0.6 < \kappa \leq 0.8$ and $0.4 < \kappa \leq 0.6$ values indicate good and moderate agreements, respectively. In contrast, $\kappa < 0.4$ is considered a poor agreement (Vieira and Garret, 2005). The interobserver variability in total score assigned (values 3 to 9) was also estimated as a correlation coefficient (Spearman rank correlation coefficient). For these tests a $P < 0.05$ was considered significant. Logistic regression was used to assess the influence of clinicopathological parameters on the odds of grading disagreement. For this analysis, a $P < 0.1$ was considered significant. All analyses were performed using R free software (R Core Team) using packages psych (Revelle, 2014) and Hmisc (Harrell, 2014).

### 3.3 Results

In this series of 46 simple CMC, mean (standard deviation) tumor size was 3.3 cm (3.1cm). At the time of diagnosis, 33% (15/46) of the tumors showed vascular invasion and/or lymph node metastases. During the follow-up period, 27% (8/30) of dogs developed progression-related events (*i.e.* recurrences or distant metastases). Grade I tumors were relatively uncommon, representing 11% to 20% of cases depending on the observer (Table 1).

**Table 1** – Individual and consensual (observers 1 and 2) grading of 46 canine mammary carcinomas using the Nottingham histological grade method.

| | **Obs 1** | **Obs 2** | ***Consensus*** **Obs 1+2** | **Obs 3** |
|---|---|---|---|---|
| Grade 1 | 5 | 9 | 6 | 8 |
| Grade 2 | 20 | 19 | 21 | 17 |
| Grade 3 | 21 | 18 | 19 | 21 |

Overall, there was an agreement percentage for tumor grading of 52%. For tubule formation, nuclear pleomorphism and mitotic counts the agreement was 61%, 50% and 54%, respectively. The agreement of the sum of scores was 24%. The interobserver variability, measured as the percentage of concordance and $\kappa$ values in paired comparisons, is illustrated in Table 2. The tumor grade $\kappa_w$ varied from 0.59 to 0.80 (mean $\kappa_w$ of all pairwise comparisons was 0.70). For tubule formation, nuclear pleomorphism and mitotic counts, the mean $\kappa_w$ of all pairwise comparisons was 0.71, 0.51, and 0.69, respectively. Higher agreement values were obtained for some of the paired comparisons: a) consensus and observer 3 (medical pathologist) for tubule formation, nuclear pleomorphism and mitotic count; and b) observer 2 *versus* observer 3 for overall tumor grade (Table 2). In general, the highest agreement between observers was seen for evaluation of tubule formation, closely followed by the mitotic count (Table 2). The agreement for nuclear pleomorphism in all pairwise comparisons was moderate. In all instances, the Spearman correlation coefficient for the overall score was higher than 0.70 ($P < 0.001$).

**Table 2** – Percentage of concordance and kappa statistics values between observers in grading parameters scores and in histological grading.

| | TF | NP | M | Grade |
|---|---|---|---|---|
| **Obs1/Obs2** | | | | |
| C | 76% | 65% | 69% | 67% |
| $\kappa_u$ | 0.61 (0.42-0.81) | 0.40 (0.17-0.63) | 0.49 (0.29-0.68) | 0.47 (0.26-0.69) |
| $\kappa_w$ | 0.71 (0.52-0.89) | 0.57 (0.41-0.74) | 0.68 (0.50-0.86) | 0.68 (0.53-0.83) |
| **Obs1/Obs3** | | | | |
| C | 70% | 65% | 61% | 59% |
| $\kappa_u$ | 0.50 (0.29-0.72) | 0.38 (0.15-0.60) | 0.32 (0.12-0.51) | 0.33 (0.10-0.55) |
| $\kappa_w$ | 0.69 (0.54-0.85) | 0.43 (0.21-0.66) | 0.55 (0.34-0.76) | 0.59 (0.42-0.76) |
| **Obs2/Obs3** | | | | |
| C | 72% | 65% | 74% | 78% |
| $\kappa_u$ | 0.55 (0.34-0.75) | 0.39 (0.15-0.62) | 0.58 (0.39-0.77) | 0.66 (0.46-0.85) |
| $\kappa_w$ | 0.66 (0.47-0.86) | 0.46 (0.21-0.72) | 0.77 (0.63-0.92) | 0.80 (0.68-0.93) |
| **Cons/Obs3** | | | | |
| C | 76% | 70% | 70% | 70% |
| $\kappa_u$ | 0.61 (0.41-0.81) | 0.45 (0.22-0.68) | 0.50 (0.31-0.69) | 0.52 (0.30-0.73) |
| $\kappa_w$ | 0.76 (0.62-0.90) | 0.58 (0.39-0.77) | 0.77 (0.67-0.88) | 0.71 (0.56-0.86) |

Legend: brackets meaning 95% confidence intervals; C – percentage of concordance; $\kappa_u$ – kappa unweighted; $\kappa_w$ – kappa weighted; Obs – observer; cons – consensus; TF – tubule formation; NP – nuclear pleomorphism; M – mitotic count.

Cases with complete agreement between the three observers for tumor grade are illustrated in Fig. 1. When disagreement existed, the pathologists always clustered their opinions around two adjacent grades and the difference in score of each parameter and the sum of scores were ±1, in the majority of cases. As the disagreement usually corresponded to adjacent scores the $\kappa_w$ was invariably higher that $\kappa_u$ (Sim and Wright, 2005).

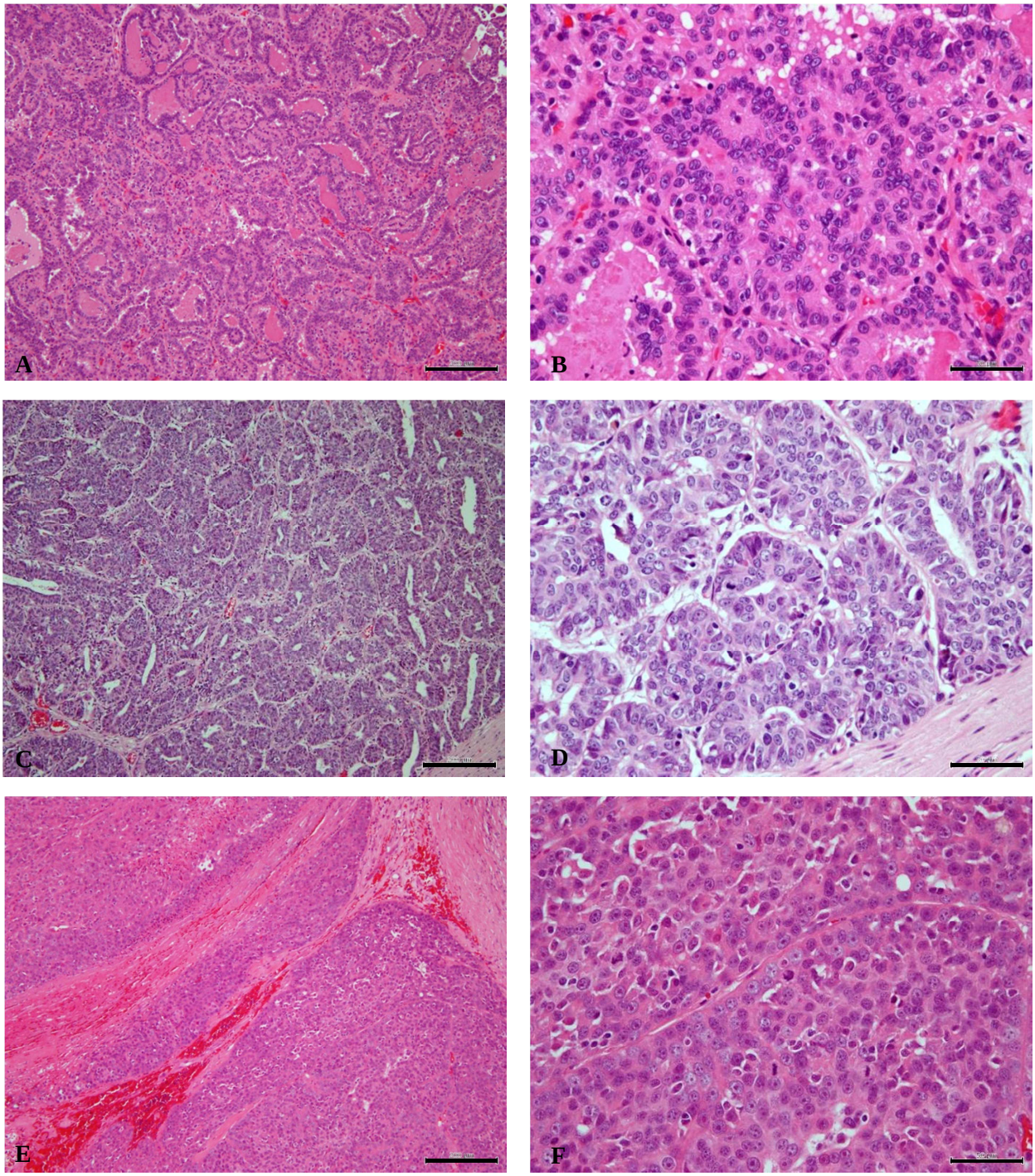


**Fig. 1** – Canine mammary simple carcinomas presenting overall grade agreement between the three observers. A and B – grade I; C and D – grade II carcinoma; E and F – grade III carcinoma. Hematoxylin-eosin. Bar 200 µm (E),100 µm (A, C), 50 µm (B, D, F).

The odds of disagreement when scoring parameters increased with tumor size: each centimeter increase in diameter accounted for 1.4 times higher odds of disagreement ($P$ = 0.065). In contrast, the odds of disagreement decreased by a factor of 0.03 when vascular invasion and/or regional lymph node metastases

were detected at diagnosis ($P$ = 0.08). The level of disagreement was similar in tumors with and without progression during the follow-up period.

### 3.4 Discussion

In the last decade, NHG has been used to CMC grading; however, its use requires adjustment for veterinary pathology (Matos *et al*. 2012; Mills *et al*., 2015). In this first study about grade reproducibility, we focused on simple CMC since they are considered most similar to the common forms of human breast carcinoma. This subtype of tumors is suitable for comparing grading assessment by veterinary and medical pathologists, which was one goal of this study. Moreover, as simple carcinomas are associated with a poorer prognosis when compared to complex and mixed carcinomas (Misdorp *et al*., 1999), it is critical, in prognostic terms, to be aware of interobserver reproducibility in grading this particular tumor subgroup.

The reproducibility observed in this study ($\kappa_w$ = 0.70 for the overall grade) is in close agreement with the human scenario ($\kappa$ of 0.30-0.70) (Meyer *et al*., 2005; Rakha *et al*., 2010). Furthermore, the higher reproducibility value in scoring tubule formation (0.71), followed by mitotic count (0.69) and finally nuclear pleomorphism (0.51) is similar to the majority of human breast studies (reviewed by Meyer *et al.*, 2005; Rakha *et al*., 2010). In grading CMC, consensus seems to be least common with scoring of nuclear pleomorphism. In human literature, various reasons have been proposed to justify this, including the qualitative nature of the scoring method and the heterogeneity of the nuclear features within a tumor (Meyer *et al.*, 2005; Longacre *et al.*, 2006; Adams *et al.*, 2009). Moreover, we recently demonstrated that CMC that scored 1 and 2 present similar mean volume–weighted nuclear volumes (Santos *et al*., 2014). Additionally, the use of the normal surrounding parenchyma as reference may jeopardize the reproducibility of nuclear pleomorphism in CMC, since the parenchyma often presents variability in nuclear features, depending on the estrous cycle stage (Santos *et al*., 2010).

The second poorest agreement was seen for mitotic count, probably due to the selection of areas for counting mitotic figures (Meyer *et al*., 2005; Longacre *et al.*, 2006). In large tumors, the high number of slides can be an additional bias,

which could explain increased odds of grading disagreement with increasing size. To decrease bias, some studies in human breast pathology have assigned designated counting areas on the slides of each tumor (Tsuda *et al*., 2000). In our study, there was no attempt to guide observers to any particular slide or tumor area. Even if this led to some of the interobserver variation, it represents more accurately the procedures of pathologists during their routine diagnostic activity (Longacre *et al*., 2006).

In this study, the Spearman correlation coefficient for the total combined score was relatively high, indicating that when an observer attributed a high score to a tumor, it was likely that the other observer would also attribute a high score.

The levels of agreement in grading parameters showed a tendency to increase when consensus between two observers was reached. This suggests that efforts to obtain a grading consensus are an effective way to compensate for potential individual bias in scoring. In human medicine it has been postulated that two or three pathologists should suffice to reach a valid consensus (Dalton *et al*., 2000).

In conclusion, this is the first study addressing interobserver agreement in grading CMC, to the authors' knowledge. The NHG when applied to simple CMC presented a level of reproducibility similar to that reported for human breast carcinomas. Future intra- and interdepartmental studies with panels of observers and different subtypes of CMC are warranted to fully ascertain the reliability of the grading method.

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# CHAPTER 4

## VALUE OF NOTTINGHAM HISTOLOGICAL GRADING PARAMETERS AND PROGNOSTIC INDEX IN CANINE MAMMARY MALIGNANT TUMORS

*[The contents of this chapter are in press in Anticancer Research.]*

**Summary**

The human Nottingham histological grade (NHG) has been adapted to canine mammary malignant tumors (CMT). Nottingham prognostic index (NPI) enables the identification of more aggressive human breast cancers. The prognostic value of each grading parameters and NPI has never been detailed in malignant CMT. The aim of the present study was to assess the prognostic value of NHG, its parameters and NPI. Univariable and multivariable analysis were used to assess the prognostic value of NHG, its parameters and the NPI adapted to malignant CMT in a cohort of fifty nine dogs. Short disease-free interval and overall survival were associated with higher NHG, particularly grade III. Only nuclear pleomorphism was significantly associated with survival. Veterinary-adapted NPI exhibited a strong predictive value for disease progression. NHG, nuclear pleomorphism and the veterinary-adapted NPI have prognostic value in CMT. Nuclear pleomorphism is an independent prognostic factor. Nuclear pleomorphism evaluation should be included in routine pathology reports.

## 4.1 Introduction

Although the body of evidence in biological markers of canine mammary tumors has increased tremendously in the last few years, the individual prognosis and clinical management of female dogs affected by the disease are still challenging topics, emphasizing both the need for an in-depth evaluation on the existing prognostic factors and the search for new ones (Matos *et al.*, 2012).

In the last decade, the human Nottingham histological grade (NHG) has been applied (with or without modifications) to malignant canine mammary tumors (CMT) (Karayannopoulou *et al.*, 2005; Gama *et al.*, 2010; Clemente *et al.*, 2010; Peña *et al.*, 2013, Santos *et al.*, 2013; Im *et al.*, 2014; Mainenti *et al.,* 2014). NHG is based in the assessment of three morphological parameters: tubule formation, nuclear pleomorphism and mitotic counts (Elston and Ellis, 1998). Each parameter of the NHG is scored 1 to 3 and the total combined score defines the grade (Fig. 1). Both tubule formation and mitotic counts are evaluated semi-quantitatively using thresholds, but nuclear pleomorphism, classified according to the size of the nuclei and their variability, is scored in a much more subjective way (Elston and Ellis, 1998; Meyer *et al.*, 2005).

Despite two recent studies demonstrated that the NHG with modfications provided independent prognostic information (Peña *et al.*, 2013; Mainenti *et al.*, 2014), others only detected an association with survival when grades have been grouped — *e.g.*, grade I plus grade II *versus* grade III (Karayannopoulou *et al.*, 2005; Santos *et al*., 2013) — thus raising the issue of the validity of a three-tier classification for malignant CMT grading. Moreover, the individual prognostic value of each of the NHG parameters has never been detailed in malignant CMT, to the author's knowledge.

In fact, it has been stressed that in order to be universally accepted in malignant CMT, the prognostic legitimacy of a grading method must be validated in various prospective cohorts (Goldschmidt *et al.*, 2011; Matos *et al.*, 2012).

In human pathology, the NHG is highly correlated to survival and has been included in a prognostic index, called the Nottingham Prognostic Index, NPI (Blamey, 1996; Lee and Ellis, 2008). This Index allows a stratification of breast cancer patients (Rampaul *et al.*, 2001), and incorporates three independent

prognostic factors: tumor size, lymph node stage and histological grade (Haybittle *et al.*, 1982; Blamey, 1996).

The tumor size corresponds to the largest diameter, determined by the pathologist during the macroscopic examination of fresh specimens or the microscopic examination of very small invasive carcinomas (Elston *et al.*, 1999). In veterinary medicine, the tumor size, also defined as the largest diameter determined either by the clinician or by the pathologist, has been integrated in the World Health Organization (WHO) staging of malignant CMT (Peña *et al.*, 1998; Rutteman *et al.*, 2001; de las Mulas *et al.*, 2005) and there is a general agreement that it has prognostic significance (Ferreira *et al*., 2009; Sorenmo *et al.*, 2011).

The lymph node staging in human breast cancer patients is also a three-tier system, depending on the number of metastatic lymph nodes and their location (Lee and Ellis, 2008). In veterinary medicine, the presence of regional lymph node metastases is also relevant for disease staging, but the number of positive nodes is disregarded (Rutteman *et al.*, 2001). Other differences reside in the diagnostic imaging of regional lymph nodes and the sentinel lymph node examination, which are routinely assessed in women but not routinely performed in veterinary medicine (Webster *et al.*, 2011). In dogs, the recommended procedures depend on the anatomical location of the primary tumor. In all cases, regional lymph nodes should be carefully evaluated during the pre-surgical physical examination (Sorenmo *et al.*, 2011); for axillary nodes, a cytological evaluation is advised when enlarged and surgery should be performed when metastatic disease cannot be excluded (Misdorp, 2002). In contrast, the superficial inguinal nodes are usually dissected during the gross examination of all the regional caudal or radical unilateral mastectomy specimens and routinely submitted for histological examination (Misdorp, 2002; Sleeckx *et al.*, 2011; Sorenmo *et al.*, 2011). Several authors have confirmed that the histological evidence of metastases in regional lymph nodes at the time of diagnosis is a significant prognostic factor (Hellmen *et al*., 1993; Yamagami *et al*., 1996; Chang *et al*., 2005).

The presence of vascular invasion has been also considered as an evidence of the metastatic potential of malignant CMT (Rasotto *et al.*, 2012). In human

breast cancer, vascular permeation closely correlates with the regional lymph node involvement and local recurrence (Elston *et al.*, 1999). According to some authors, vascular invasion may be a valuable surrogate for the lymph node stage, when nodes were not removed, while others have stressed that vascular invasion adds prognostic information to histological grade and tumor size in women with node negative breast carcinoma (Elston *et al.*, 1999; Lee *et al*., 2006). In malignant CMT, the presence of neoplastic emboli in vessels also has an independent role in survival (Sarli *et al.*, 2002), and recently it was reported that vascular invasion (associated or not with regional lymph node metastases) was associated with short survival times (Santos *et al.*, 2014). Therefore, it is reasonable to assume that the assessment of vascular/lymph node invasion at the time of diagnosis may be a valuable estimator of the tumor metastatic capacity (Rasotto *et al.*, 2012; Rasotto *et al.*, 2014; Santos *et al.*, 2014).

Whilst no study has ever reported a prognostic index in malignant CMT, it has been suggested that a combination of factors could strengthen the prognostic information (Peña *et al.*, 1998; Sarli *et al.*, 2002). Moreover, a comprehensive evaluation of the individual prognostic value of each NHG grading parameter in CMT has never been performed, as far as we know. Therefore, in this study we aimed to: 1) evaluate the prognostic value of NHG in a cohort of female dogs with malignant CMT; 2) analyze each of the three grading parameters regarding their individual prognostic impact; and 3) compute an adaptation of NPI and assess its sensitivity and specificity to predict post-surgical disease progression in malignant CMT.

## 4.2 Material and methods

### *4.2.1 Selection of cases, histological analysis and follow-up*

A cohort of fifty nine female dogs with spontaneous malignant CMT treated at ICBAS-University of Porto was retrospectively selected. Dogs included in this study underwent surgery as the only treatment. Owners provided consents for surgery with curative intents and 2 years follow-up, as well as for the use of the material for research purposes. Of these female dogs, forty nine were selected based on the presence of a single malignant tumor (Matos *et al.*, 2012). In the subgroup of 10 dogs with multiple malignant tumors, a reference lesion was

assigned in accordance to published approaches for synchronous breast cancers and CMT (Beckmann *et al.*, 2011; Santos *et al.*, 2011; Schmid *et al.*, 2011; Santos *et al.*, 2013). In brief, the reference lesion was considered as the tumor presenting vascular invasion (primary criterion) or the one with largest diameter (secondary criterion). The selection of cases and their histological study were blinded to survival outcomes, thus following the recent guidelines in veterinary oncology (Webster *et al.*, 2011). Dogs with distant metastases at the time of the diagnosis were excluded.

The histological diagnosis was reviewed by two pathologists (MS and PDP) using the criteria of the WHO classification (Misdorp *et al.*, 1999). For each case, the tumor size and the histological evidence of vascular invasion (defined as the presence of tumor emboli within endothelial-lined spaces irrespective of the type and number of invaded vessels) and/or regional lymph node metastases [lymph nodes were evaluated in routine slides and after immunolabelling with pancytokeratin AE1/AE3 and cytokeratin 14, as previously described by Matos *et al.* (2006a)] at the time of diagnosis were recorded. The tumor size was categorized according to WHO criteria (T1 < 3 cm, T2 3-5 cm and T3 > 5 cm) and to a previous defined cut-off (< 2.9 cm and ≥ 2.9 cm) (Santos *et al.*, 2014). The NHG (Fig. 1) was performed by 2 pathologists and, when discrepancies occurred on a particular parameter, a consensus was reached. Only epithelial cells were considered for NHG.

NPI was adapted from (Haybittle *et al.*, 1982), and computed as: NPI = [tumor size (cm) x 0.2] + NHG (1, 2 or 3 respectively for I, II and III grades) + evidence of vascular invasion and/or regional lymph node metastases (1 or 2, respectively if absent or present).

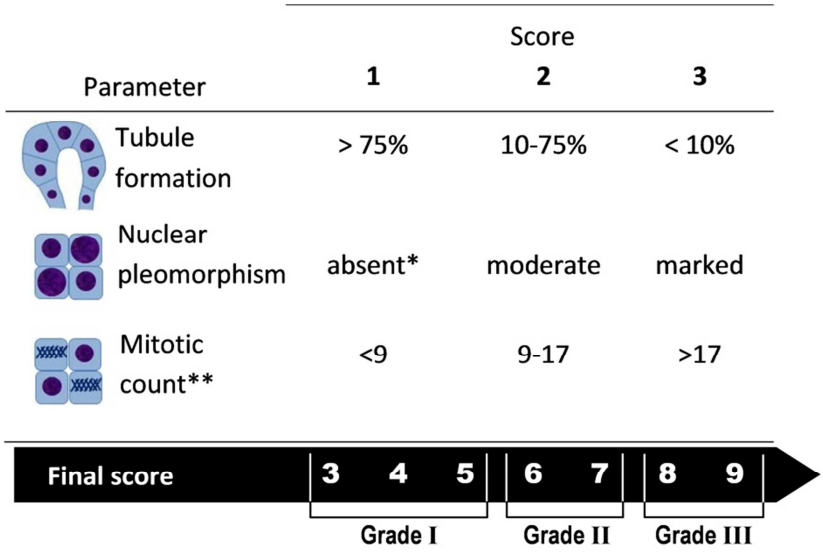


**Fig. 1** – Nottingham histological grade method: criteria for scoring each grading parameter (adapted from Elston and Ellis, 1998). *cells similar to normal surrounding parenchyma; ** counted in 10 high power fields (400x), field diameter of the microscope = 0.55 mm.

The schedule and the protocol of clinical evaluations before the surgery and during the follow-up period were performed as previously described (Santos *et al.*, 2013). The disease-free interval (DFI) was calculated from the date of surgery to the diagnosis of disease progression (recurrence or metastasis, with cytological or histological confirmation). Overall survival (OS) was calculated from the date of surgery to the date of animal death/euthanasia due to metastasis. Animals that died or were euthanized for unrelated causes and those that were lost to follow-up were censored, respectively, at the time of death and at the data of their last follow-up examination. Complete necropsies were performed, after the owner consent, in all the dogs that died spontaneously or were euthanized, and suspected metastatic lesions were confirmed by histopathological examination.

### *4.2.2 Statistical analysis*

The association between histological subtypes and grade was assessed using the Fisher test. Disease-specific survivals were determined using Kaplan-Meier product-limit estimates, with log-rank (Mantel-Cox) tests used to estimate differences in survival fractions according to the NHG (I, II and III) and the score of each grading parameter (1, 2 and 3). A similar approach was performed

considering the final combined grading scores (3 to 9), used to categorize tumors of low grade (final score ≤ 6) and of high grade (final score ≥ 7). Regarding mitotic score, we also considered tertile values (or 3-quantiles, *i.e.,* the two thresholds that divide the ordered distribution of mitotic counts values into three parts) as alternative cut-offs [*i.e.*, scores 1, 2 and 3, respectively for < 11, 11-23 and > 23 mitotic figures per 10 high-power field (each field with a diameter of 0.55 mm)]. Receiver operating characteristic (ROC) curves were used to assess the sensitivity and specificity of the veterinary-adapted NPI for predicting tumor progression at 12 months post-surgery. Optimal cut-off was defined according to the maximization of sensitivity and specificity. Using the optimal cut-off, Kaplan-Meier curves were generated and compared using the Log-rank test. Cox hazard's regression model was used to evaluate the independent prognostic role of various pathological factors in a multivariable analysis. As vascular invasion, tumor size and NHG were components of veterinary-adapted NPI, when this index was included in the multivariable analysis their components were excluded from the model, in order to avoid colinearity.

In all cases, a *P* value < 0.05 was considered significant. Statistical analyses were performed with R Development Core Team software, version 2.7.1 (Vienna, Austria) and IBM SPSS Statistics, version 22 (IBM, New York, USA).

### 4.3 Results

Fifty nine female dogs aged from 6 to 18 years (mean of 10.9 years) were included. Of these, 38 animals presented simple carcinomas (18 tubulopapillary, 17 solid, 1 anaplastic, 1 squamous cell and 1 mucinous carcinoma), 18 had complex carcinomas and 3 had carcinosarcomas. The largest diameter of the tumors ranged from 0.5 to 15 cm, being equal or larger than 2.9 cm in 26 cases (44%). According to the WHO tumor size system, 35 were T1, 11 were T2 and 13 were T3. At the time of diagnosis, 17 cases (29%) presented vascular/regional lymph node invasion. Post-surgical progression was diagnosed in 17 cases (in 14 out 17 the diagnosis of recurrence and/or metastases occurred within the first 12 months of the follow-up period). Tumors that progressed included different histological subtypes: 7 solid carcinomas, 5

complex, 3 tubulopapillary, 1 anaplastic and 1 carcinosarcoma. During the follow-up, 14 dogs (24%) died or were euthanized due to progressive disease, 29 (49%) were alive and free of disease 24 months after the surgery, whilst 16 dogs (27%) were censored before the end of the follow-up period, being lost to follow-up or died from non-malignancy-related causes (in these, the median follow-up time was 13 months).

Animals older than 10.9 years (median age in this series) had increased risk for developing recurrence and metastases *(P* = 0.01). The WHO category of tumors T1 < 3 cm was associated with better survival times, but no significant difference seemed to exist between the two other size categories regarding DFI (Fig. 2).

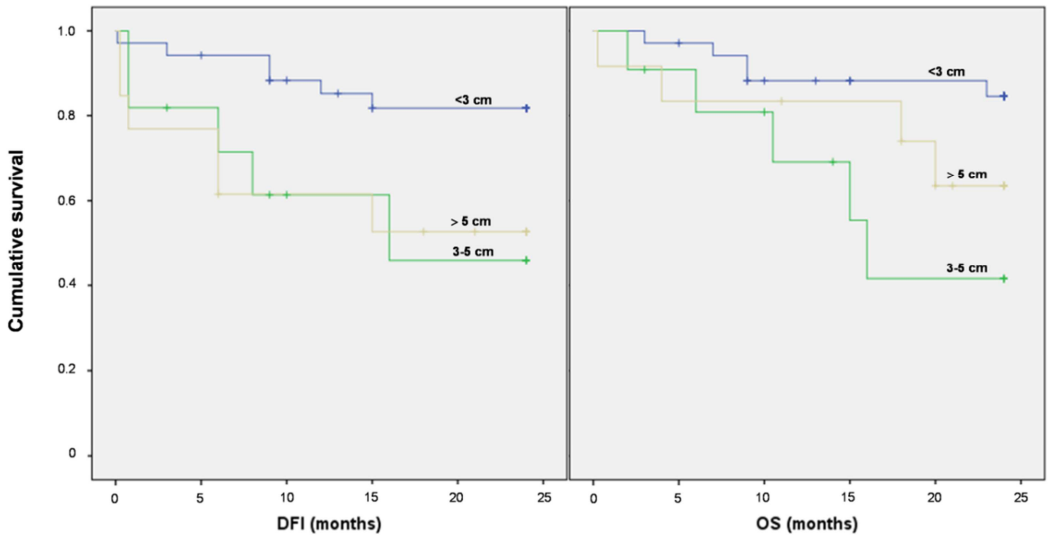


**Fig. 2** – Kaplan-Meier curves of disease-free interval (DFI) and overall survival (OS) of cases included in each WHO tumor size category. Female dogs with tumor largest diameter < 3 cm had better survival outcomes but no statistical difference seemed to exist between the other two categories regarding DFI (log-rank test, *P* = 0.03 for DFI and *P* = 0.04 for OS). Censoring is indicated by vertical marks.

The cut-off 2.9 cm allowed a stratification of tumors with significant differences in survival: size < 2.9 cm had 21 and 22 months of DFI and OS, respectively, whereas size ≥ 2.9 cm had 15 and 18 months of DFI and OS (*P* = 0.009 for DFI and *P* = 0.02 for OS) (Fig. 3). The evidence of vascular invasion and/or regional lymph node metastases at the time of diagnosis was associated with shorter DFI and OS (*P* < 0.0001).

Regarding the NHG, 14 (24%) tumors were grade I, 22 (37 %) grade II and 23 (39%) were grade III. A final combined score ≤ 6 (considered as low grade tumors) was computed in 22 cases (38%) whilst 37 (62%) cases had a combined score ≥ 7 (considered as high grade tumors). The distribution of grades in each histological subtype and the scores of NHG parameters are presented in Table 1 and 2, respectively. An association between the histological subtypes and grade was observed (*P* = 0.01). Notably, all the solid carcinomas and carcinosarcomas were graded II and III. However, no significant association existed between histological subtypes and survival, even when the simple carcinomas were compared with all the other groups together.

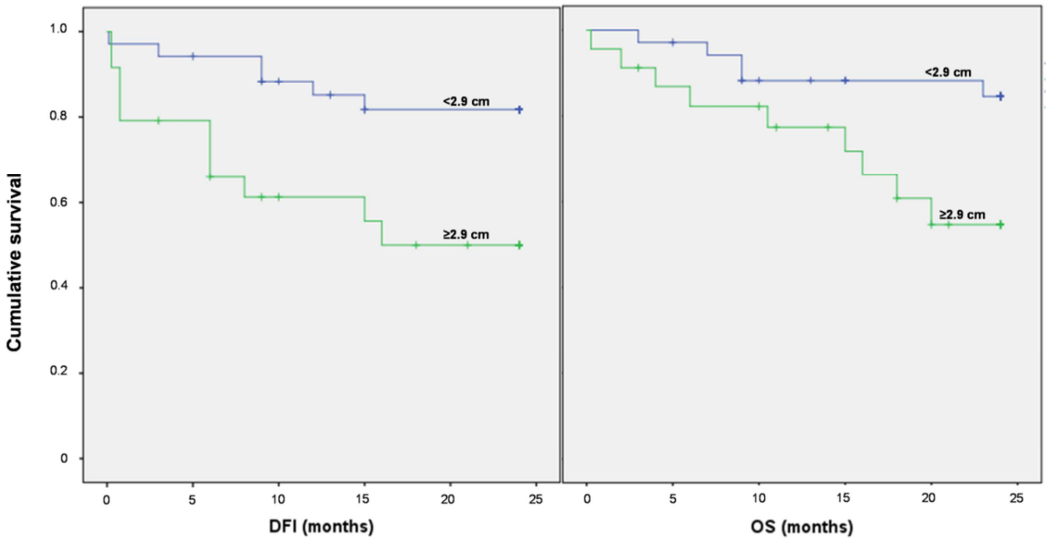


**Fig. 3** – Kaplan-Meier plots comparing the disease-free interval (DFI) and overall survival (OS) according to tumor largest diameter. Cases with ≥ 2.9 cm were associated with poor survival (log-rank test, *P* = 0.009 for DFI and *P* = 0.02 for OS). Censoring is indicated by vertical marks.

**Table 1** – Distribution of grades by the histological subtypes of 59 malignant canine mammary tumors.

| Histological subtype | Grade | | | Total |
|---|---|---|---|---|
| | I | II | III | |
| TP | 6 | 9 | 3 | 18 |
| Solid | 0 | 4 | 13 | 17 |
| Complex | 8 | 5 | 5 | 18 |
| Carcinosarcoma | 0 | 2 | 1 | 3 |
| Others* | 0 | 2 | 1 | 3 |
| Total | 14 | 22 | 23 | 59 |

*anaplastic (1), squamous cell (1) and 1 mucinous carcinoma (1)

DFI and OS were significantly related to NHG ($P$ = 0.002 and $P$ = 0.02, respectively) (Fig. 4). The mean DFI in grades I, II and III was 23, 21 and 14 months, respectively, whereas their mean OS was 24, 21 and 17 months. When evaluating the DFI, the prognosis strength of a two-tier classification (high and low grades determined by the final score) was slightly lower ($P$ = 0.04) than that of the original three-tier system of NHG ($P$ = 0.001), and that classification was not significantly associated with OS ($P$ = 0.1). When cases graded I and II were grouped and compared with those of grade III, the former presented significantly longer DFI ($P$ < 0.0001) and OS ($P$ = 0.008) (Fig. 5).

**Table 2** – Scoring (and % of distribution) of the each grading parameter of the Nottingham histological grading method in 59 malignant canine mammary tumors.

| Parameter | Score | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Tubule formation | 11 (19%) | 29 (49%) | 19 (32%) |
| Nuclear pleomorphism | 5 (9%) | 32 (54%) | 22 (37%) |
| Mitotic count | 15 (25%) | 15 (25%) | 29 (50%) |

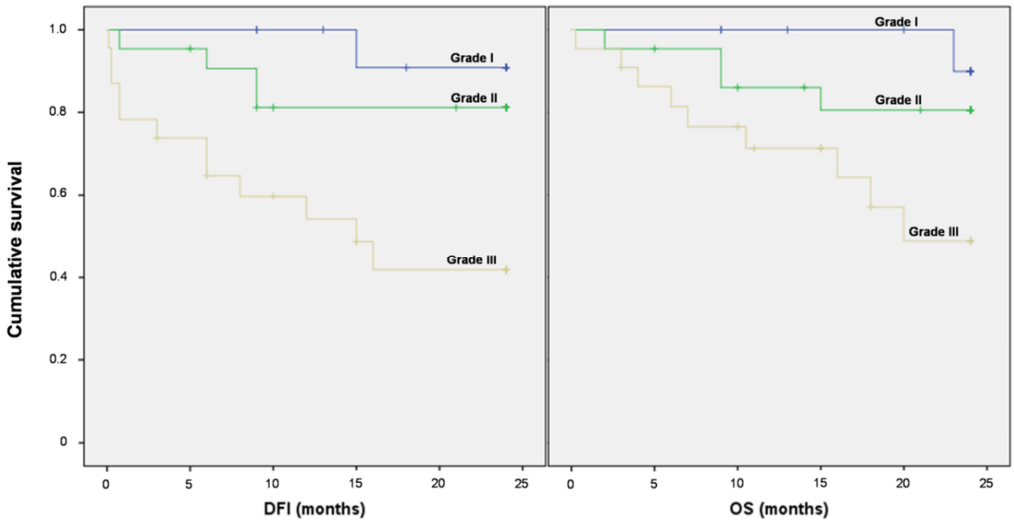


**Fig. 4** – Disease-free interval (DFI) and overall survival (OS) of female dogs with grade I, grade II and grade III mammary malignant tumors. Significant differences existed between the three curves (log-rank test, *P* = 0.002 for DFI and *P* = 0.02 for OS). Censoring is indicated by vertical marks.

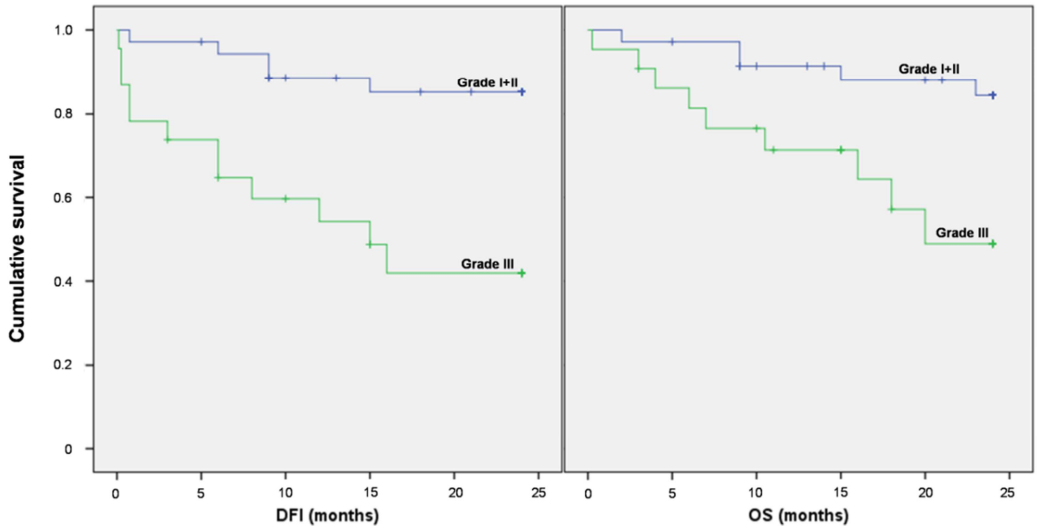


**Fig. 5** – Kaplan-Meier analyses of disease-free interval (DFI) and overall survival (OS) among 59 female dogs with malignant mammary tumors. Animals with tumors graded I or II had a significant longer DFI and OS when compared with animals with tumors graded III, according to Nottingham histological grading method (log-rank test, *P* < 0.0001 for DFI and *P* = 0.008 for OS). Censoring is indicated by vertical marks.

Among the NHG components, nuclear pleomorphism emerged as a statistical significant predictor of the outcome, with longer OS (*P* = 0.001) and DFI (*P* < 0.0001) in animals bearing tumors with nuclear pleomorphism scores of 1 or 2, as opposed to score 3 (Fig. 6). Tubule formation and mitotic counts had no

prognostic significance, even when scores 1 and 2 were grouped and compared to score 3 or when score 1 was compared to scores 2 plus 3. No association with outcome was observed when tumors were scored according to the tertile values of the mitotic counts.

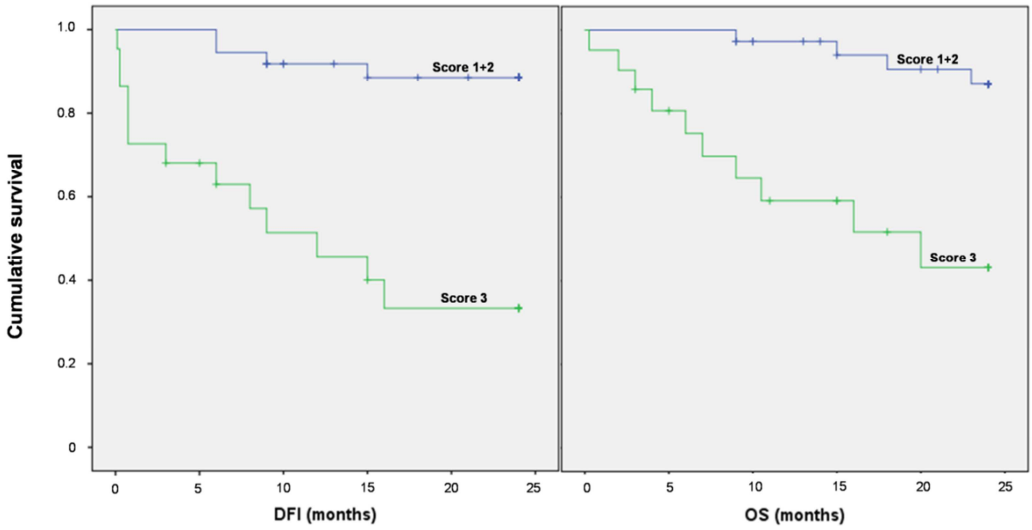


**Fig. 6** – Disease-free interval (DFI) and overall survival (OS) curves of female dogs with tumors scored 1 or 2 in nuclear pleomorphism compared to female dogs with tumors scored 3 in this parameter. Nuclear pleomorphism was significantly associated with survival (log-rank test, $P < 0.0001$ for DFI and $P = 0.001$). Censoring is indicated by vertical marks.

The mean veterinary-adapted NPI was 4.2 ± 1.4, ranging from 2.1 to 7. According to the ROC curve results, the performance of this index to identify tumors progressing in the first 12 months post-surgery was moderately accurate as the area under the ROC curve (AUC) was 0.80 (95% CI: 0.68 - 0.92) (Fig. 7) (Greiner *et al*., 2000). An optimal veterinary-adapted NPI cut-off value of 4.25 was defined based on the maximal pair of sensitivity and specificity values (sensitivity of 88% and specificity of 67%). High cut-off levels were associated with high specificity, with an obvious decrease of sensitivity (for instance, a veterinary-adapted NPI cut-off of 5.3 was associated with 91% specificity and 47% sensitivity). In order to perform a survival analysis, cases were grouped by the previously determined veterinary-adapted NPI cut-off value. Cases with a veterinary-adapted NPI ≥ 4.25 were associated with poorer outcome (Fig. 8).

Taking DFI and OS as dependent variables and selecting a set of independent pathological variables including tumor size (either defined as a continuous variable or with categories defined by the 2.9 cm cut-off), vascular invasion, nuclear pleomorphism (score 1 plus 2 *versus* score 3), and NHG (I plus II *versus* III) — the multivariable Cox hazard's regression selected two independent covariates, in decreased order of significance: 1) nuclear pleomorphism ($P < 0.0001$ for DFS and $P = 0.002$ for OS; 2) vascular invasion ($P = 0.01$ for DFS and $P = 0.03$ for OS). When veterinary-adapted NPI and nuclear pleomorphism were used as covariates on the Cox regression model, the two variables were independent predictors of survival.

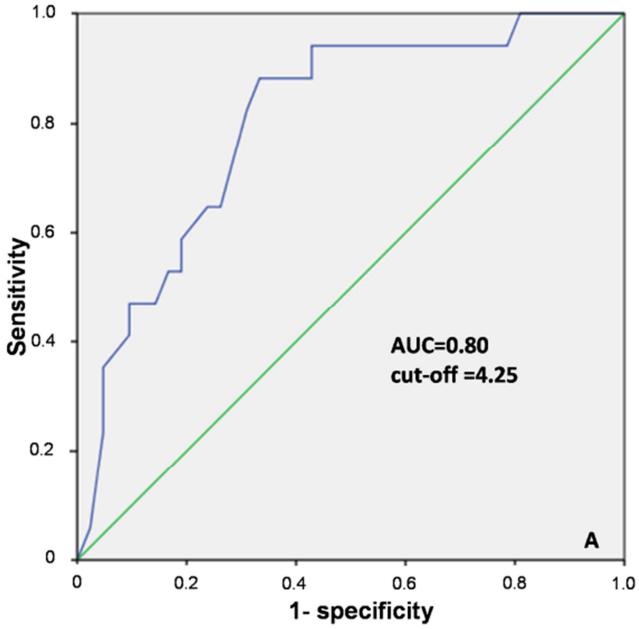


**Fig. 7** – Receiver operating characteristic (ROC) curve for tumor progression in 59 female dogs based on the veterinary-adapted Nottingham prognostic index (NPI). The NPI had good discriminative power between tumors that progressed within the first 12 months post-surgery and those that did not progressed.

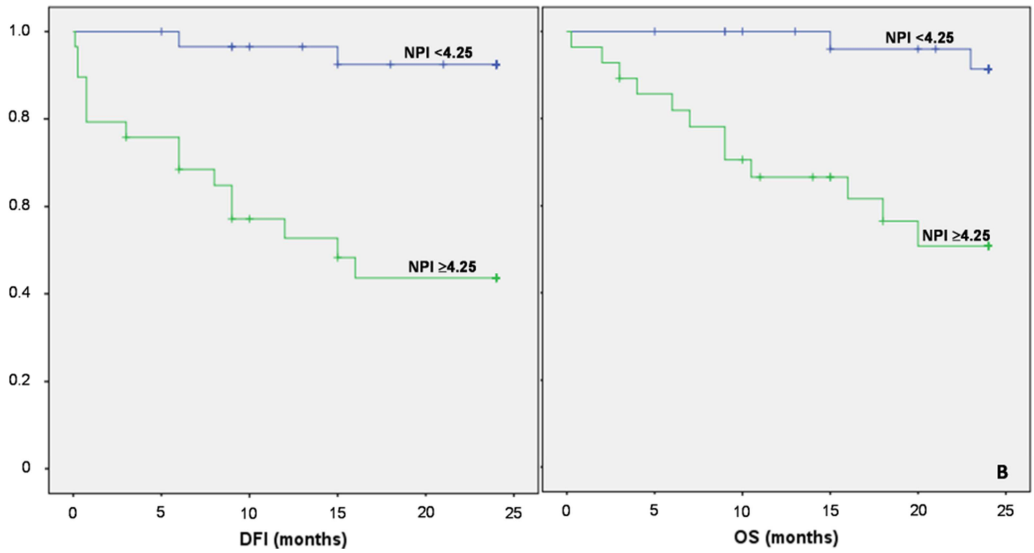


**Fig. 8** – Kaplan-Meier plots of disease-free interval (DFI) and overall survival (OS) among 59 female dogs with malignant mammary tumors grouped by the veterinary-adapted NPI cut-off. NPI ≥ 4.25 was associated with poor survival outcomes (log-rank test, $P < 0.0001$ for DFI and $P = 0.001$ for OS curves). Censoring is indicated by vertical marks.

### 4.4 Discussion

When examining a malignant CMT, the pathologist is expected to provide relevant information with prognostic significance. This goal could be achieved by tumor grading, since it is known for long that the microscopic appearance of tumors and their biological behavior are associated (Greenough, 1925; Elston *et al.*, 1999). In the latest years, the NHG (with or without modifications) has been applied for malignant CMT grading by several investigators (Karayannopoulou *et al.*, 2005, Matos *et al.*, 2006, Clemente *et al.*, 2010, Gama *et al.*, 2010, Santos *et al.*, 2011, Rasotto *et al.,* 2012, Manuali *et al.*, 2012, Guimarães *et al.*, 2014, Im *et al.,* 2014, Mainenti *et al.*, 2014), but data regarding its prognostic value is still relatively limited, namely when comparing to the data in human breast cancer.

This study analyzed the relation between the NHG and survival in a cohort of animals with malignant CMT and, to the best of our knowledge this is the first study where the association between survival and each parameter of the NHG was systematically assessed. In our cohort, a high proportion of tumors were graded II and III, which is in line with previous studies (Gama *et al.*, 2010; Santos *et al.*, 2013). In contrast, other survival studies had a higher proportion

of grade I tumors (Karayannopoulou *et al.*, 2005; Peña *et al.*, 2013; Mainenti *et al.*, 2014). Since the selection of cases was completely blinded to histological features and follow-up data, it is likely that the high proportion of grades II and III tumors represents an intrinsic feature of this cohort. At the same time, it should be stressed that in our cohort there was an overrepresentation of female dogs with a single malignant CMT and, eventually, this could represent a bias of selection. This should not affect our results, because solitary and multiple tumors have no significant differences in survival (Hellmén *et al.*, 1993; Philibert *et al.*, 2003). In fact, the prevalence of tumor progression (29%) and cancer-associated death (24%) observed in our study are within the published range of values (Hellmén *et al.*, 1993; Karayannopoulou *et al.*, 2005; Sassi *et al.*, 2010; Peña *et al.*, 2013).

Herein, the histological type and NHG were significantly associated, corroborating previous studies (Karayannopoulou *et al.*, 2005; Peña *et al.*, 2013; Im *et al.*, 2014). Such association has been justified with the evaluation of tubule formation that is typically scored 3 in solid carcinomas — a histological type often associated with a worse prognosis (Peña *et al.*, 2013).

Regarding the NHG, it was associated with disease progression and cancer-related death, especially when grade I and II tumors were compared to grade III; this is also in line with previous findings (Karayannopoulou *et al.*, 2005; Santos *et al.*, 2013). However, in this study we assessed for the first time the role of each NHG parameter. In this regard, only nuclear pleomorphism stood as relevant, with a significant differences in survival between animals bearing tumors scored 1 plus 2 and those affected by score 3. Notably, the multivariable survival analysis also highlighted the nuclear pleomorphism as an independent prognostic factor, in detriment of NHG. This suggested that only two scores of nuclear pleomorphism should be considered for an accurate prognostic assessment of malignant CMT. The use of a two-tier system for classifying nuclear pleomorphism could also solve the modest interobserver agreement associated with the use of NHG three scores criteria (Santos *et al*., 2014). Our results also suggested that tubule formation and/or mitotic counts may dilute rather than strengthen the prognostic value of the NHG. The absent relation between mitotic counts and patient outcomes corroborates previous findings

(Sarli *et al.*, 2002; Santos *et al.*, 2013), although it seems to contradict other reports, where higher proliferative activities have been associated with poorer outcomes (Peña *et al.*, 1998; Nieto *et al.*, 2000; Sarli *et al.*, 2002; Matos *et al.*, 2006b). However, the latter studies used immunohistochemistry techniques for the detection of proliferating markers (*e.g.*, Ki-67 and PCNA) rather than mitotic counts. In routinely stained slides it may be difficult to identify mitotic figures, particularly in tumors with high apoptotic activity or in those with large areas of necrotic tissue (Ladekarl, 1998; Meyer *et al.*, 2005). Furthermore, the mitotic count of the NHG is largely dependent on the sampling strategy (selection of high power fields by the observer) and the cellularity of the tumor (Ladekarl, 1998). Considering that the distribution of the mitotic count scores was skewed (half of the cases were score 3), we also analyzed this feature with tertiles boundaries — following an approach previously used in human and veterinary pathology (Frkovis-Grazio and Bracko, 2002; Mills *et al.*, 2015). Nevertheless, such analysis also failed to improve the separation of survival plots obtained with the original thresholds of NHG.

Tubule formation is closely related with the differentiation of epithelial cells (Misdorp, 2002) and with the histological diagnosis (Peña *et al.*, 2013). To the best of our knowledge, no other study ever assessed the association between tubule formation and prognosis in malignant CMT. In the veterinary literature, only a recent study devoted to feline mammary carcinomas reported a weak association between tubule formation and survival (Mills *et al.*, 2015).

In this cohort of dogs we also assessed the NPI — to the best of our knowledge this is the first report of its use in malignant CMT. Notably, this index when adapted to malignant CMT exhibited a strong discriminative power for identifying cases which progress in the first 12 months after surgery. Therefore, it seems to be useful for malignant CMT, and it could also be included in the pathologist report. In our case, we introduced some modifications to the original NPI: considering that vascular invasion was as an independent prognostic factor in our cohort, we surrogated the lymph node stage from the original formula, by the histological evidence of vascular invasion and/or regional lymph metastases; all the remaining items of veterinary-adapted NPI (size and NHG) were maintained. It should be noted that the influence of tumor size on survival

is well acknowledged (Sorenmo *et al.*, 2011). Even if we showed the utility of a veterinary-adapted NPI, we anticipate that further modifications to the NPI could be introduced. According to our multivariable analysis, the inclusion of nuclear pleomorphism could increase its prognostic value. At this point, we already highlighted the value of this index, but a formula more fitted to malignant CMT must be validated, and perhaps upgraded by larger prospective studies.

In conclusion, this study demonstrated that the NHG is associated with survival outcomes in malignant CMT. Regarding its components, nuclear pleomorphism was the only parameter associated with survival, retaining its prognostic significance in multivariable analysis. We also showed that a veterinary-adapted NPI provided valuable data for the prediction of post-surgical disease progression. It is suggested that nuclear pleomorphism should be classified as two-tier system and its score should be included in routine pathological reports. Efforts would be helpful to further validate the use of veterinary-adapted NPI (or other similar prognostic indices) in malignant CMT, so that adjuvant therapies could be assigned to properly selected cases, similar to the current standards in human breast cancer care.

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# CHAPTER 5

## STEREOLOGICAL ESTIMATION OF A CELLULARITY-RELATED PARAMETER IN CANINE MAMMARY MALIGNANT TUMORS

*[This chapter has been submitted.]*

**Summary**

Grading of canine mammary carcinomas (CMC) is associated with subjective assessments made by the pathologists. Due to its unbiased nature, stereology can be used to objectively quantify morphological parameters associated with grading and malignancy. However, the use of stereology in CMC has not been fully disclosed. The nuclear numerical density [$N_V$ (nuclei, tumor)] is a cellularity-associated parameter that can be estimated by the optical disector. Herein, it was estimated in 44 CMC and its association with clinicopathologic factors — such as tumor size, histological subtype and grade, vascular/lymph node invasion, nuclear pleomorphism and survival — was evaluated. Considering all the cases, the mean $N_V$ (nuclei, tumor) was $1.6x10^6 \pm 0.5x10^6$ nuclei $mm^{-3}$. Lower values were attained in complex carcinomas, comparing to simple carcinomas, in tumors smaller than 5 cm, with low mitotic activity and in those with high nuclear pleomorphism. No statistically significant association with grade or vascular/lymph node invasion was observed, but tumors with disease progression had lower nuclear numerical densities. The $N_V$ (nuclei, tumor) and the correlated parameters fairly mirror those in human breast cancer, suggesting an interesting interspecies agreement. This first estimation of the nuclear numerical density in CMC highlights the feasibility of the optical disector and their utility for objective morphological assessments in CMC. The association between nuclear numerical density and disease progression warrants future studies.

## 5.1. Introduction

The level of knowledge in canine mammary carcinomas (CMC) has increased considerably in recent years, with breakthroughs in prognosis assessment. Several putative prognostic factors have been pointed (Sleeckx *et al*., 2011), but it is still recognized that the definition of prognosis in CMC is difficult due to the marked clinical as well as morphological heterogeneity (Matos *et al*., 2012).

The emphasis on highly sophisticated techniques in oncology and in the so-called *omics* has led to an overlooking of tumor morphology, at least to some extent (Pinder *et al*., 1995). Still, it is consensual that the histopathological assessment of tumor features is somehow subjective and this can jeopardize the biological conclusions, namely in terms of prognosis, which can be retrieved from the study of CMC (Sørensen, 1992). Such subjectivity may be overcome by quantitative morphological parameters assessed by suitable morphometrical or stereological methods (Marcos *et al*., 2012). These methods are substantially different: while morphometry describes quantitatively what is seen in conventional sections [at the microscope or in two-dimensional (2D) images], using a caliper and sometimes benefiting from image-analysis software, stereology uses probes or test-systems in 2D images or virtual optical z-planes, aiming to obtain the three-dimensional (3D) information inherent of all biological tissues (Geuna, 2005; Marcos *et al*., 2012). Stereology can be used in histological sections of tumors, allowing objective and unbiased estimates (in relation to the 3D reality) of many parameters, such as absolute or relative volumes of the cells or their nuclei and numerical nuclear densities (Sørensen, 1992; Ladekarl, 1998).

Stereological studies have been performed in human breast cancer and estimates of nuclear volumes (volume and number-weighted mean nuclear volumes) and of numerical density ($N_V$) of nuclei and mitotic figures have been correlated with prognosis (Sørensen, 1992; Ladekarl and Sørensen, 1993a; Artacho-Pérula and Roldán-Villalobos, 1997; Ladekarl, 1998). In CMC, the use of stereology is still very incipient (Casteleyn *et al*., 2014), but it already started to solve issues related with the subjective assessment of nuclear pleomorphism in grading of CMC (Santos *et al*., 2014).

The relative proportion of neoplastic glandular structures, the so-called tubule formation is one parameter of the NHG, being scored 1, 2 or 3, respectively,

when more than 75, 10-75% or less than 10% of neoplastic cells are arranged in ductal structures, with an obvious lumen (Elston and Ellis, 1991). It has been suggested that highly cellular CMC, *i.e*., solid subtypes, are associated with a poorer prognosis comparing with tubulopapillary tumors (Misdorp, 2002; Sorenmo, 2003). However, cellularity assessed by pathologists tends to be purely qualitative (high *versus* low) and may be highly subjective. To the best of our knowledge, a quantitative evaluation of a cellularity parameter, such as the $N_V$, has never been performed in CMC. Such an evaluation can be performed by the optical disector (Sterio, 1984; Geuna, 2005). Instead of counting nuclear cell profiles, which not only depend on the cell number but also on the size, shape, and spatial orientation and distribution of nuclei, the disector uses a 3D counting cube with inclusion and exclusion sides that allows counting nuclei in proportion to their real number (Marcos *et al*., 2012; Gundersen *et al*., 2013).

The primary aims of this study were to estimate the $N_V$ (nuclei, tumor) in CMC and their relation with other clinicopathological parameters, namely tumor size, histological diagnosis, vascular/lymph node invasion and Nottingham histological grade (NHG) parameters (*i.e.*, tubule formation, nuclear pleomorphism and mitotic count). Ultimately we intended to evaluate the prognostic utility of the $N_V$ (nuclei, tumor) in CMC.

## 5.2 Material and methods

### *5.2.1 Selection of cases and histological analysis*

Forty four spontaneous CMC treated at veterinary clinics of ICBAS-University of Porto were retrospectively selected, blinded to clinical and other pathological data. The animals were submitted to surgical resection of the tumors with the owner's consent. Follow-up data were collected over two years following the protocol detailed in Santos *et al.* (2013) was available for twenty seven cases. The histological diagnosis and grading was reviewed by two pathologists (MS and PDP) using the criteria of the World Health Organization classification (Misdorp *et al*., 1999) and the NHG (Elston and Ellis, 1991). For this, routine 4-5 μm thick sections resulting from the largest cross slab of the tumor (slab with on average 0.5 cm in thickness) were retrieved and screened. For every case, the tumor size and the histological evidence of vascular invasion and/or regional

lymph node metastases [lymph nodes were evaluated in routine slides and after immunolabelling with pancytokeratin AE1/AE3 and cytokeratin 14, as previously described by Matos *et al.* (2006)] were recorded. As to tumor size, it was categorized according to WHO criteria (T1< 3 cm, T2 = 3-5 cm and T3 > 5 cm).

*5.2.2 Sectioning and stereological analysis*

Of every case, a thick section (30 µm thick) from all the paraffin blocks was obtained. To avoid chatter, the surface of the paraffin block was warmed (by breathing on) immediately before cutting each section. After being picked from the water-bath, the sections were covered with a cotton cloth and gently pressed against the slide with a finger, for ensuring adhesion. The sections were mounted on precleaned slides primed with aminopropyltriethoxy-silane. Finally, sections were dried overnight at 37°C and then stained with hematoxylin-eosin.

For the stereological analysis we used a workstation comprising: 1) a microscope (Olympus BX-50, Japan) equipped with a 100x oil-immersion lens (Olympus Uplan NA = 1.35) and a matching condenser; 2) a microcator (Heidenhain MT-12, Traumrent, Germany), to control the movements and position in the Z-direction (0.5 µm accuracy); 3) a motorized stage (Prior, United Kingdom) for stepwise displacement in the x–y directions (1 µm accuracy); 4) a CCD video camera (Sony, Tokyo, Japan) connected to a 17″ PC monitor (Sony); and 5) a computer with a stereology software (CAST-Grid, version 1.5, Olympus, Denmark). At the monitor, a final magnification of 4750x allowed an accurate recognition of the nuclei of the neoplastic cells. The first field of vision was randomly selected by the software. Thereafter, fields were sampled systematically by stepwise movements of the stage in the x- and y-directions, so that a minimum of 40 fields were examined per tumor. Throughout the disector height — 16 µm, *i.e.,* Z-distance used for counting within the 30 µm thick section — a software generated counting frame was superimposed, having a defined area of 253 $\mu m^2$ and inclusion and forbidden lines (Fig. 1), to prevent the edge effect counting biases (Gundersen, 1977).

Nuclei were counted when two conditions were met: (1) at the plane of focus, they were within the counting frame or touching the inclusion lines and not touching the forbidden lines or their extensions; (2) the rim of the nucleus was in

perfect focus at a plane below 4 µm and above or equal to 20 µm in the Z-axis (Fig. 1). The potential bias from lost caps was avoided by having upper and lower guard heights (of 4 and 10 µm, respectively) (Marcos *et al.,* 2012). Spindle-shaped nuclei were excluded from the counts.

The $N_V$ (nuclei, tumor) was estimated using the formula (Gundersen *et al*., 1988):

$$N_V\,(\text{nuclei, tumor}) = \Sigma Q^{-}/[h \times a(\text{frame}) \times \Sigma P]$$

where $\Sigma Q^{-}$ corresponded to the total number of neoplastic cells counted in the sampled fields, and a(frame), *h* and ΣP were, respectively, the area of the counting frame, height of the disector and the total number total number sampled fields within the reference space. Since the reference space defined was the parenchyma of the tumor, fields that were empty, containing large vessels, stroma, or necrotic areas were excluded. The coefficient of error (CE) of the estimations of $N_V$ (nuclei, tumor) was determined using the formula (Gundersen *et al*., 2013):

$$CE(N_V) = \sqrt{\frac{\Sigma u^2}{\Sigma u.\Sigma u} + \frac{\Sigma v^2}{\Sigma v.\Sigma v} - 2\frac{\Sigma u.v}{\Sigma u.\Sigma v}}$$

where *u* and *v* stands for the number of nuclei counted ($Q^{-}$) and total number sampled fields within the reference space (P), respectively.

The CE of the $N_V$ estimations was then compared with the observed relative variance among cases, $OCV^2$, according to the formula (Gundersen *et al*., 2013):

$$OCV^2 = BCV^2 + CE^2(N_V)$$

where $BCV^2$ is the inherent biological relative variance of the $N_V$ in tumors and $CE^2$is the mean square of the individual estimates of the CE of $N_V$.

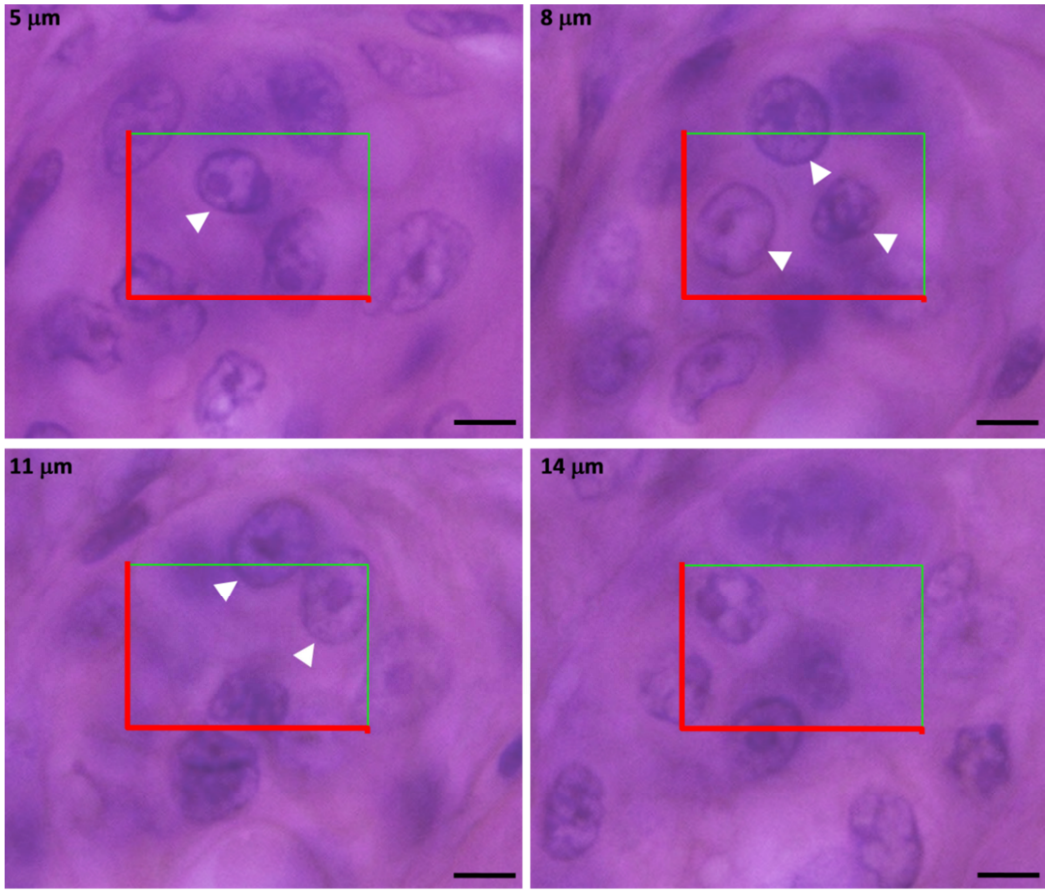


**Fig. 1** – Series of light micrographs from a thick section (30 µm) of a canine mammary carcinoma that form an optical disector (the depth of each optical plane is indicated in the upper left corner). Nuclei of neoplastic cells are counted if they are seen within the counting frame or touching the inclusion (green) lines, but not touching the exclusion (red) lines. In this illustrative field, 6 nuclei are counted (arrowheads); Bar 6 µm.

*5.2.3 Statistical analysis*

To test if the data followed a normal distribution the Shapiro-Wilk and Kolmogorov-Smirnov tests were used. For skewed data, a logarithmic transformation was applied. The associations between the NV (nuclei, tumor) and: 1) NHG grade (grade I, II and III); 2) grading parameters — tubule formation, nuclear pleomorphism and mitotic counts scores; 3) WHO size categories; 4) histological subtypes, were tested with one-way ANOVA, followed by Tukey post-hoc tests. The differences in $N_V$ (nuclei, tumor) in tumors presented or not vascular/lymph node invasion were assessed with the *t*-test for independent samples. Regarding the histological subtype, the tumors were also grouped and compared, as a) simple *versus* complex carcinomas and b) solid carcinomas *versus* all the other subtypes, using the *t*-test for independent

samples with F test for checking the homogeneity of variances. The association degree between the NV (nuclei, tumor) and the volume-weighted mean nuclear volume [previously assessed by point sampled intercepts (Santos *et al*., 2014)] was evaluated by Pearson correlation test. In all cases, a *P* value $< 0.05$ was considered significant. Statistical analyses were performed with the IBM SPSS Statistics, version 22 (IBM, New York, USA).

### 5.3 Results

Thirty out 44 tumors were diagnosed as simple carcinomas (11 tubulopapillary, 16 solid, 2 squamous cell and 1 mucinous) and 14 were complex carcinomas. The tumor largest diameter ranged from 0.5 to 15 cm, being 7 cases T1, 25 cases T2 and 12 cases T3. At the time of diagnosis, 12 cases (27%) presented vascular/regional lymph node invasion. Regarding NHG, 9 cases were grade I, 15 were grade II and 20 were grade III. As to tubule formation, score 1, 2 and 3 were present in seven, 20 and 17 tumors, respectively. Follow-up data was available for 27 female dogs, and during this period, 30% (8/27) of animals presented progression of the disease (defined as recurrence and/or metastases *di novo*). Of the remaining, 55% (15/27) were alive and clinically disease-free at 24 months after the surgery, whilst 15% (4/27) were censored for being lost to follow-up or for non-malignancy-related death.

Regarding the stereological estimation, an average of 259 nuclei was counted per tumor, (≈ 6 cells per disector); the mean section thickness was 28.9 µm (coefficient of variation = 0.1). The mean CE of the $N_V$ (nuclei, tumor) estimations was 0.07 (CE estimations varied from 0.04 to 0.11). This means that the estimation methodology was responsible for 5% of the total observed variance. Therefore, the biological variability was by far the most important component of the observed variability of the $N_V$ (nuclei, tumor) estimations. The mean (SD) and median $N_V$ (nuclei, tumor) were $1.6x10^6$ nuclei $mm^{-3}$ ($0.5x10^6$) and $1.4x10^6$ nuclei $mm^{-3}$, while the minimum and maximum values were $0.8x10^6$ to $3.2x10^6$ nuclei $mm^{-3}$ (Fig. 2).

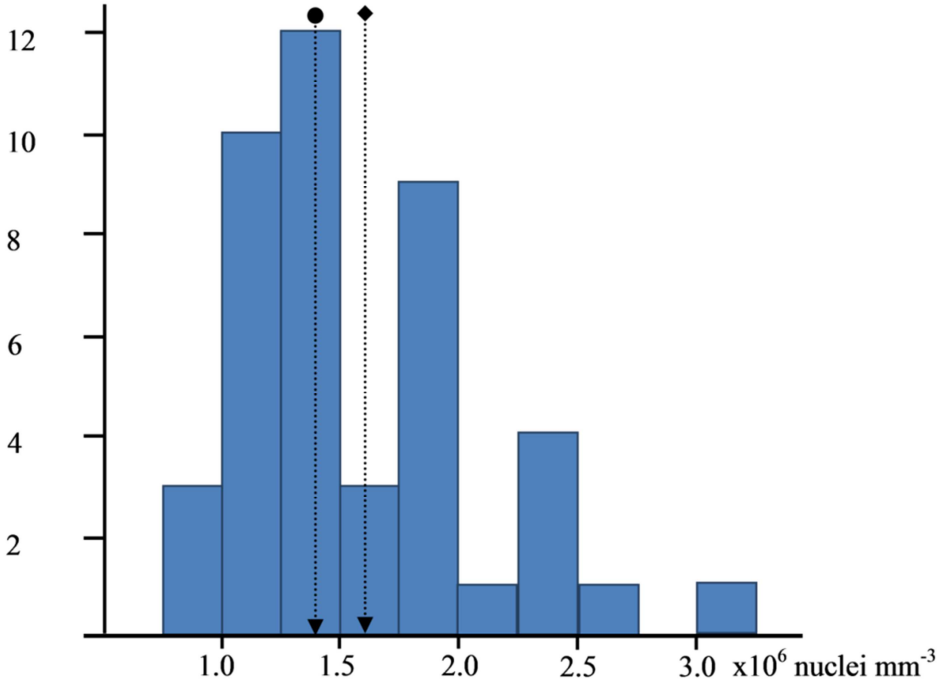


**Fig. 2** – Histogram of the mean $N_V$ (nuclei, tumor) values in the 44 canine mammary carcinomas; lozenge-arrow: mean value; circle-arrow: median value.

The $N_V$ (nuclei, tumor) was significantly higher in simple carcinomas [mean=1.7x10$^6$ (0.5x10$^6$ nuclei mm$^{-3}$)] comparing to complex carcinomas [mean=1.3x10$^6$ (0.2x10$^6$ nuclei mm$^{-3}$)] (*t*-test, *P* = 0.002), but no statistical difference existed when solid carcinomas were compared with the other subtypes (including when compared with the tubulopapillary carcinomas). The $N_V$ (nuclei, tumor) was 1.3x10$^6$, 1.7x10$^6$ and 1.6x10$^6$ nuclei mm$^{-3}$ in grade I, II, III tumors, respectively; with no significant differences. Regarding the NHG parameters (tubule formation, nuclear pleomorphism and mitotic counts), the $N_V$ (nuclei, tumor) did not differ with the tubule formation scoring, but an association with nuclear pleomorphism was observed — tumors scored 3 for nuclear pleomorphism presented lower $N_V$ (nuclei, tumor) compared to tumors scored 2 (Tukey test, *P* = 0.02). Similarly, a significant increase in numeric nuclear density existed from tumors scored 1 or 2 to those scored 3 in mitosis counts (Tukey test, *P* = 0.006 score 1 *versus* score 3 and *P* = 0.013 score 2 *versus* score 3). With respect to tumor size, no difference in $N_V$ (nuclei, tumor) was observed in tumors of each three WHO size categories. However, when tumors larger than 5 cm were compared with smaller ones, the former presented a significant higher $N_V$ (nuclei, tumor) (*t*-test, *P* = 0.03).

The $N_V$ (nuclei, tumor) was weak-to-moderate, negatively and linearly correlated (r = -0.34; *P* = 0.03) to the nuclear size pleomorphism, quantified herein as the volume-weighted mean nuclear volume — *i.e.,* the $N_V$ (nuclei, tumor) tended to be lower in tumors presenting higher nuclear size pleomorphism.

As to vascular/lymph node invasion status, the $N_V$ (nuclei, tumor) was similar in tumors with and without evidence of invasion. On the other hand, the eight cases that showed post-surgical disease progression during the follow-up period tended to have a lower $N_V$ (nuclei, tumor) ($1.4x10^6$ nuclei $mm^{-3}$) when compared with cases without evidence of metastases and/or recurrence ($1.8x10^6$ nuclei $mm^{-3}$) (*t*-test, *P* = 0.047).

### 5.4 Discussion

Studies over the last thirty years have built a consensus on the value of quantification for improving the prognostic value of morphological parameters in malignant tumors (Baak *et al*., 1985; van der Linden *et al*., 1986; Ladekarl and Sørensen, 1993a,b; Ladekarl, 1995; Ladekarl, 2004; Nedergaard *et al*., 2007). Stereological methods not only achieve such quantification, but have additional advantages of unbiasedness and reproducibility (Marcos *et al*., 2012). These have been applied for long in breast pathology (Ladekarl, 1998; Ladekarl, 2004), but their use in the veterinary oncology is still incipient (Casteleyn *et al*., 2014).

In this study we used the optical disector to assess the $N_V$ (nuclei, tumor) in CMC. Notably, the mean value for CMC ($1.6x10^6$ nuclei $mm^{-3}$) was higher (but in the same order of magnitude) than that reported for human breast cancer ($0.4x10^6$ nuclei $mm^{-3}$) (Artacho-Pérula and Roldán-Villalobos, 1997). Interspecies differences may be at the basis of this discrepancy, along with eventual influences of technicalities, particularly variations in definition of the reference space (for example, we excluded stromal areas). Still, our data suggest that CMC present a higher density of nuclei than breast carcinomas. Despite the differences in figures between our and human studies, some observations in breast cancers were fairly mirrored in CMC. In both cases, there was no significant association between $N_V$ (nuclei, tumor) and histological grade, but a significant negative correlation was noted between the $N_V$ (nuclei,

tumor) and the volume-weighted mean nuclear volume — r= -0.34, -0.63 and -0.31, respectively in our study and in two breast cancer studies (Artacho-Pérula and Roldán-Villalobos, 1997; Ladekarl, 2004).

Another interesting finding in both dogs and humans is that cancers with worst survival outcomes had a lower $N_V$ (nuclei, tumor) (Artacho-Pérula and Roldán-Villalobos, 1997). At a first glance, this is an unexpected observation that appears to contradict the traditional concept that highly cellular tumors are associated with poorer prognosis (Misdorp, 2002). However, it should be kept in mind that any numerical density is a relative parameter (*i.e.*, a fraction) that can be influenced by the number of nuclei/cells or by changes in the reference space (*i.e.*, decreases in numerator or increases in the denominator). A decrease in the $N_V$ (nuclei, tumor) can occur in different scenarios, namely when cells get larger, or more distant (*e.g.*, due to an increase in extracellular matrix as it probably occurs in complex carcinomas, or due to the loss of epithelial adhesion), or when an increased nuclear/cellular pleomorphism exists (Fig. 3). The latter is more likely to occur in CMC, since we have previously described that the volume-weighted mean nuclear volume (which, we recall, estimates the nuclear size pleomorphism) was significantly higher in more aggressive tumors (Santos *et al.*, 2014), and herein there is a negative correlation between that volume parameter and the $N_V$ (nuclei, tumor).

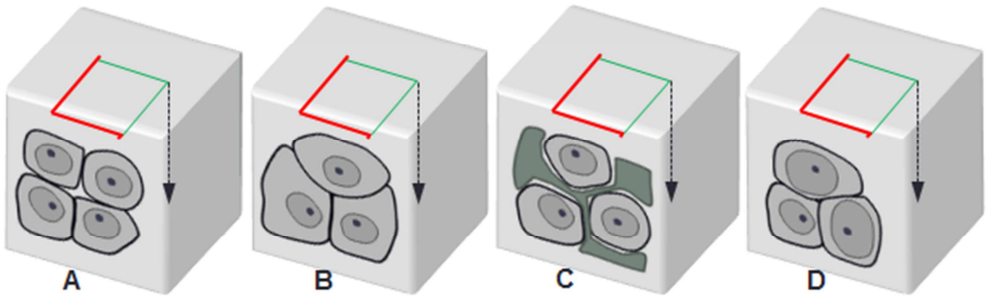


**Fig. 3** – Potential (theoretical) explanations for the changes in the $N_V$ (nuclei, tumor). For the sake of illustration consider a reference space (gray cube) holding particles that are counted through the optical disector (A). From B to D the $N_V$ (nuclei, tumor) decreases through different mechanisms. In (B) cells enlarge, thus few nuclei are counted, whereas in (C) cells are apart, due to extracellular matrix deposition or loss of intercellular adhesion. In (D) cells are highly pleomorphic, some cells are considerably larger, so few nuclei are counted in the disector.

Paraffin shrinkage during tissue processing can influence the reference space and, therefore, lead to overestimations of the $N_V$ (Mandarim-Lacerda, 2003; Marcos *et al*., 2012). Since the cases of this series were handled by the same surgical team, with similar fixation times and processing protocol, it would be reasonable to assume that the shrinkage in x-y would be alike in all cases. In fact, it should be stressed that the possibility of bias related to tissue handling when stereology is applied to routine diagnostic material (as in this study) should not cloud the advantages of stereology over traditional 2D techniques (Kamp *et al*., 2009). These latter are not only affected by shrinkage, but are also severely (uncontrolled) influenced by the shape, orientation and size of the particles (nuclei, cells) being counted (Wanke, 2002; Marcos *et al*., 2012; Gundersen *et al*., 2013).

Herein, we observed that the $N_V$ (nuclei, tumor) was not associated with the qualitative assessment of tubule formation, which is integrated in the NHG. Indeed, we failed to observe differences between solid and tubulopapillary carcinomas regarding this stereological parameter. This supports that the presence of luminal structures in routine sections is not directly correlated with cellularity at 3D level. According to our present data, both solid and tubulopapillary carcinomas are heterogeneous regarding the 3D densities of nuclei; other studies of survival and immunohistochemistry status of solid and tubulopapillary tumors also pointed to the existence of heterogeneity in these groups of CMC (Santos *et al*., 2013; Yoshimura *et al*., 2014). Yet, this study evidenced that complex carcinomas have decreased $N_V$ (nuclei, tumor). A possible explanation for this could reside in the presence of small portions of myxoid matrix, typical of these tumors (Misdorp, 2002). When being surrounded by that extracellular matrix, cells tend to appear separated and thus fewer neoplastic cell nuclei would be counted in the disector (Fig. 3C).

As a final methodological appraisal, in this first approach to the $N_V$ (nuclei, tumor) of CMC we obtained a small CE, much below the 0.1 threshold (Gundersen *et al*., 2013), and the error due to the methodology was also low. For future studies and for practical purposes this CE could be optimized, by counting fewer nuclei per tumor. In this vein, counting 20 fields per tumor would suffice and this would significantly reduce the time needed for the analysis (for forty fields, around 30 minutes were needed).

In conclusion, we showed that an objective and unbiased estimation of a cellularity-related parameter — expressed as $N_V$ (nuclei, tumor) — in CMC can be obtained by stereological methods. This study better characterizes CMC by offering new 3D-relevant data not influenced by the uncontrolled biases inherent to the simplistic 2D counts. The lower $N_V$ (nuclei, tumor) in cases that progressed seems to be a promising finding, but further follow-up studies with large series are needed to fully clarify the association between $N_V$ (nuclei, tumor) and survival outcomes.

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# CHAPTER 6

## SYSTEMATIC SAMPLED *VERSUS* SELECTIVE MITOTIC COUNTS IN CANINE MAMMARY MALIGNANT TUMORS

**Summary**

Mitotic activity has been proven to be a strong prognostic factor in human breast carcinomas, and different methodologies to counting mitosis have been scrutinized over the years. In malignant canine mammary tumors (CMT) the mitotic figures are considered a diagnostic and grading parameter. Mitotic counts performed in ten high-power fields with the highest (subjectively) perceived proliferative activity are included in the grading systems adapted to malignant CMT. The individual prognostic significance of this grading component has been scarcely studied in malignant CMT and conflicting results have been reported. In human pathology, selective mitotic counts have been criticized for supposed low reproducibility. Preliminary data indicated that this may also be true in malignant CMT. This study investigated the prognostic value of the mitotic count performed using the Nottingham grade method and whether the prognostic value could be improved by a systematic random sampling procedure, combined with a correction for the area of the epithelium present. Routine histological sections of fifty seven malignant CMT of different histological types were studied. For forty seven cases two years follow-up data was available. The selective *versus* systematic mitotic counts showed a weak positive correlation ($r = 0.35$, $P = 0.03$). The first estimation was higher in larger tumors and solid carcinomas, and no association between the systematic count and the clinicopathological parameters existed. Also, both types of mitotic counts did not provide prognostic information. In this vein, the inclusion of mitotic activity as a grading parameter in malignant CMT should be revised. Moreover, veterinary pathologists should explore alternative proliferation markers and, eventually, other quantification strategies for prognostic purposes in malignant CMT.

## 6.1 Introduction

The capability to sustain proliferative activity is considered one of the fundamental hallmarks of cancer (Hanahan and Weinberg, 2011). Ninety years ago, the first study addressing malignancy grading of human breast carcinoma, recognized the presence of mitoses as a key feature (Greenough, 1925). Afterwards, all the important grading systems developed for breast carcinomas contemplated mitotic figures as a relevant parameter (Patey and Scarff, 1928; Bloom and Richardson, 1957; Elston and Ellis, 1991). Initially, the mitotic and hyperchromatic nuclei were both considered in the grading systems, but after a modification was introduced, only unequivocal mitotic figures were counted and scored according to specific cut-offs based on the microscope field diameter (Elston and Ellis, 1991). Several reports favored mitotic count as the main prognostic component of the grade method of breast carcinomas, being especially important in the decision of adjuvant treatment for lymph node-negative breast cancer patients (*e.g.*, Meyer *et al*., 2005; Volpi *et al*., 2004; Skaland *et al*., 2008).

Despite this, mitotic counting has been criticized due to its highly selective and subjective nature (Jannink *et al*., 1995). According to the grading recommendations, mitotic figures should be counted in the most proliferating part, which usually comprises the peripheral area of tumors (Elston and Ellis, 1991). However, mitotic heterogeneity has been reported, and without sampling the whole tumor it is difficult to know where to begin (Jannink *et al*., 1996a,b; Tsuda *et al*., 2000; Meyer *et al*., 2005). Additionally, two other pitfalls regarding mitotic counting should be kept in mind. In one hand, the variability on the proportions of neoplastic cells in each high power field is not taken into account in the grading criteria, and, on the other hand, it is often difficult to distinguish a mitotic figure from apoptosis, pyknosis or even irregularities of nuclear staining (which can occur with routine staining) (Meyer *et al*., 2005).

Several approaches have been recommended to overcome these limitations and to improve the reproducibility of mitotic count in human breast carcinomas. Some authors recommended a simplistic approach that consists in counting more than 10 high-power fields either in heterogeneous tumors or in those with low mitotic activity (O'Leary and Steffes, 1996; Elston and Ellis, 1998; Meyer *et*

*al*., 2005). Others researchers used morphometrical or stereological methods to objectivize the estimation of the mitotic count. Over 25 years ago, a method to standardize the counting of mitotic figures in tumors was developed, aiming to circumvent the problem associated with the varying amount of neoplastic tissue in each field (Haapasalo *et al*., 1989). This method included the simultaneous counting of the mitotic figures and estimation of the relative volume of neoplastic tissue, by a point-counting method in each field, thus generating the so-called volume corrected mitotic index (M/V index) (Haapasalo *et al*., 1989). The M/V index in breast carcinomas has been reported to be powerful in predicting survival (Lipponen *et al*., 1991; Aaltomaa *et al*., 1991, 1992; Jannink *et al*., 1995; Kronqvist *et al*., 1998). In one of these studies, the M/V index was also estimated by systematic random sampling (Jannink *et al*., 1995). The latter mitotic index gave an assessment of the mitotic activity throughout the whole tumor, presenting also prognostic value in breast carcinomas (Jannink *et al*., 1995). Anyway, it seems that the different mitotic estimates (*i.e.,* related to volume, area or number of nuclear profiles) in breast cancer tended to be highly correlated (Jannick *et al*., 1995; Ladekarl, 1998; 2004).

In malignant canine mammary tumors (CMT), the number of mitosis is also used to assist in diagnosis and grading (Misdorp *et al*., 1999; Misdorp, 2002; Peña *et al*., 2013). The prognostic significance of mitosis estimations in CMT is scarcely studied and the existing reports are controversial, with two studies reporting no association with prognosis (Sarli *et al*., 2002; Santos *et al*., 2013), and a more recent study reporting the opposite (Mainenti *et al*., 2014). As far as we know, the use of systematic sampling for selecting the fields for mitotic counts has never been implemented in malignant CMT.

Because the systematic sampling guarantees the generation of representative samples, when compared to schemes not grounded on probabilistic theories, like choosing fields of view by arbitrarily decisions of a pathologist, we hypothesized that using solid sampling schemes may improve the value of mitotic counts. Accordingly, the aims of the present study were to: 1) count mitotic figures in the whole tumor, using a systematic sampling approach and estimating the area of epithelial cells in each counting field; 2) compare mitotic counts per epithelial area with mitotic counts obtained by the histological grade

method criteria; 3) estimating the prognostic value of these mitotic figures estimations.

## 6.2 Material and methods

### *6.2.1 Selection of cases and histological study*

Fifty seven spontaneous malignant CMT surgically removed with curative intents in the veterinary clinics of the ICBAS-University of Porto were retrospectively selected from the Pathology laboratory archives. The selection of cases was blinded to clinical data. In the clinical archives, two years follow-up data was available for forty seven cases. The follow-up protocol was detailed elsewhere (Santos *et al.,* 2013). Owners gave informed consent for both surgery and follow-up.

The histological diagnosis and grade was performed by two observers (MS and PDP) using the WHO classification and the Nottingham grade method (Elston and Ellis, 1991; Misdorp *et al*., 1999; Karayannopoulou *et al*., 2005). Cases with score discrepancies in grading parameters were reviewed using a multi-head microscope, in order to obtain a consensual score. The tumor size (*i.e.,* largest diameter measured with a caliper before surgery) and the histological evidence of vascular invasion (defined as the presence of tumor emboli within endothelial-lined spaces) and/or regional lymph node metastases [lymph nodes were evaluated in routine slides and after immunolabelling with pancytokeratin AE1/AE3 and cytokeratin 14, as previously described by Matos *et al.* (2006)] at the time of diagnosis were recorded. All the slides resulting from the largest cross section were screened.

### *6.2.2 Assessment of mitotic activity*

Regarding selective mitotic count (sMC), the mitotic figures were counted in 10 high-power fields (400x) and scored using the cut-offs defined by the field diameter of the microscope (field diameter of 0.55 mm; field area of 0.238 $mm^2$) — score 1 (≤ 8 mitotic figures), score 2 (9 to 17 mitotic figures); score 3 (≥ 18 mitotic figures) — thus assuring equivalence with assessments made by Elston and Ellis (Elston and Ellis, 1998; Karayannopoulou *et al*., 2005). The selection of the high-power fields for mitotic counting was performed in what the

observers judged to be the most mitotically active parts of the tumor, which generally corresponded to peripheral areas (Elston and Ellis, 1991). The mean value of the mitotic count performed by the two observers (MS and PDP) was obtained and used to estimate the number of mitotic figures per the total area of tumor.

Systematic random mitotic counting (rMC) was performed by one observer (MS) in a workstation comprising: 1) a microscope (Olympus BX-50, Tokyo, Japan) equipped with a UPlan Apo 100x (1.25 NA) oil-immersion lens (Olympus); 2) a motorized stage (Prior, United Kingdom) for stepwise displacement in the x–y directions (1 μm accuracy); 3) a CCD video camera (Sony, Tokyo, Japan) connected to a 17″ PC monitor (Sony); 4) a computer with a stereology software (Olympus Denmark, CAST-Grid, version 1.5). At the monitor, a final magnification of 4750x allowed an easy and accurate recognition of neoplastic cells and mitotic figures. In each tumor, a minimum of 40 fields of vision were sampled systematically random that, is, the selection of the first field was random and the subsequent fields were sampled systematically by stepwise movements of the stage in the x- and y-directions roughly proportional to the overall area of the tumor (Fig. 1). In each field, the area of the neoplastic epithelium was estimated using a probe of test points (points hitting the epithelial cells were counted). Simultaneously, the mitotic figures were counted in a frame with an area = 2533 $\mu m^2$ (Fig. 2).

For both sMC and rMC, only the unequivocal mitotic figures were considered, excluding both hyperchromatic and apoptotic nuclei (Fig. 2).

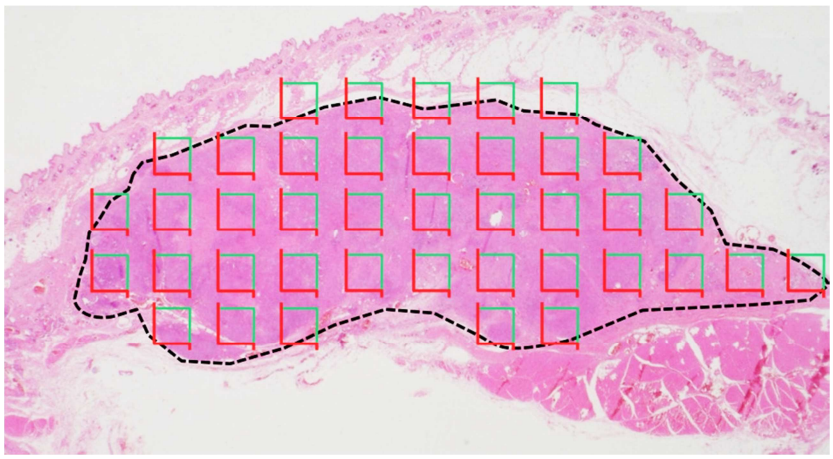

**Fig. 1** – Schematic representation of the systematic random sampling used for mitotic count. After outlining the tumor (hatched line) and setting the number of aimed fields, the software selects randomly the first field and the next ones are automatically equally spaced within the tumor, by stepwise movements of the stage in the x- and y-directions.

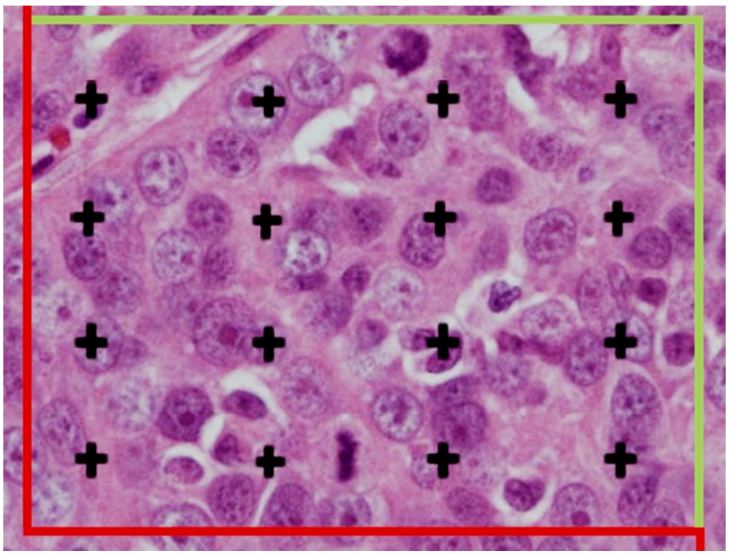

**Fig. 2** – Light microscopy image of a solid canine mammary carcinoma, with the test system used for counting the mitotic figures and assessing the area of neoplastic epithelium. It consists of a counting frame with inclusion lines (green), exclusion lines (red), and points (16 equidistantly spaced points). Mitotic figures were counted provided they are inside the counting frame or touch the green lines but not the red ones. For assessing the reference area, points hitting the neoplastic epithelium are counted. In this illustrative field, 2 mitotic figures and a total of 13 points hitting the neoplastic epithelium were counted.

### *6.2.3 Statistical analysis*

The homogeneity of variances and normal distribution of the sMC, rMC and tumor size (in cm) values were verified using the Shapiro-Wilk and Kolmogorov-

Smirnov tests, respectively. For skewed data, such as the sMC and rMC, a logarithmic transformation was applied.

The correlation between the sMc and rMC was assessed using the Pearson correlation test. The same approach was used to evaluate the relation between the mitotic counts and the tumor size. The relation between each mitotic count and the WHO size categories was tested with one-way ANOVA, followed by the Tukey post-hoc tests. A similar approach was used to test the relation between each mitotic count and the histological subtypes of tumors. The differences in mitotic estimates in tumors < 2.9 cm and tumors ≥ 2.9 cm (in the largest diameter), and in tumors with or without vascular/lymph node invasion, were assessed with the *t*-test for independent samples. Tumors were also grouped according to their histological subtypes (solid plus anaplastic carcinoma *versus* tubulopapillary plus complex) and compared using the *t*-test for independent samples.

The differences in survival between tumors of the three NHG scores regarding mitotic count were graphically represented using the Kaplan-Meier curves, and compared by the log-rank test. A similar analysis was performed for the score of sMC and rMC defined by their tertiles thresholds values (sMC: score 1 ≤ 6; score 2: 7-12; score 3 ≥ 13 mitotic figures $mm^{-2}$; rMC: score 1 ≤ 10, score 2 : 11-24 mitotic figures $mm^{-2}$; score 3 ≥ 25 mitotic figures $mm^{-2}$).

In all cases, a *P* value < 0.05 was considered statistically significant. Statistical analyses were performed with the IBM SPSS Statistics, version 22 (IBM, New York, USA).

### 6.3 Results

In this series, 45 out 57 tumors were diagnosed as simple carcinomas (19 tubulopapillary, 21 solid, 2 squamous cell and 3 mucinous carcinomas), 8 were complex carcinomas and 4 carcinosarcomas. The largest tumor diameter ranged from 0.5 to 15 cm, being more than 2.9 cm in 24 cases (42%). Nineteen cases (33%) presented vascular/regional lymph node invasion at the time of the diagnosis. Regarding histological grading, 9 cases were grade I, 24 were grade II and another 24 were grade III. As to sMC grading, score 1, 2 and 3 were present in 11, 15 and 31 tumors, respectively. Follow-up data was available for

47 female dogs; overall 36% developed recurrence and/or metastases and 24% died or were euthanized due to progressive disease.

An average of 8 mitoses $mm^{-2}$ and 15 mitoses $mm^{-2}$ was determined in the sMC and rMC methods, respectively, being the range of values wider in rMC (Table 1). In 21 out 57 the rMC was less than the sMC and, in 16 cases of those cases the rMC was less than 1 mitotic figure $mm^{-2}$. In the other cases the rMC was higher than the respective sMC: in these cases, the correction to the area of the neoplastic epithelium increased the rMC values by ≈ 53%, on average. These two mitotic indexes were weakly but significantly correlated (r = 0.35; *P* = 0.03). The sMC, but not the rMC, was also positively correlated with tumor size (r = 0.42; *P* = 0.001). Tumors with a size ≥ 2.9 cm presented a significantly higher sMC (*t*-test, *P* = 0.008). A similar result was obtained when the tumor size was categorized according to the WHO criteria: tumors with size larger than 3 cm presented high rMC compared to smaller tumors (Tukey test, *P* = 0.005), as well as tumors with more than 5 cm [higher sMC than tumors sized 3-5 cm (Tukey test, *P* = 0.001)].

The sMC were significantly different in tubulopapillary compared to solid carcinomas (Tukey test, *P* = 0.003) (Table 1); but no differences were detected in rMC of tumors with different histological classification. When solid plus anaplastic carcinomas were compared with tubulopapillary plus complex tumors (excluding carcinosarcomas, squamous and mucinous carcinomas), the first ones presented a significantly higher sMC (14 *versus* 8 mitosis $mm^{-2}$) (*t*-test, *P* = 0.001).

No differences in sMC and rMC were observed in tumors with or without vascular/lymph node invasion. The log-rank test showed no significant association between the DFI or the tumor-related OS and the sMC values (scored either according to the NHG or by the tertiles of their distribution). A similar result was obtained when the rMC were scored according to tertiles. In fact, sMC and rMC were on average similar in tumors that showed post-surgical progression and those without progression (cases with a minimum follow-up of 15 months).

**Table 1** – Median and mean ± standard deviation (SD) of selective mitotic count (sMC), expressed as number of mitoses per $mm^{-2}$, and of systematic random mitotic count corrected for the area of the neoplastic epithelium (rMC), given as number of mitoses per $mm^{-2}$.

| Tumor | sMC | | rMC | |
|---|---|---|---|---|
| | Median | Mean ± SD | Median | Mean ± SD |
| Simple carcinoma | 8.5 | 11 ± 8 | 15 | 20 ± 20 |
| ▪ Tubulopapillary carcinomas | 6 | 7 ± 5 | 15 | 16 ± 17 |
| ▪ Solid carcinomas | 13 | 13 ± 8 | 13 | 21 ± 22 |
| Complex carcinomas | 6.5 | 9 ± 5 | 14.5 | 15 ± 15 |
| Carcinosarcomas | 7 | 6 ± 2 | 14 | 17 ± 5 |
| All cases | 8 | 10 ± 8 | 15 | 19 ± 19 |

## 6.4 Discussion

Mitotic counting is one of the oldest but also one of the most practical methods to assess proliferation in a tumor (van Diest *et al*., 2004). In malignant CMT, the role of mitotic count as a prognostic factor is sparsely studied and a profound discussion regarding the methodology of mitotic counting in these tumors is still missing. Sarli *et al.* (1999) introduced a correction of the mitotic counts for the area of the nuclei of the neoplastic epithelium presented in each field of vision, but their study did not include survival analysis. The same group established a mitotic index expressed as the number of mitoses per 1000 neoplastic cells, in 10 high-power fields selected subjectively in the area with the highest mitotic activity. However, this index was not useful for prognostic purposes (Sarli *et al*., 2002). Similarly, our group failed to detect significant associations between sMC and survival outcomes (Santos *et al*., 2013; Santos *et al*., 2015a in Chapter). However, a more recent study reported that mitotic count, defined by the threshold of 10 mitotic figures in 10 high-power fields, was an independent prognostic factor (Mainenti *et al*., 2014). We hypothesized that the low reproducibility of the counting method and/or high variation of cellularity in malignant CMT could contribute to the discrepancy regarding the prognostic significance of the mitotic count. Accordingly, we assessed the mitotic count in

malignant CMT by using the classic approach of counting 10 high-power fields in the peripheral area of the tumors and, for the first time, by a systematic random sampling of fields throughout the tumor, associated with a point-counting method to estimate the area of the neoplastic epithelium. In approximately 30% of the cases, the rMC was less than 1 mitotic figure per $mm^2$, while the sMC was higher. This indicates that in those cases the observer was able to select fields with increased mitotic activity for performing the sMC. The correction for the neoplastic epithelium area increased the rMC by 50%, on average, indicating that in those cases there was a high proportion of non-epithelial tumoral elements, such as stroma or necrotic areas.

In cases where the rMC was higher than the sMC, the possibility that the higher magnification used for the latter allowed a better identification of mitotic figures could not be excluded. Some mitotic figures, when observed with a smaller magnification, would not be differentiated from apoptotic nuclei and thus would not be counted. In human breast cancer, mitotic count perform in 10 randomly selected fields and corrected for the relative volume of neoplastic epithelium was generally lower that the sMC, even when the latter was also corrected for the relative volume of neoplastic cells (Jannink *et al*., 1995).

Our results indicate the existence of a high variability regarding mitotic activity in malignant CMT. The heterogeneity in the mitotic activity within the tumor surely troubled the pathologist's task of unambiguously identifying the most proliferative areas. This caveat would justify the interobserver variation in scoring sMC in malignant CMT (Santos *et al*., 2015b in Chapter 3), and backs the need for a consensus regarding the best methodology for assessing proliferative activity.

In the studied series, the association between mitotic activity with other clinicopathological factors, such as histological type and tumor size, was only observed for the sMC. Nevertheless, both sMc and rMc were not associated with survival outcomes. The values obtained with both estimates were quite similar in the cases that showed progressive events (including fatal cases) during the follow-up and in those that did not. In this vein, no real advantage was shown by using the systematic random sampling protocol, even when a correction for the area of the neoplastic epithelium was simultaneously

performed. The absence of prognostic value of mitotic counts in malignant CMT contrasts with the existing evidence for breast cancer (Jannink *et al*., 1995; Baak *et al*., 2005). The canine mammary gland is well acknowledged for its profound cyclic proliferative and regressive changes that take place in each estrous cycle phase (Santos *et al*., 2010). Mitotic figures and high expression of proliferative markers (*e.g.*, Ki-67) have been detected during the estrous cycle phases associated with raised progesterone (Santos *et al*., 2010). So, at this point, we cannot exclude the hypothesis that the normal cyclic hormonal influences in the canine mammary parenchyma could also affect the tumor cellularity and kinetics; *viz.* being partially responsible for the mitotic activity observed within the tumor. This would justify, to some extent, the heterogeneity of mitotic activity between cases and also the absence of prognostic value of mitotic counts in malignant CMT. As part of the mitotic activity would be a normal physiologic response, rather that an intrinsic aggressive neoplastic feature.

Our results regarding sMC support previous evidences that solid carcinomas are associated with a high proliferative activity (Sarli *et al*., 1999; Yoshimura *et al*., 2014). However, variation in the amounts of neoplastic epithelium per each high-power field, between solid, tubulopapillary (presenting luminal and interpapillary spaces) and also complex tumors (presenting areas of myxoid matrix), could also contribute to that difference. Actually, in the solid carcinomas it is more likely to observe mitotic figures in each field, since it bears a larger area of neoplastic cells. Accordingly, Sarli *et al.* (1999) reported no differences between the solid and the other simple carcinomas by correcting the mitotic figures number per the area of the nuclei of the neoplastic epithelium counted in 10 high-power fields selected in the zone with the high proliferative activity.

The absence of prognostic significance of both types of mitotic counts supports a classical report by Owen (1979), where the author considered, in a comparative perspective, that the mitotic counts in dogs might not be as important as in women. Meanwhile, more recent studies also failed to demonstrate a prognostic role for mitotic figures estimates (Sarli *et al*., 2002; Santos *et al*., 2013).

An important methodological issue related to mitotic counts is the reported lack of precision of the estimations in tumors with low proliferative activity (Ladekarl

*et al*., 1997); some tumors included in this study seemed to fit with this description. It was suggested that an increased precision of the proliferation measure would be achieved by using immunohistochemical staining against proteins expressed during different phases of the cell cycle, as for example PCNA and Ki-67 (Ladekarl *et al*., 1997). In malignant CMT high indexes of these proteins have been associated both with aggressive behavior and poor prognosis (Klopfleisch *et al*., 2011). Despite their proven prognostic value, these immunomarkers are still not included in the routine protocol for malignant CMT (Klopfleisch *et al*., 2011).

Considering the state of the art of the methods used to assess mitotic counts, diverse refinements that can provide more realistic estimations of the number of mitosis could be performed. Despite practical in operational terms, the tested approaches inherently have a number of biases. In first place, the estimates were merely 2D and therefore depend on the size and orientation of the mitotic nuclei, as well as the section thickness. Modeling of the rMC values could be attempted to overcome the bias against smaller particles (in these case smaller mitotic nuclei) that occurred when using a thin histological section. Different model-based corrections such the proposed by Weibel and Gomez (1962) could be used to estimate the 3D number of mitoses. In order to apply such corrections, assumptions regarding the shape of the mitotic nuclei should be made and therefore, the estimations would still not be free of bias (Lemley *et al*., 2013). Additionally, to carry out this method an estimation of the volume fraction of the mitotic nuclear profiles would be needed. This will be tested in the near future; however, at this point, alternative proliferative markers, as previously stated, seem to be more rewarding tools for studying malignant CMT.

In conclusion, at least with the currently tested methodologies, grading mitotic activity seems not to be important for prognosis definition of malignant CMT. Estimating mitotic figures over the whole tumor section and correcting the count for the amount of epithelium do not add prognostic significance. The results markedly contrast with that observed in human breast cancer, and raises further doubts on the adoption of 2D-based mitotic counts in the grading criteria for malignant CMT.

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# CHAPTER 7

## SEARCHING FOR POTENTIAL GRADING PARAMETERS IN CANINE MAMMARY MALIGNANT TUMORS

**Summary**

In recent years, the histological grade of malignant canine mammary tumors (CMT) has been assigned using the human Nottingham histological grade method as basis. When applied to CMT, the grade seemed to be useful for prognostic purposes; however the contribution of each grading parameter is unclear. Recently, it was reported that nuclear pleomorphism, when scored as a two-tier (score 1 plus 2 *versus* score 3) was the only grading parameter associated with survival. This finding incited the search for potential alternative morphological grading parameters. Herein, the association between eight morphological parameters and survival was investigated. Micropapillary structures, necrosis, squamous differentiation, nucleolar pattern, inflammatory infiltrates, scirrhous stroma, abnormal nuclei, and chromatin vesiculation were assessed. The parameters were evaluated qualitatively (presence *versus* absence) and semi-quantitatively (as a percentage) in 59 malignant CMT, for which two years follow-up data was available. The association between the parameters and the survival was investigated by univariable analyses. Additionally, multivariable Cox proportional hazards model was used to analyze the effect of different parameters on disease-free interval and overall survival. Nuclear pleomorphism (classified as two-tier system), vascular invasion and tumor size were also included as co-variables in Cox models. Necrosis, squamous metaplasia, abnormal nuclei were associated with short DFI and OS. Moreover, the predominance of a pattern of atypical nucleoli was a marker of increased odds for post-surgical progression, while the presence of scirrhous stroma was associated with tumor-related death. In the multivariable model, nuclear pleomorphism, vascular invasion and abnormal nuclei arise as independent factors for shorter DFI, while nuclear pleomorphism and necrosis emerged as independent prognostic factors for shorter OS. According to these results the pathologist should routinely report the nuclear pleomorphism score, the existence of vascular invasion, the percentage of necrosis and the proportion of cells with abnormal nuclei in all cases of malignant CMT, in order to provide relevant prognostic information to the clinician.

## 7.1 Introduction

In recent years, the histological grading of malignant CMT has been performed following the standard method used for human breast cancer (*e.g.*, Karayannopoulou *et al*., 2005; Clemente *et al*., 2010; Rasotto *et al*., 2012), namely by using the so-called Nottingham histological grade method (NHG) or the Elston and Ellis modification of Bloom and Richardson method. This system included the assessment of three parameters, being scored 1 to 3 and the overall grade determined by the final combined score, as detailed in Chapter 4 (Elston and Ellis, 1991). Veterinary pathologists have been using the original method or a recent proposed modification (Peña *et al*., 2013). However, the prognostic role of histological grade is less established in dogs than in humans (Matos *et al*., 2012). Some issues related to histological grading, as the interobserver variation and the role of each grading parameter, remain fairly unknown in malignant CMT. Recently, the interobserver reproducibility in histological grading, as well as the individual prognostic value of each grading parameters were evaluated (Santos *et al*., 2015a; b in Chapters 3 and 4). For those studies a cohort of female dogs was used for which the two years follow-up data was available. According to the results, the grade is prone to an interobserver variation similar to that observed in human breast cancer. Nuclear pleomorphism was the least consensual parameter among observers, followed by the mitotic counts. However, on a prognostic perspective the nuclear pleomorphism, classified as a two-tier system (score 1 plus 2 *versus* score 3) was also the only grading parameter associated with clinical outcomes (Santos *et al.*, 2015b in Chapter 4). Neither tubule formation, nor mitotic count provided relevant prognostic information. A higher overall grade was associated with worst survival, especially when grade I plus II were compared to grade III (Santos *et al*., 2013; Santos *et al*., 2015b in Chapter 4). This evidence and similar ones provides raises questions regarding possible refinements of the method, eventually by adding or replacing some grading parameters. The search for morphological parameters, easily assessed by the pathologists in routine material, which can be associated with survival outcomes, represents a good investment in veterinary medicine (de las Mulas *et al*., 2005; Dagli, 2008). This is especially true when we consider that the more sophisticate diagnostic techniques are not affordable by most dog owners in many parts of the world. It

should be noted that the traditional factors, such as the grade or the pathological stage of the regional lymph nodes, still remain as cornerstones for prognosis definition in human breast cancer (Elston *et al*., 1999; Rakha *et al*., 2010); even if the level of knowledge, regarding genetic and molecular markers, of human breast cancer is incomparably higher than in malignant CMT.

This study aims to evaluate qualitatively and semi-quantitatively morphological parameters in malignant CMT, using histological routine sections and to disclose their prognostic value regarding DFI and OS in univariable and multivariable analyses.

## 7.2 Material and methods

### *7.2.1 Selection of cases and follow-up*

A cohort of fifty nine female dogs with spontaneous malignant CMT, treated at the veterinary clinic of ICBAS-University of Porto, was retrospectively selected. Dogs underwent surgery as the only treatment. Owners provided consents for surgery with curative intents, two years follow-up, and for the use of the material for research purposes. Of the female dogs, forty nine were selected based on the presence of a single malignant tumor. In the subgroup of 10 dogs with multiple malignant tumors, a reference lesion was assigned in accordance to published approaches for synchronous breast cancers and CMT (Beckmann *et al.*, 2011; Santos *et al.*, 2011; 2013; Schmid *et al.*, 2011). This reference lesion was considered as the tumor presenting vascular invasion (primary criterion) or the one with largest diameter (secondary criterion). The selection of cases and their histological study were blinded to survival outcomes, thus following the recent guidelines in veterinary oncology (Webster *et al*., 2011). Dogs with distant metastases at the time of the diagnosis were excluded.

The schedule and the protocol of clinical evaluations, before the surgery and during the follow-up period, were performed as previously described (Santos *et al*., 2013). The DFI was calculated from the date of surgery to the diagnosis of disease progression (recurrence or metastasis, with cytological or histological confirmation). As to the OS, it was calculated from the date of surgery to the date of animal´s death due to metastases. Animals that died or were euthanized for unrelated causes and those that were lost to follow-up were censored,

respectively, at the time of death and at the date of their last follow-up examination. Complete necropsies were performed in all dogs that died spontaneously or were euthanized, and suspected metastatic lesions were confirmed by histopathological examination.

*7.2.2 Assessment of morphological parameters*

Two observers reviewed the diagnosis, in a multi-headed microscope using the WHO classification of CMT (Misdorp *et al*., 1999) and considering all the slides resulting from the largest tumor cross section. The tumor size [in cm and categorized by the previously established cut-off (Santos *et al*., 2014)], vascular invasion [irrespective of the type and number of invaded vessels (Fig. 1A)], histological grade and scores of the grading parameters were retrieved from a previous study (Santos *et al*., 2015 in Chapter 4). The same observers assessed by consensus and, considering all the sectional area of the tumor presented in the slides, the following tumoral characteristics: 1) presence of micropapillary structures (Fig. 1B); 2) percentage of necrosis; 3) percentage of squamous differentiation; 4) nucleolar morphological pattern [pattern 1: indistinct nucleoli; pattern 2: small but prominent nucleoli; pattern 3: large, multiple or atypical nucleoli (Fig. 1C)]; 5) presence and type of inflammation; 6) presence of scirrhous stroma (either intra- or peritumoral); 7) percentage of abnormal nuclear forms [defined as relative number of nuclei not round or oval, that presented indentations, angularity or irregularities (Fig. 1D)]; 8) percentage of chromatin pallor or vesiculation. The definition criteria for the nucleolar pattern, abnormal nuclei and chromatin vesiculation followed a description for feline mammary carcinomas (Mills *et al.*, 2015). A nucleolar pattern was assigned to a tumor when at least half of the cells presented that type of nucleoli. It is opportune to mention that the evaluation of the nuclear and nucleolar features was assessed in the neoplastic luminal epithelial cells.

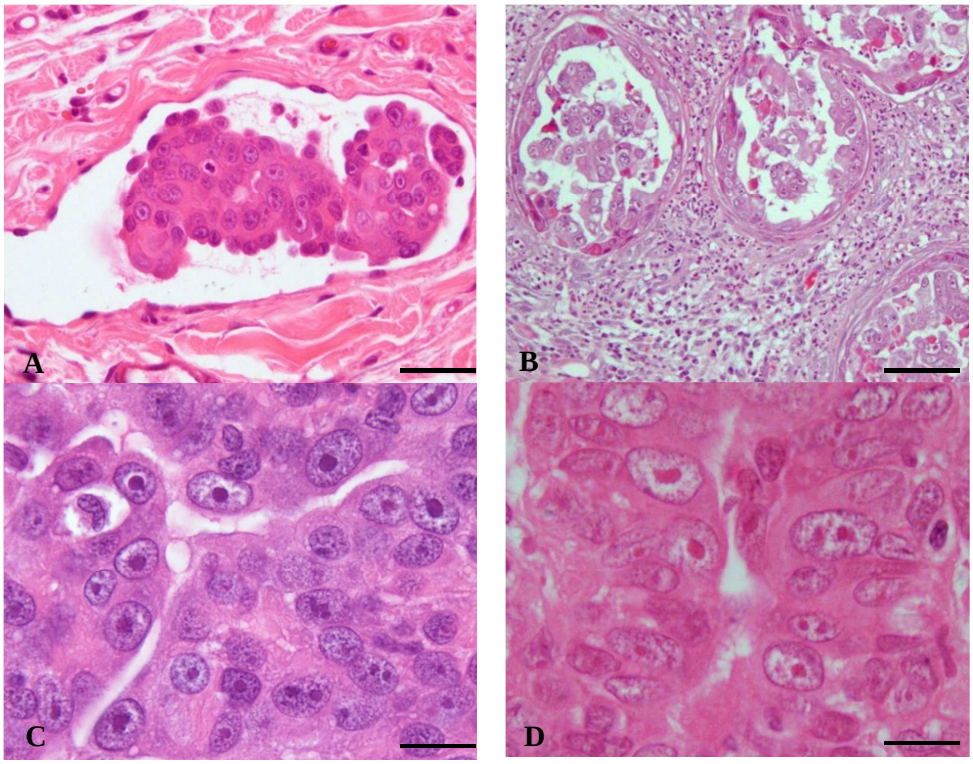


**Fig. 1** – A: vascular invasion corresponding to the presence of an obvious embolus within a endothelial-lined structure; B: micropapillary structures corresponding to small clusters of cells lacking a fibrovascular core within a duct; C: nucleolar morphology pattern 3, in which the majority of cells have large, multiple and atypical nucleoli; D: abnormal nuclei with irregular forms and with areas of pallor corresponding to chromatin vesiculation; Hematoxylin-eosin, bar 35 µm (A), 50 µm (B) and 20 µm (C,D).

Categorization of the percentage of the morphological parameters was tested using different cut-offs, namely the median value, tertiles (or 3-quantiles, *i.e.,* the two thresholds that divide the ordered distribution of percentage values into three parts) and quartile values for statistical purposes.

*7.2.3 Statistical analysis*

Disease-specific survivals were determined using Kaplan-Meier product-limit estimates, with log-rank (Mantel-Cox) test used to estimate differences in survival fractions according to the presence and the score of each morphological parameter. Univariable and multivariable analyses were done using Cox proportional hazards model to investigate the association between clinicopathological parameters and DFI and OS. The morphological parameters that proved to be significant in the univariable regression analysis (at a *P* lower than 0.10) were selected to enter the multivariable model. To control for

collinearity the correlation between all independent variables was tested and when the correlation between two variables was strong, only one was allowed to enter the multivariable model. In this latter, backward stepwise selection was performed. Briefly, in this selection method, all the candidate variables are included in the first step. Then, in each following step the least significant variable was removed and the significance of the remaining variables rechecked. The deletion of variables was performed until no further improvement of the model was possible (achieving a *P* value lower than 0.05 for all variables in the model). Logistic regression coefficients were used to estimate the hazard of each independent variable of the model. The 95% confidence interval is given for all variables that figured in the final model. Statistical analyses were performed with R Development Core Team software, version 2.7.1 (Vienna, Austria) and IBM SPSS Statistics, version 22 (IBM, New York, USA).

### 7.3 Results

Fifty nine female dogs aged from 6 to 18 years (mean of 10.9 years) were included in this study. Of these, 38 animals presented simple carcinomas (18 tubulopapillary, 17 solid, 1 anaplastic, 1 squamous cell and 1 mucinous carcinoma), 18 had complex carcinomas and 3 had carcinosarcomas. The largest diameter of the tumors ranged from 0.5 to 15 cm (mean 3.5 cm). At the time of diagnosis, 15 cases (25%) presented vascular invasion. Post-surgical progression was diagnosed in 17 cases (including 7 solid carcinomas, 5 complex, 3 tubulopapillary, 1 anaplastic and 1 carcinosarcoma). During the follow-up, 14 dogs (24%) died due to progressive disease.

The evaluation of the eight new morphological parameters was possible in all the tumors. The presence of micropapillary structures was not related to any survival time. On the other hand, the existence of necrotic areas within the tumors was marginally associated with short DFI (log-rank test, $P = 0.06$). When this parameter was scored using the median value (20%) as threshold, it allowed a good discrimination of tumors with significant differences in survival. Animals bearing tumors with less than 20% of necrosis necrosis had a DFI of 22

months and an OS of 23 months, while those cases where necrosisexceed 20% had a 15 and 18 months of DFI and OS, respectively (Fig. 2).

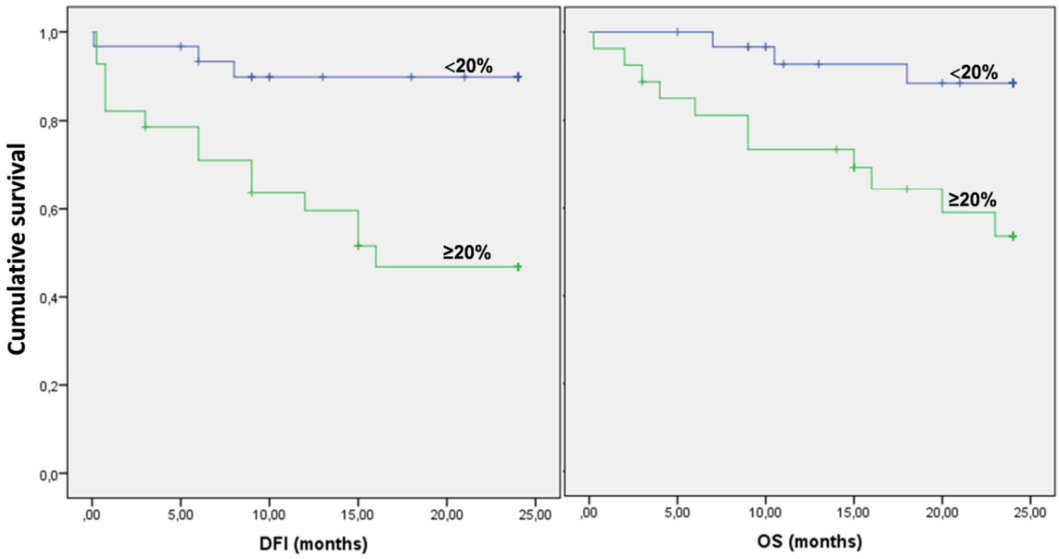


**Fig. 2** – Kaplan-Meier plots comparing the disease-free interval (DFI) and overall survival (OS) according to the proportion of necrosis. Short DFI an OS was associated with the presence of necrosis ≥ 20% of the tumor area (log-rank test, *P* = 0.001 for DFI and *P* = 0.005 for OS). Censoring is indicated by vertical marks.

Tumors presenting squamous differentiation were significantly associated with short OS (log-rank test, *P* = 0.02) and marginally associated with short DFI (log-rank test, *P* = 0.05); the fourth quartile cut-off of 10% of squamous cells allowed a better identification of progressive and fatal cases (Fig. 3).

Tumors with a predominance of nucleolar morphology pattern 3 (large, multiple and atypical nucleoli) were also associated with short DFI (log-rank test, *P* = 0.04) (Fig. 4), but this parameter had no influence on the OS.

The presence of inflammatory infiltrates of any kind, including lymphoplasmacytic ones, within and around the tumor, was not significantly related to DFI or OS.

The presence of more than 25% (median value) of neoplastic cells with abnormal nuclear forms was associated with short DFI (*P* = 0.02). When the tertiles values were used for scoring this parameter, the first tertile value (15%) appeared as a better cut-off than the median value, showing the best separation of the curves (Fig. 5).

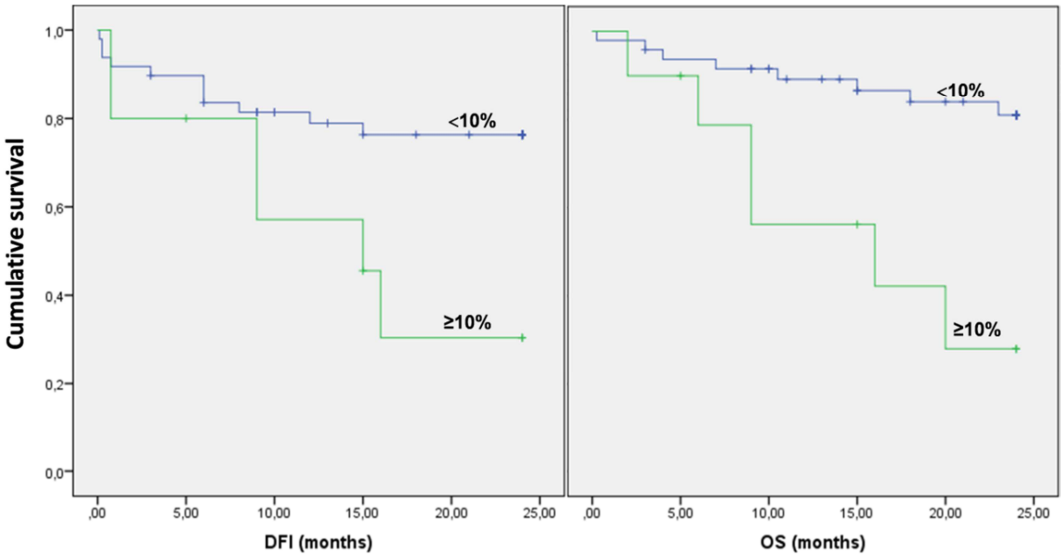


**Fig. 3** – Kaplan-Meier curves. Cases that presented 10% or more of squamous cells had significant shorter disease-free interval (DFI) and overall survival (OS) (log-rank test, *P* = 0.02 and *P* = 0.001, respectively). Censoring is indicated by vertical marks.

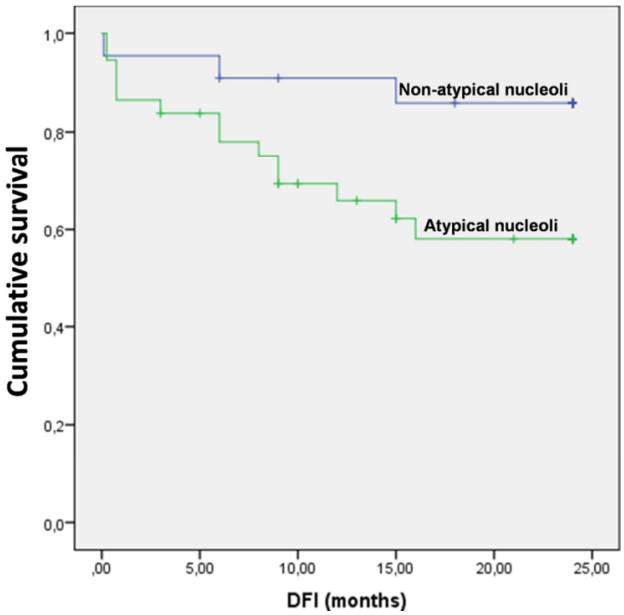


**Fig. 4** – Kaplan-Meier curves showing that cases with pattern 3 of nucleolar morphology (large, multiple and atypical) were associated with worst disease-free interval (DFI) comparing to cases without that type of pattern (log-rank test, *P* = 0.04).

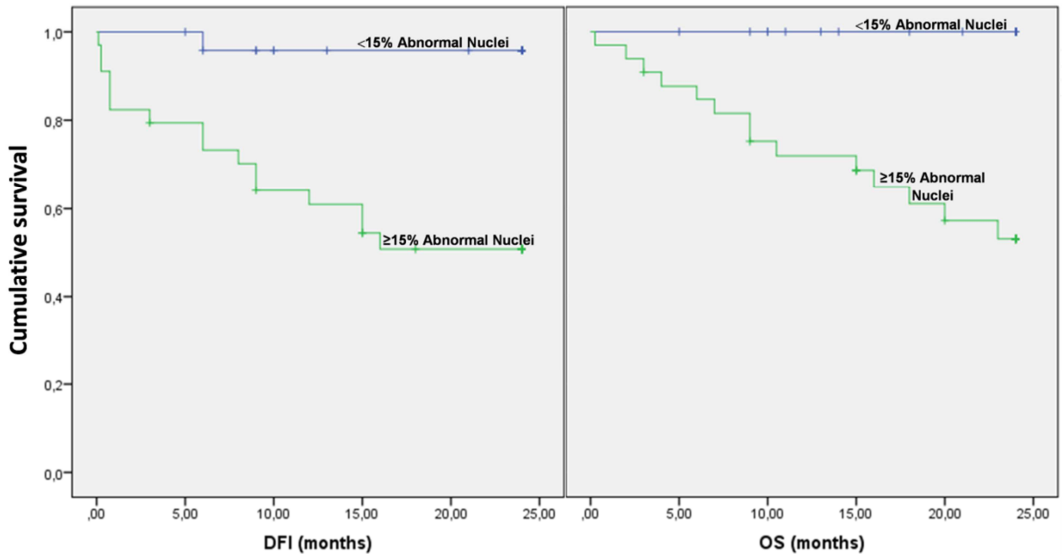


**Fig. 5** – Kaplan-Meier plots of disease-free interval (DFI) and overall survival (OS) according to the percentage of abnormal nuclei: 15% or more nuclei with abnormal morphology was associated with poorer survival times (log-rank test, *P* = 0.001 for DFI and *P* < 0.001 for OS).

The presence of neoplastic cells with chromatin vesiculation (even when different types of categorization were attempted) was not related to survival. On the other hand, the existence of dense collagenous/scirrhous stroma within or around the tumors was associated with short OS (log-rank test, *P* = 0.04) (Fig. 6).

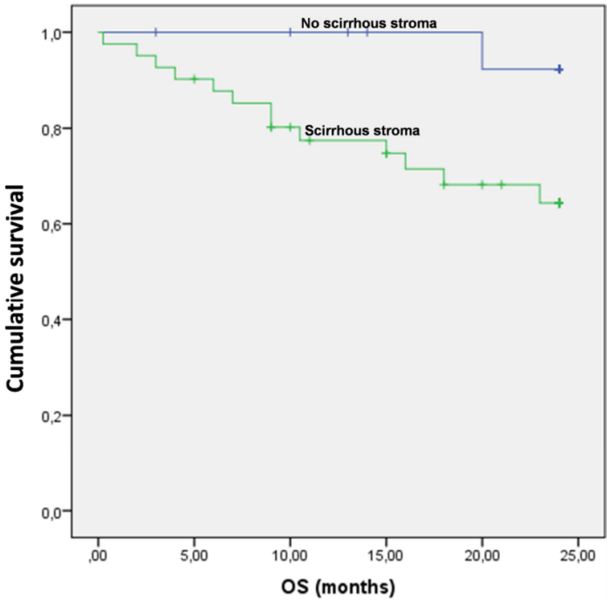


**Fig. 6** – Animals bearing malignant mammary tumors with a scirrhous stroma had shorter cancer-specific overall survival (OS) compared to those without that connective tissue reaction (log-rank test, *P* = 0.04).

Results of univariable and multivariable Cox regression analysis are summarized in Tables 1 and 2. Considering DFI as dependent variable, the combined effect of necrosis (scored according to the median value of 20%), squamous differentiation (scored by the cut-off of 10%), nucleolar pattern 3 (presence *versus* absence), scirrhous stroma (presence *versus* absence), abnormal nuclei (scored by the cut-off of 15%), tumor size (cm), vascular invasion (presence *versus* absence), and nuclear pleomorphism (evaluated according to the NHG criteria and classified as score1 plus 2 *versus* score 3) was evaluated by multivariable analysis. In the final multivariable model and, after the exclusion of non significant variables, the nuclear pleomorphism, vascular invasion and abnormal nuclei (≥ 15%) emerged as independent factors ($P$ = 0.01, $P$ = 0.007, $P$ = 0.04, respectively) (Table 2). A similar approach was done for OS as a dependent variable with the same set of variables included in the multivariable model for DFI, except the abnormal nuclei that were scored by the median value (because with the cut-off 15% the model did not converged). In the final multivariable model nuclear pleomorphism and necrosis ≥ 20% emerged as independent factors for predicting OS ($P$ = 0.002 and $P$ = 0.02, respectively) (Table 2).

**Table 1** – Univariable Cox regression analyses of disease-free interval (DFI) and overall survival (OS) as dependent variables in 59 cases of malignant canine mammary tumors.

| | **Univariable analysis** | | | | | |
|---|---|---|---|---|---|---|
| | **DFI** | | | **OS** | | |
| **Variables** | **β** | **SE** | ***P*** | **β** | **SE** | ***P*** |
| Micropapillae [1] | 0.1 | 0.53 | *ns* | 0.43 | 0.56 | *ns* |
| Necrosis [1] | 1.7 | 1.03 | 0.102 | 1.56 | 1.04 | 0.134 |
| Necrosis [2] | 1.8 | 0.64 | 0.004 | 1.64 | 0.65 | 0.012 |
| Squamous differentiation [1] | 0.9 | 0.50 | 0.057 | 1.22 | 0.56 | 0.030 |
| Squamous differentiation [3] | 1.1 | 0.51 | 0.028 | 1.63 | 0.55 | 0.003 |
| Inflammation [1] | 0.8 | 0.50 | 0.130 | 0.50 | 0.54 | *ns* |
| Scirrhous stroma [1] | 1.3 | 0.75 | 0.077 | 1.87 | 1.03 | 0.072 |
| Atypical nucleoli [1] | 1.2 | 0.64 | 0.054 | 1.10 | 0.65 | 0.091 |
| Abnormal nuclei [4] | 1.1 | 0.49 | 0.028 | 0.98 | 0.53 | 0.068 |
| Abnormal nuclei [5] | 2.6 | 1.03 | 0.010 | *nc* | *nc* | *nc* |
| Chromatin vesiculation [1] | 0.03 | 0.49 | *ns* | -0.36 | 0.54 | *ns* |
| Chromatin vesiculation [6] | -0.3 | 0.36 | *ns* | -0.33 | 0.592 | *ns* |
| Vascular invasion [1] | 1.5 | 0.49 | 0.003 | 1.37 | 0.538 | 0.011 |
| Tumor size (cm) | 0.1 | 0.06 | 0.058 | 0.09 | 0.069 | *ns* |
| Nuclear pleomorphism [7] | 2.1 | 0.58 | <0.001 | 1.97 | 0.598 | 0.001 |

Legend: [1] presence *versus* absence; [2] scored by median value of 20%, [3] scored by 4$^{th}$ quartile of 10%; [4] scored by the median value of 25%; [5] scored by the 1$^{st}$ tertile value of 15%; [6] scored by the median value of 5%; [7] NHG score 1 plus 2 *versus* 3; β – beta coefficient; nc – model did not converged; ns – not significant (*P* > 0.1); SE – standard error.

**Table 2** – Multivariable survival analyses (Cox proportional hazards regression) of disease-free interval (DFI) and overall survival (OS) as dependent variables in 59 cases of malignant canine mammary tumors.

| Dependent variable | Independent variables | β | SE | Hazard Ratio | *P* | 95% CI |
|---|---|---|---|---|---|---|
| **DFI** | Abnormal nuclei [1] | 2.14 | 1.06 | 8.46 | 0.04 | 1.06-67.5 |
| | Vascular invasion [2] | 1.38 | 0.51 | 3.98 | 0.007 | 1.45-10.9 |
| | Nuclear pleomorphism [3] | 1.48 | 0.60 | 4.39 | 0.01 | 1.36-14.1 |
| **OS** | Necrosis [4] | 1.45 | 0.66 | 4.29 | 0.02 | 1.18-15.6 |
| | Nuclear pleomorphism [3] | 1.83 | 0.60 | 6.26 | 0.002 | 1.9-20.6 |

Legend: [1]scored by the 1st tertile value of 15%; [2]presence *versus* absence; [3]NHG score 1 plus 2 *versus* 3; [4]scored by median value of 20%, β – beta coefficient; CI – confidence interval; SE – standard error.

## 7.4 Discussion

Despite the promising value of more sophisticated techniques for studying malignant CMT, the most attractive prognostic factors remain those that can be assesed using easy, accessible and economic methods (de las Mulas *et al*., 2005). Morphological parameters evaluated in routine sections of tumors certainly meet those criteria. Indeed, when examining malignant CMT pathologists frequently deal with the challenge of providing reliable prognostic data in their routine reports (Dagli, 2008). Assigning the grade to a tumor could be a simple way to achieve that goal (Betz *et al*., 2012).

In the last decade, it was shown that grade of malignant CMT based on the NHG criteria (with or without some modifications) was associated with survival outcome (Karayannopoulou *et al*., 2005; Peña *et al*., 2013; Santos *et al*., 2013; Santos *et al*., 2015b in Chapter 4). However, the histological grade has less prognostic value than its parameter nuclear pleomorphism by itself, indicating that tubule formation and mitotic counts may dilute, rather than strengthen, the prognostic value of the histological grade (Santos *et al*., 2015b in Chapter 4). Based on these results, searching for alternative grading parameters in malignant CMT is warranted.

In this study, eight morphological parameters were evaluated qualitative and semi-quantitatively (by defining a percentages and, whenever possible, categorized by cut-offs) in 59 malignant CMT of different histological subtypes. Of those parameters, necrosis, squamous differentiation, nucleolar morphology pattern, presence of scirrhous stroma and presence of abnormal nuclei all showed a significant association with at least one survival time, in univariable analyses. In multivariable models the set of variables included nuclear pleomorphism, vascular invasion and tumor size, whose prognostic value has been previously highlighted (Santos *et al*., 2014; Santos *et al*., 2015b in Chapter 4). Upon multivariable analysis, nuclear pleomorphism, vascular invasion and abnormal nuclei emerged as independent prognostic factors regarding DFI, whereas for OS, the independent prognostic factors were nuclear pleomorphism and necrosis (in more than 20% of the area of the tumor) (Table 2).

In this series, the existence of a component of micropapillary structures (*i.e.,* small clusters of cells lacking fibrovascular cores within a lumen) was not associated with prognosis. Micropapillary carcinoma is an aggressive variant of breast cancer, well known for its lymphotropism and poor prognosis (Zekioglu *et al*., 2004). Some evidence has been produced that this variant should also be considered in CMT (Gama *et al*., 2008; Gamba *et al*., 2013) and a recent classification scheme included this particular subtype of CMT (Goldshmidt *et al*., 2011). In the present study, micropapillae were diagnosed in 17 tumors but no significant association with vascular invasion/lymph node metastases (data not shown) or with survival times were detected. We hypothesize that the prognosis of malignant CMT could be related to the level of micropapillary differentiation, rather than to the presence *versus* absence of those structures. Nevertheless, further studies are needed to completely elucidate this issue. The discrepancy of our results, and those previously reported (Gamba et al., 2013), could also be caused by different interpretation regarding the description of micropapillary structures.

Necrosis has been regarded as a morphological marker of the growth rate of malignant tumors and its presence represents a sign of a discrepancy between tumor growth and blood supply (Leek *et al*., 1999). The prognostic significance of necrosis in malignant CMT was noted by other authors in univariable analysis

(*e.g.*, de las Mulas *et al*., 2005). According to our findings, the score of 20% of necrosis seemed valuable for prognosis definition in malignant CMT.

Occasionally, malignant CMT present components of non-glandular epithelial elements with squamous appearance, similarly to human breast cancer. In breast pathology their presence defined a metaplastic carcinoma, and has been considered an independent marker of worst prognosis (Rakha *et al*., 2015). Likewise, the squamous cell carcinoma of the mammary gland is considered a highly infiltrative and invasive tumor in female dogs (Misdorp *et al*., 1999). In the majority of cases in this series, the squamous elements were admixed with neoplastic glandular epithelium. Even if this mixed appearance (glandular / squamous) has been recognized for long (Hampe and Misdorp, 1974), the significance of the squamous component for the prognosis of malignant CMT has been less studied. In this study, the presence of squamous differentiation in a mixed pattern with other different neoplastic arrangements of the cells (tubular, papillary, solid) constituted a marker of short survival, especially when the squamous component accounted for more than 10% of the total of the cells. A similar threshold has been used by human pathologists to define metaplastic squamous carcinomas (Rakha *et al*., 2015). Previous studies were unable to correlate squamous metaplasia with malignancy and prognosis of malignant CMT (Monlux *et al*., 1977; Santos *et al*., 2013). In this vein, further studies are needed to confirm the association of squamous differentiation and a poor prognosis.

Nucleoli are one of the parameters used to assist in the nuclear grading definition (Elston and Ellis, 1991). However, the individual role of the morphology of the nucleoli, when assessed in routine stained sections, as a prognostic marker in malignant CMT has never been detailed. According to our results, large, multiple and atypical nucleoli, when presented in at least half of the neoplastic cells, represented a morphological predictor for high recurrence and metastization rates. Previous studies in CMT reported conflicting results as to the prognostic value of nucleolar features. Two studies did not observe an association between tumor recurrence or metastization and the number of nucleoli and/or the counts of the nucleolar organizer regions (AgNOR) (Bratulic *et al*., 1996; Löhr *et al*., 1997). AgNOR correspond to nucleolar proteins

detected by a silver stain as discrete black dots, and their area is directly related to the total nucleolar area and to the level of transcriptional activity (Derenzini *et al*., 2009). In other study, AgNOR counts (area and number per cell) in malignant CMT were associated with cancer-free survival in univariable analysis (Sarli *et al*., 2002). Considering our results, and those focusing on AgNOR estimations, further analyze nucleoli as putative prognostic markers in malignant CMT is advisable and unbiased quantitative methods would be of interest in this context. The nucleolar volume and its variability, similarly to the nuclear size pleomorphism, could be estimated in routine sections of the tumors using the stereological method point sampled intercepts (PSI) (Sørensen, 1992). This method has been successfully applied to estimate the nuclear size pleomorphism in both canine and human mammary carcinomas (Ladekarl *et al*., 1998; Santos *et al*., 2014 in Chapter 2) and nucleolar size and its variation in human melanomas (Sørensen *et al*., 1993).

According to our results the presence of intratumoral and/or peritumoral inflammation, including lymphoid infiltrates, was not of prognostic significance in malignant CMT. Still, the role of lymphoid infiltrates in the behavior of malignant CMT remains unclear (Kim *et al*., 2013). These infiltrates were either associated with lower recurrence rate (Kurzman and Gilbertson, 1986), or with the opposite, *i.e.,* higher histological grade and lymphatic invasion and shorter OS (Carvalho *et al*., 2011; Kim *et al*., 2013). In human breast pathology, the prognostic significance of lymphoplasmacytic infiltration within and around invasive carcinomas has been, and continues to be a subject of intense debate (Hoda, 2014). An infiltrate rich in plasma cells is usually observed in medullary carcinomas (Ellis *et al.*, 2003). The majority of non-medullary carcinomas with a prominent lymphocytic reaction tend to be poorly differentiated (Hoda, 2014). Some investigators have found that human breast carcinomas with a lymphoplasmacytic host response have a relatively favorable prognosis; while others found no difference or even a less favorable outcome (Ellis *et al.*, 2003).

In this study the observation of increased, fibrous and highly eosinophilic stroma around or forming septa within the tumor was associated with short OS. The scirrhous stroma, also known as desmoplastic reaction, has been regarded as a morphologic sign of a defense against invasive neoplastic cells in human breast

cancer (Parham *et al*., 1988). Interestingly, the amount of fibrous tissues produced has also been considered an indicator of the "age of the tumor", with longstanding tumors presenting larger amounts of stroma (Parham *et al*., 1988). These two hypotheses (highly invasive and longstanding tumors) are both compatible with a short cancer-specific OS observed in this study.

As regarding abnormal nuclei, they were defined as those with irregular shape and contour, following a similar description for feline mammary carcinomas (Mills *et al*., 2015). The individual prognostic value of abnormal nuclei in malignant CMT has never been reported, as far as we know. Using morphometrical methods on cytological smears of CMT of different subtypes, Simeonov and Simeonova (2007) reported a higher nuclear perimeter (expected in larger and irregular nuclei) in solid and anaplastic carcinomas comparing to benign tumors and tubulopapillary carcinomas. These authors also observed an increased nuclear morphometrical estimator (so called nuclear roundness) closely related to shape from benign to malignant tumors (Simeonov and Simeonova, 2006). However, none of these studies included follow-up data or survival analysis. Therefore the prognostic value of such cytological features could not be determined.

Despite the auspicious findings regarding the prognostic value of some morphological parameters, this study has some limitations. One of the major disadvantages of using morphological parameters for prognostic assessment is the existence of high intratumoral heterogeneity regarding a specific parameter (Ladekarl, 1998; Meyer *et al*., 2005). In this vein, the prognostic value of the parameters should not only be validated in larger prospective cohorts, but also submitted to a rigorous evaluation regarding their reproducibility. In order to cope with this caveat, the assessments in this study were made by two pathologists in a discussion microscope. Still, the assessment of intra- and interobserver variation remains mandatory for validation of their prognostic value (Webster *et al*., 2011). Moreover, methodologies allowing an objective and unbiased assessment of morphological parameters should be tested in future studies. In this perspective, stereological methods should be evaluated, as a point-counting technique to estimate relative volumes of necrosis,

squamous cell differentiation or scirrhous stroma or the PSI method to estimate the mean nucleolar volume and its variability, as previously described.

In conclusion, this study reinforced the independent prognostic value of nuclear pleomorphism and vascular invasion. The presence of necrotic areas in more than 20% of the tumor was also a marker of short OS. Regarding nuclei, not only the increased of the nuclear size and its variation seemed important for prognostic purposes, but also the presence of irregularities in their shape and outline. According to these results, the pathologist should routinely report the nuclear pleomorphism score, the existence of vascular invasion, the amount of necrosis and the proportion of cells with abnormal nuclei. Despite further prospective studies are needed to clarify their role for prognostic purposes, atypical nucleolar morphology, the existence of squamous cell differentiation and the presence of a desmoplastic reaction should also be described by the veterinary pathologists in their routine reports.

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# CHAPTER 8

## CANINE MAMMARY MALIGNANT TUMORS: NEW INSIGHTS IN THEIR GENETIC BACKGROUND

**Abstract**

Despite neoplastic mammary disease in female dogs represents a major health concern for owners and veterinarians, a step towards target and individualized therapy of the affected animals still has not been done. The growing knowledge regarding malignant canine mammary tumors (CMT) led to the view of CMT as a model for human breast cancer. However, the genetic basis of CMT and its homology with human breast cancer is still poorly characterized. In this study we performed high resolution oligonucleotide array comparative genomic hybridization (aCGH) to assess copy number changes in ten malignant CMT from seven female dogs. The series included synchronous multiple tumors from three female dogs. In eight tumors genomic imbalances were detected, with wider variation of losses, deletions and gains. The chromosomes CFA 9, 22, 27, 34 and X were more frequently affected. In two tumors aberrations on the Xp21.3-p11.21 segment, which includes the PCDH11X gene also called protocadherin 11 X-linked were detected. Dissimilar aCGH ratio profiles were noted in synchronous tumors of the same animal and this provided preliminary evidence for a possible independent pathogenesis of multiple CMT. By analysing two samples of the different halves of two tumors, it was noticed that regional differences in the number of genomic imbalances could exist in larger tumors. Despite the small number of cases, this study already demonstrated that malignant CMT of different histological subtypes can displayed a wide range of genomic aberrations affecting different chromosomes.

## 8.1 Introduction

Cancer represents the leading health concern among dogs and research on anticancer treatments is of utmost relevance for animal health (Morris Animal Foundation 2005). Mammary gland tumors are the most frequent neoplasia in female dogs in countries where the spaying is not routinely performed early in life (Sorenmo, 2003). Approximately half of the tumors are classified as malignant based on the histopathological examination (Lana *et al*., 2007). However the evidence of malignant morphological features does not always imply an aggressive biological behavior with development of distant metastases. The establishment of reliable markers for predicting malignant canine mammary tumors (CMT) metastatic capacity is an unresolved issue that limits the implementation of new therapeutic options (Matos *et al*., 2012).

The heterogeneity both at the clinical and morphological level is an idiosyncratic characteristic of the CMT (Sorenmo *et al*., 2011). CMT can appear as a solitary nodule or, more frequently, as multiple synchronous nodules, with variable macroscopic and microscopic features (Perez-Alenza *et al*., 2000; Hellmén, 2005; Sorenmo *et al*., 2009). Despite the common occurrence of synchronous multiple CMT, little is known about the interrelation between the lesions. The hypothesis that malignant CMT could arise from pre-existing benign tumors has been debated (Sorenmo *et al*., 2009). In human oncology, clarifying the relation between multiple breast nodules is considered of chief importance for establishing therapeutic strategies (Bendifallah *et al*., 2010). Genetic tests represent a suitable methodology to assess the relatedness of multiple synchronous tumors (Teixeira *et al*., 2002; 2004; Agelopoulos *et al*., 2003). For instance, radical surgical procedures would be recommended when intramammary spreading of neoplastic spreading cells seemed to be the mechanism responsible for the multiplicity of tumors (Bendifallah *et al*., 2010).

At the microscopic level CMT are also high heterogeneous. Accordingly, tumors can be composed of luminal epithelial cells exclusively, or can have a biphasic and triphasic phenotype, with myoepithelial cells and metaplastic mesenchymal elements such as cartilage or bone tissue. Mammary tumors involving different morphological cell populations are more frequent in dogs than in humans

(Misdorp *et al*., 1999; Misdorp, 2002; Lakhani *et al*., 2012; Reis-Filho *et al*., 2012).

Human breast cancer is well characterized at the genomic level, primarily through classical cytogenetic and comparative genomic hybridization (CGH), techniques that provide a global picture of the whole genome organization (Teixeira *et al*., 2002; 2004). The latter is a method that detects unbalanced structural changes resulting in a physical change in copy number of a genomic region or in the whole chromosome (Reis-Filho *et al*., 2005). The resolution of CGH has been improved by the introduction of array based approaches (Costa *et al*., 2008; Tan and Reis-Filho, 2008). The usefulness of CGH technology in the classification, grading and prognosis assessment of human breast cancer is described in detail in several reviews (Reis-Filho *et al*., 2005; van Beeers and Nederlof, 2006; Climent *et al*., 2007). Moreover, CGH offers the opportunity to evaluate the degree of clonal association between multiple synchronous tumors, as well as the presence of intratumoral genetic heterogeneity (Agelopoulos *et al*., 2003; Teixeira *et al*., 2004; Wa *et al*., 2005; Torres *et al*., 2007).

To date only two studies described genomic aberrations in CMT (Beck *et al*., 2013; Liu *et al*., 2014). Canine microarray-based CGH has been developed in the last years (Thomas *et al*., 2007; 2008; Breen and Thomas, 2012). A high resolution canine oligonucleotide array CGH (aCGH) platform is now available (Breen and Thomas, 2012), offering valuable opportunity to identify DNA copy number imbalances associated with carcinogenesis in different tissues. Additionally, the study of the type and nature of recurrent genomic DNA copy number aberrations (CNAs) in CMT should contribute to identify regions of canine genome containing genes associated with the mammary carcinogenesis process, as demonstrated for other canine malignancies (Thomas *et al*., 2007; Breen and Thomas, 2012)

The aim of this study was to identify CNAs associated with malignant CMT using aCGH analysis. For this purpose a series of spontaneous malignant CMT of different histological type were chosen to reflect the usual heterogeneity observed in the diagnostic routine. In order to detect eventual different clonal populations within tumors, two paired samples were collected from the same

tumor whenever possible. Moreover, multiple synchronous CMT of the same female dog were also analyzed, aiming to evaluate their clonal relatedness.

## 8.2 Material and methods

### *8.2.1 Clinical cases, histologic and immunohistochemical study*

In this study ten spontaneous malignant CMT and one normal mammary parenchyma sample from seven female dogs that underwent surgical treatment in the UPvet, veterinary clinics of the University of Porto were included. From three of those female dogs, tissue samples from two macroscopically independent synchronous mammary tumors were analyzed. Additionally, for two tumoral lesions two paired samples from separate halves of the mass were studied. The owners provided informed consent and the study protocol was approved by the Ethics Committee of the ICBAS. Fresh frozen samples were stored at -80ºC until processed for DNA extraction by standard methods.

The remaining tumoral specimens were routinely processed and slides stained with hematoxylin-eosin (H&E) were used for histopathological classification and grading. The histological diagnosis and grade was performed by two observers (MS and PDP) using the official classification and the Nottingham grade method, respectively (Elston and Ellis, 1991; Misdorp *et al*., 1999, Karayannopoulou *et al*., 2005). Immunostaining using a panel of epithelial, mesenchymal and myoepithelial cells markers was performed (Peña *et al*., 2014) to confirm the histological diagnosis and assess cellular heterogeneity within the tumors (Table 1).

### *8.2.2 Array comparative genomic hybridization*

Recurrent CNAs were assessed by analysis of extracted DNA using ~180,000-feature microarray (design 025522, Agilent Technologies, Santa Clara, CA) comprising repeat-masked ~60-mer oligonucleotides distributed at approximately 13kb intervals throughout the dog genome sequence assembly (canFam version 2.0, May 2005) (Thomas *et al*., 2014). The reference DNA for each array comprised an equimolar mix of blood-derived DNA from six unrelated healthy dogs of the same gender and breed as the patient. Array image files were processed using Feature Extraction version 10.7 (Agilent

Technologies) and raw data were filtered to exclude probes exhibiting non-uniform hybridization or signal saturation. Data were imported into Nexus Copy Number version 7 (Biodiscovery, El Segundo, CA) and recurrent CNAs within each tumor were defined using the FASST2 segmentation algorithm, based on a minimum of three consecutive probes with log2 tumor:reference values > 0.2 (gain) or ≤ -0.2 (loss), resulting in an effective resolution of ~26kb (two intervals of ~13kb). High level gains and losses within individual tumors were defined using default log2 tumor: reference values of > 1.14 and ≤ -1.1, respectively. These data were narrowed using the 'peaks only' function to report the minimal regions within these segmented CNAs that show the highest penetrance within this series. Genes and uncharacterized coding sequences within regions of CNA were defined using the UCSC canine genome sequence browsers (www.genome.ucsc.edu/) and the Gene database (www.ncbi.nlm.gov/gene).

**Table 1** – Panel of antibodies used for immunohistochemical evaluation of canine malignant mammary tumors.

| **Antibody** | **Clone** | **Dilution** | **Antigen retrievel / Incubation** | **Source** |
|---|---|---|---|---|
| Pancytokeratin | AE1-AE3 | 1:300 | Water bath with Target Retrieval Solution (Dako) / Overnight | Zymed |
| CK 14 | LL002 | 1:10 | Water bath with Target Retrieval Solution (Dako) / Overnight | Serotec |
| α-Actin | HHF35 | 1:300 | Water bath with Target Retrieval Solution (Dako) / Overnight | Dako |
| Vimentin | V9 | 1:500 | Water bath with Target Retrieval Solution (Dako) / Overnight | Dako |
| p63 | 4A4 | Ready-to-use | Pressure cooker with sodium citrate buffer / Overnight | Ventana |

## 8.3 Results

In this series, five complex carcinomas (CC), three simple carcinomas (SC) and two carcinosarcomas (CS) were included. The age and breed of the female dogs and macroscopic characteristics of the tumors are provided in Table 2. Relevant histopathological features of the tumors are summarized in Table 3. In

all the cases, tumor cells presented invasive features of the surrounding stroma. Simple carcinomas were characterized by a proliferation of luminal epithelial cells with some resting myoepithelial cells. In the complex tumors some degree of non-atypical myoepithelial proliferation, corresponding to p63 positive cells was observed. In the two carcinosarcomas, atypical, fusiform, vimentin positive, mesenchymal cells and/or foci of metaplastic immature cartilage (with endochondral ossification in one case) were observed. In all tumors, except two (MS3T2 and MS33T2) CK14 positive luminal epithelial cells were observed.

**Table 2** – Epidemiological and clinical characteristics of the seven female dogs with malignant mammary tumors.

| Case | Age (years) | Breed | Tumor | Mammary gland pair | Tumor size (cm) |
|---|---|---|---|---|---|
| I – MS10 | 13 | Mixed-breed | T1 | M3 right | 6 |
| II – MS27 | 10 | Mixed-breed | T1 | M4 left | 1.5 |
| | | | T2 | M4-M5 left | 1.5 |
| III – MS3 | 11 | Mixed-breed | T2 | M4 left | 2.5 |
| IV – MS33 | 12 | Retriever Labrador | T2 | M4 right | 2 |
| | | | T3 | M5 left | 4 |
| V – MS4 | 10 | Siberian Husky | T1 | M5 right | 1 |
| | | | T2 | M4 right | 0.5 |
| VI – MS7 | 10 | Mixed-breed | T1 | M4 right | 6 |
| VII – MS5 | 16 | Mixed-breed | T2 | M2 left | 2.5 |

**Table 3** – Histopathological features and aCGH copy number changes (CNAs) of 10 canine mammary malignant tumors.

| Case | Tumor | Diagnosis | Grade | Nuclear grade | Samples | CNA's |
|---|---|---|---|---|---|---|
| I – MS10 | T1 | CC | II | 3 | T1a | Highly complex 26 + del of 11, 19 and 22 and gain of 24 in <25% of the cells. |
| II – MS27 | T1 | CC | II | 2 | T1a | gain of 4, 9 and 13, also starting to show loss of 12 and 27 |
| | | | | | T1b | gain of 4, 9 and 13, also starting to show loss of 12 and 27 |
| | T2 | SC | II | 2 | T2b | Broad baseline |
| III – MS3 | T2 | CC | II | 2 | T2b | del 22, gain 34 (with del) |
| IV – MS33 | T2 | CC | I | 3 | T2b | del 22, gain 34 (with del) |
| | T3 | CS | II | 2 | T3c | loss on Xp |
| V – MS4 | T1 | CC | II | 2 | T1a | no major CNAs |
| | T2 | SC | II | 2 | T2a | no major CNAs |
| VI – MS7 | T1 | CS | II | 1 | T1a | loss 3, loss 5, del 6 dist, gain 9 (with del), gain 12 dist, loss 15, gain 17, loss 18, gain 24, loss 27, gain on Xp |
| | | | | | T1b | gain on Xp |
| VII – MS5 | T2 | SC | II | 2 | T2 | Broad baseline, complex 15, 18, 23, 26 |

High-resolution aCGH revealed that the DNA sample from the normal parenchyma (collected from MS7 animal) showed no CNAs. In all but two tumors genomic imbalances were detected (Table 3); on average three alterations per tumor sample were detected (range 0-11, excluding two samples with broad baseline and wider aberrations). Those aberrations were distributed throughout the karyotype and more commonly involving segments of different chromosomes. Most of the CNAs detected were whole chromosome aneuploidy with a mixed pattern of gains and losses identified in the majority of the samples; only two samples presented with a single chromosomal imbalance (Table 3). Losses/deletions (n=21) were more frequent that gains (n=13) and more complex changes (segmental aneuploidy) being even less common (n=8).

The most common CNAs were detected on CFA22 (three tumors showed loss of segments), CFAX (MS7T1 showed gain in Xp21.3-p11.21 and MS33T3 showed deletion of the same region; both showed gain in the Xq21.2), CFA27 (two tumors showed loss), CFA34 (MS3T2 and MS33T2 exhibited similar complex pattern with gains and losses) and CFA9 (MS27T1 showing gain and MS7 showing gain with deletion). In four samples complex chromosome with simultaneous gains and deletions were detected (Table 3).

Both tumors that exhibited aberrations in the Xp21.3-p11.21 and Xq21.2 were carcinosarcomas presenting chondroid elements with high cellularity and immature matrix (Fig. 1 and Fig. 2).

A similar pattern of CNAs was detected in two complex carcinomas from different patients (MS3 and MS33) corresponding to deletion of CFA22 and gain with deletion on CFA34; these tumors had some level of phenotypical concordance, namely the presence of myxoid matrix, squamous metaplasia in some epithelial malignant cells and the absence of CK14 positive epithelial cells (Fig. 3).

In two complex carcinomas (MS10 T1 and MS27T1) additional chromosome gains and losses were detected in less than 25% of the total cell population.

The two synchronous contra-lateral tumors of case MS33 shared few morphologic similarities (different histological subtype, different histological grade and different nuclear pleomorphism grade) and did not share genomic imbalances. In case MS27, the T2 produced wider spreader of the data points suggestive of poor quality DNA sample, which jeopardized a reliable

interpretation. However, side-by-side inspection of the ideograms showed that the CNAs of T1 (gain of CFA 4, 9 and 13) were not precisely matched in T2. Both tumors of case MS4 had no detectable CNAs.

The paired samples from the two halves of T1 from MS27 female dog revealed identical aCGH ratio profiles, while the paired samples of T1 from MS7 female dog shared only genomic gains on segments of CFAX (Xp21.3-p11.21 and Xq21.2), with one sample presenting a much larger number of chromosomes imbalances (Fig. 4).

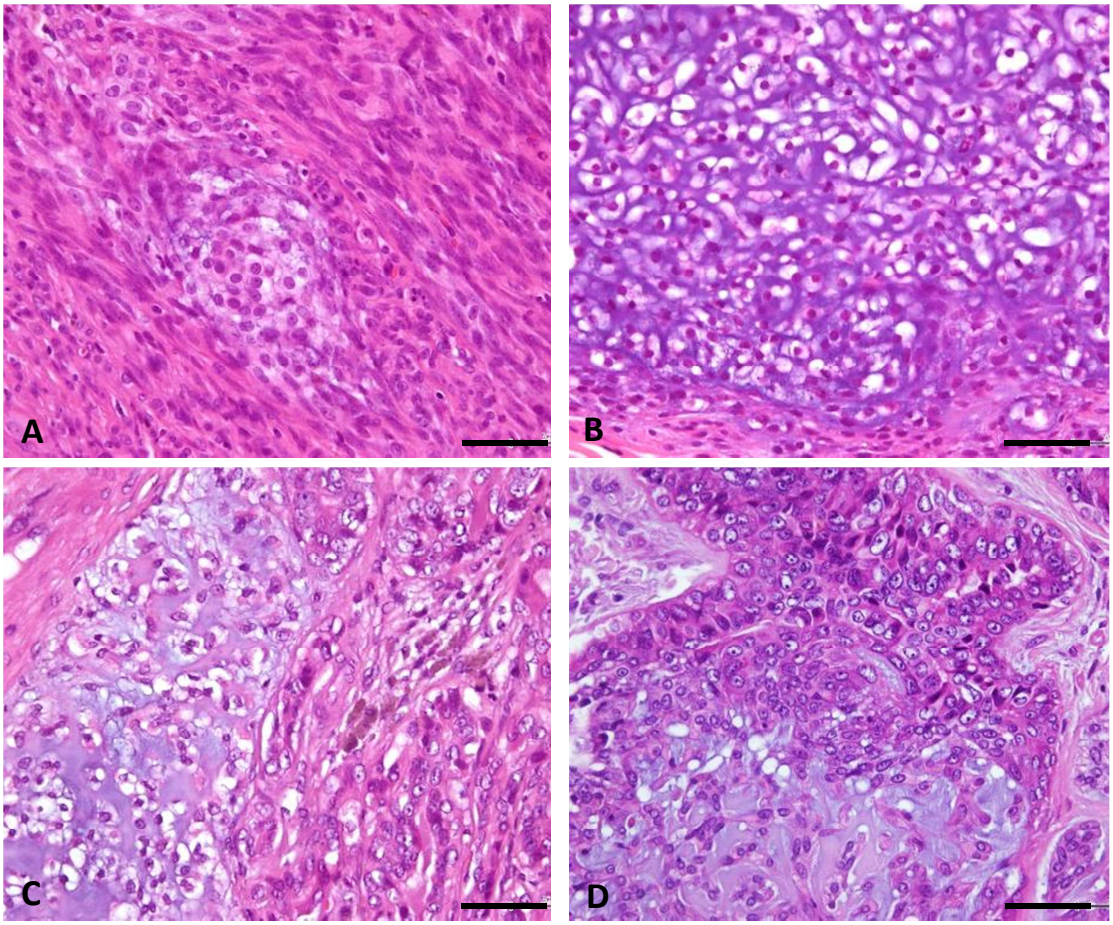


**Fig. 1** – A and B – Carcinosarcoma (MS7 T1); A – mesenchymal fusiform cells are intermixed with foci of round tightly packed cells that resemble condroblasts; B – presence of immature cartilage with high cellularity and scarce basophilic matrix. C and D – Carcinosarcoma (MS33 T3); C – foci of immature cartilage and epithelial tubular structures; D – detail of a focus of pre-cartilaginous tissue showing continuity with luminal epithelial cells. Hematoxylin-eosin; Bar 50 µm (A, B and D) amd 100 µm (C)

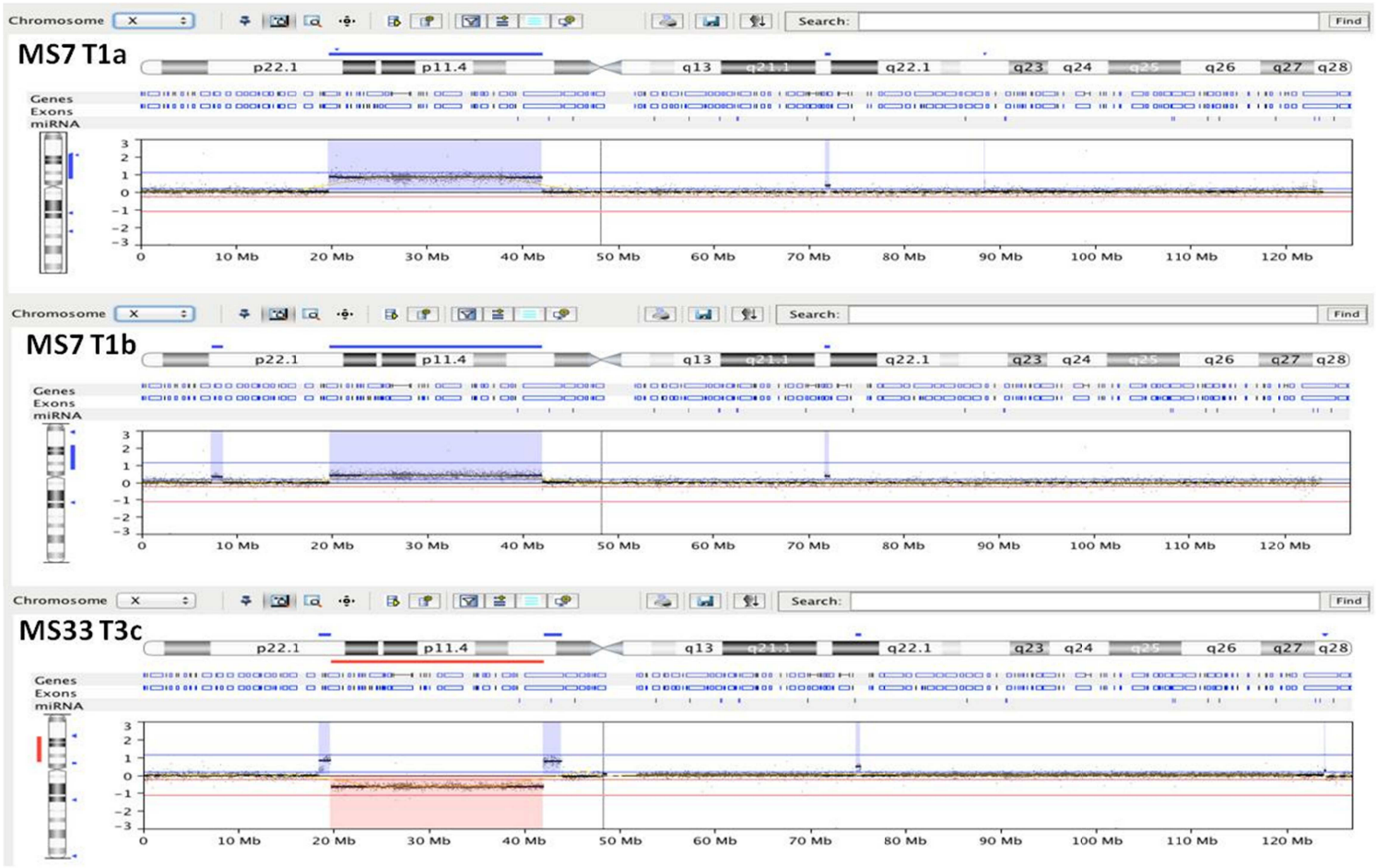


**Fig. 2 –** aCGH profiles of chromosome CFAX in two canine mammary carcinomas. The same region of Xp (19.7Mb – 42Mb) was gained (blue) in both samples of tumor T1 from MS7 animal and was deleted (red) in the tumor T3 from animal MS33. On the other hand the segment Xq21.2 was gained in both tumors.

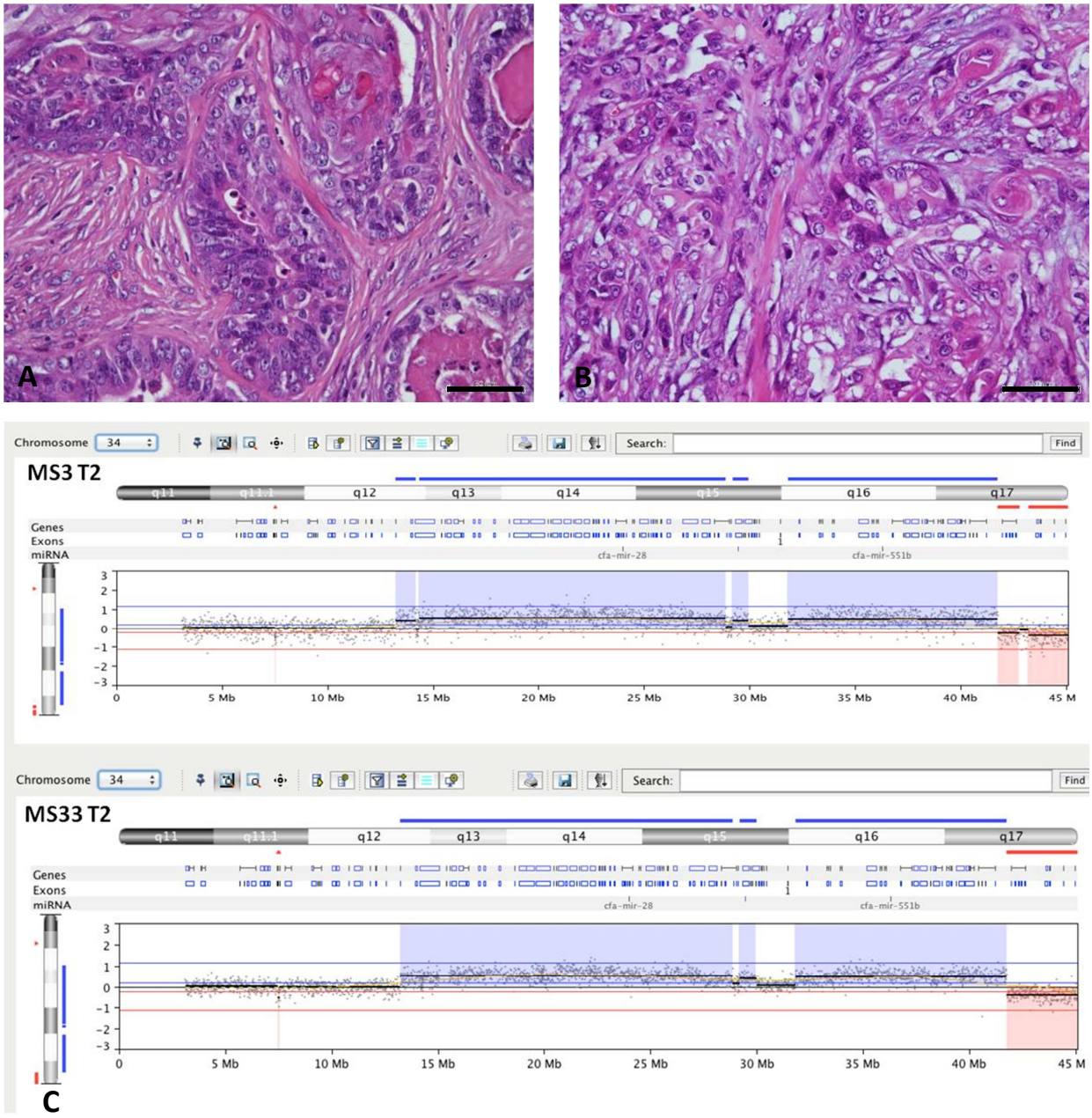


**Fig. 3** – Complex carcinomas from two female dogs (A – MS3T2; B – MS33T2) that completely shared copy number aberrations and some phenotype features, such as squamous metaplasia. C – aCGH profile along the length of the CFA34 in both tumors showing a similar pattern of gains (blue) and deletion (red). Hematoxylin-eosin; Bar 50 µm (A and B).

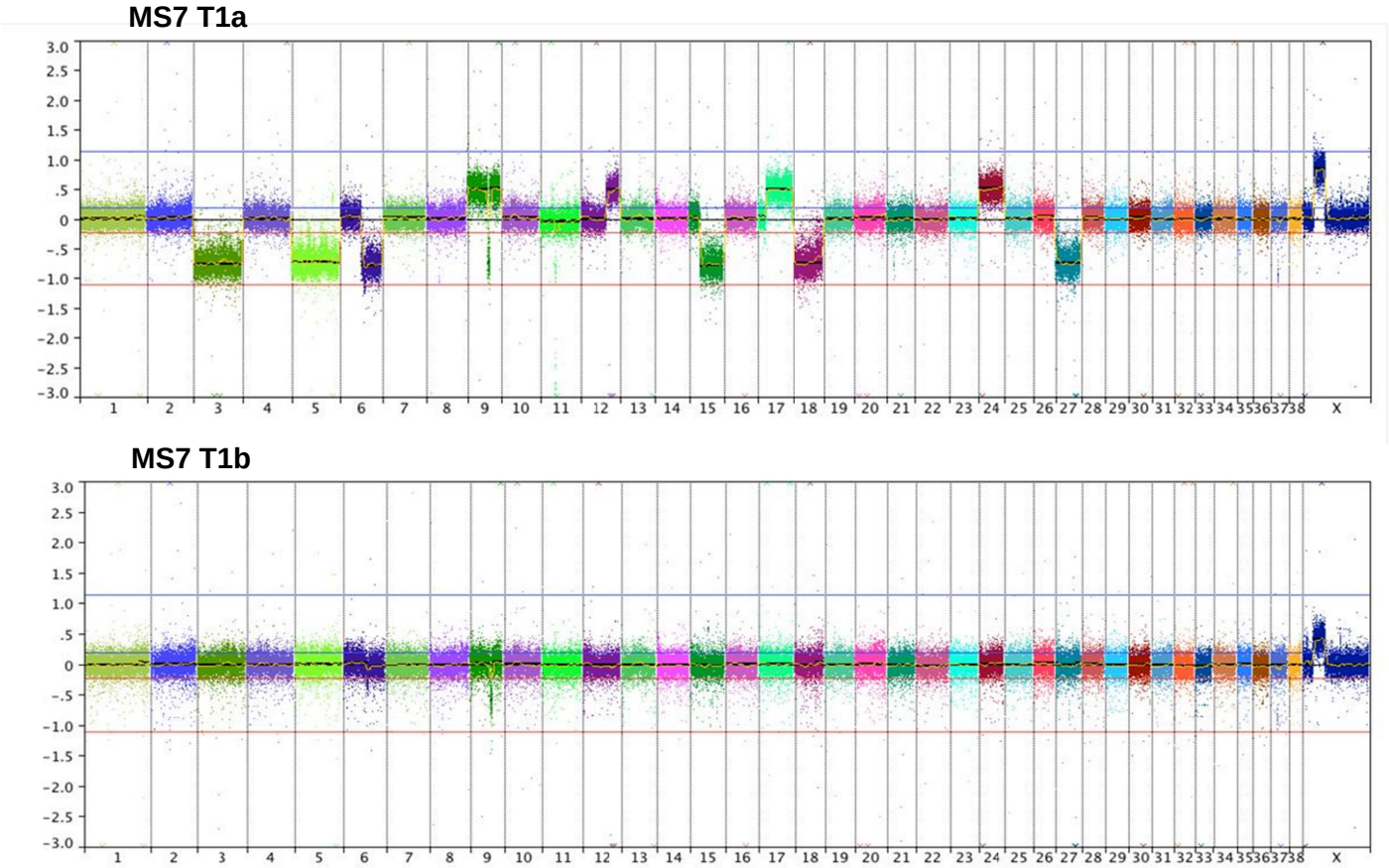


**Fig. 4** – Whole genome aCGH profiles of the samples from the two halves of the tumor T1 from animal MS7. The log 2 ratio representing cut-offs of genomic gains and losses are indicated by the horizontal bars immediately above and below the midline black line, which represents normal copy number. The two samples shared only the genomic aberration of CFAX.

## 8.4 Discussion

Malignant CMT constitute a highly heterogeneous disease both in terms of morphology and biological behavior (Sleeckx *et al*., 2011), which certainly contributes to the challenge for prognostic stratification faced by veterinary oncologists and pathologists (Matos *et al*., 2012). Based on the similarity in some clinicopathologic and epidemiologic features, spontaneous CMT have been regarded as potential models for human breast cancer (Queiroga *et al*., 2011). Molecular studies provided some evidence that some drivers of the mammary neoplastic process are shared by dogs and humans (Rivera and von Euler, 2011), however the amount of evidence supporting the dog as a model for human cancers, including breast cancer, is still limited (Breen, 2008; Rotroff *et al*., 2013).

Few conventional cytogenetic studies in CMT have been published (Mellink *et al*., 1989; Mayr *et al*., 1990; 1993). Classic cytogenetic characterization of tumor-associated chromosomal abnormalities is challenging because of the large number of canine chromosome, their small size and acrocentic morphology (Tap *et al*., 1998; Thomas *et al*., 2008). Abnormalities in CFA 8 or 11, 13 or 15, 37, 38 and X, including loss, formation of isochromosomes and centric fusion were reported by using fluorescence in situ hybridization technique (Tap *et al*., 1998). The development of cytogenetically-validated genome integrated bacterial artificial clones for each dog chromosome, allowing application of CGH techniques, which overcome the limitations of classic cytogenetic analyses (Thomas *et al*., 2007; 2008). CGH has been employed to elucidate the molecular events involved in the pathogenesis of different canine tumors, including lymphoma, osteosarcoma, venereal transmissible tumor, histiocytic sarcoma, among others (Breen and Thomas, 2012; Thomas *et al*., 2014). Very few studies addressed the genomic-wide basis of the canine mammary carcinogenesis using modern and high-resolution techniques (Beck *et al*., 2013; Liu *et al*., 2014). In this study we used aCGH to perform a global analysis of DNA copy number changes in malignant CMT of different histologic subtypes. The aCGH analysis demonstrated a broad spectrum of chromosome losses and gains distributed throughout the genome. Even within each histologic subtype group a heterogeneous pattern of genomic aberrations was

identified. The high variation of genomic aberrations and the absence of commonalities in CNAs in CMT of the same histologic subtype has already been reported (Beck *et al*., 2014), suggesting that the clinical and morphologic variability of CMT may reflect heterogeneity at genomic level.

Two of the ten cases in this study presented with no detectable CNAs. A similar finding was previously reported by two other research groups in five complex carcinomas and one simple carcinoma (Beck *et al*., 2013; Liu *et al*., 2014). However, it should be kept in mind that the absence of numerical chromosome changes in aCGH analysis does not exclude the possibility of structural genomic aberrations, which are undetected by CGH (van Beeers and Nederlof, 2006; Climent *et al*., 2007). Moreover, epigenetic events such as methylation or histone modifications could also be involved in canine mammary carcinogenesis (Liu *et al*., 2014). We should also stress that the absence of CNAs in two tumors could be related to sampling bias (those tumors were the smaller ones of this series and were admixed and surrounded by a hyperplastic mammary parenchyma – data not shown). Factors such as contamination of the tumor sample with normal/hyperplastic surrounding cells or intratumoral stromal cells could be an explanation for the absence of major CNAs in those tumors.

This is the first report of numerical aberrations in chromosomes 22 and 34. Corroborating previous findings, we also observed losses of CFA 3, 5, 18 and 27, however the frequency of losses of CFA 27 in our series was lower (2/10 cases) than that previously reported (Beck *et al*., 2013). Accordingly, our results did not support the hypothesis that malignant CMT are associated with a recurrent deletion of CFA 27 (Beck *et al*., 2013). Herein genomic imbalances were detected in all, except one complex carcinomas, including well (grade I) and intermediately (grade II) differentiated cases. In this vein, our results did not support a previous report where major CNAs were not observed in complex carcinomas (Liu *et al*., 2014).

In human breast invasive carcinomas recurrent genomic alterations included gain of 1q, 8q, 20q, 17q and loss of 16q and 17p (Reis-Filho *et al*., 2005; Torres *et al*., 2007). Gains of 3q, 8q, 17q has been associated with poor prognosis, whereas loss of 16q correlated with a favorable prognosis (Reis-Filho *et al*., 2005; Horlings *et al*., 2010). Interestingly, we observed gains in the canine

chromosomes homologues (CFA 9, 13, and 24) to the human chromosome segments 17q, 8q and 20q. Similarly, loss of CFA 5 that had homology with the human 16q and 17p segments was identified in a case included in our series (Breen *et al*., 1999).

Particularly worthy of note was the detection of genomic imbalances (gains) in the Xq21.2 segment. This region including the PCDH11X gene also called protocadherin 11 X-linked. This gene belongs to the protocadherin gene family, a subfamily of the cadherin superfamily (Berx and van Roy, 2009). The encoded protein consists of an extracellular domain containing 7 cadherin repeats, a transmembrane domain and a cytoplasmic tail that differs from those of the classical cadherins (Yang *et al*., 2005). Protocadherin 11 X-linked is highly homologous to protocadherin 11 Y-linked, which is found exclusively in man (Berx and van Roy, 2009). Deletions involving different protocadherin genes (but not PCDH11X) have been reported in malignant CMT (Liu *et al*., 2014). The role of the protocadherin 11 X-linked in CMT, as well as in breast cancer is unknown, to the author's knowledge. However there are evidences that PCDH11Y is up-regulated in human prostate cancer cell lines and could be a proto-oncogene (Berx and van Roy, 2009). In human prostate cancer cell lines, the expression of PCDH11Y was correlated with the occurrence of nuclear β-catenin (Yang *et al*., 2005). Interestingly the aberrations of the segment containing the PCDH11X gene were detected in carcinosarcomas presenting p63 positive cells and foci of immature metaplastic cartilage. In the light of the human classification system these tumors would be considered as metaplastic carcinomas with mesenchymal elements or mixed metaplastic carcinomas (Reis-Filho *et al*., 2012; Brogi *et al*., 2014). Immunohistochemistry studies have demonstrated that a subgroup of human metaplastic carcinomas displayed nuclear β-catenin expression, which is indicative of Wnt pathway activation and of epithelial-to-mesenchymal activation (Lacroix-Triki *et al*., 2010; Geyer *et al*., 2011). Moreover alterations in β-catenin expression and perturbation of Wnt pathway have also been reported in malignant CMT (Restucci *et al*., 2007; Uva *et al*., 2009).

It is still not clear whether multiple CMT have an independent origin or represent multifocal clonal-related neoplastic lesions. More often the multiple CMT

present different clinical (*e.g.*, size and consistency) and morphological features, including different histological classification (Sorenmo *et al*., 2009). Despite the fact that we have analyzed only three cases of multiple synchronous CMT, our morphologic data tended to support that observation. However, the independence or relatedness of multiple synchronous tumors should be confirmed at the genome level (Weiss *et al*., 2003). In this study, the synchronous tumors of two female dogs (excluding tumors of case MS4 that produced balanced profiles) revealed dissimilar aCGH ratio profiles and this provided preliminary evidence for a probable independent pathogenesis of multiple CMT. In human pathology the use of CGH (including array-based) has determined the clonal relationship between synchronous *in situ* and invasive breast carcinomas (Agelopoulos *et al*., 2003; Hwang *et al*., 2004; Teixeira *et al*., 2004; Wa *et al*., 2005). The current evidence for women is that synchronous ipsilateral breast carcinomas seem to be clonally related, whereas bilateral multiple tumors represent clonally independent tumors (Agelopoulos *et al*., 2003; Teixeira *et al*., 2004; Wa *et al*., 2005). Further genetic studies are warranted to fully ascertain the relation between multiple synchronous CMT. This issue would have major implications in treatment protocol and thus would be of great clinical importance.

Although our analysis was limited to two cases, it preliminary demonstrated that related sub-clones could coexisted in some malignant CMT. Notably, in the larger (size > 5 cm) and highly heterogeneous tumor, the samples collected from the different halves of the mass shared only one CNA, while the two samples of the smaller (size < 3 cm) and less heterogonous tumor harbored a similar pattern of CNAs. At this point our results show that a single sample from large tumors may fail to detect all existing DNA copy number changes. Moreover, the hypothesis that clonal evolution in malignant CMT goes along with the increased of tumor size and increased phenotype heterogeneity deserves further investigation. It is important to recall that increased tumor size was associated with malignancy of CMT and it has been consistently considered a prognostic marker in malignant CMT (Sorenmo *et al*., 2009; 2011; Santos *et al*., 2014).

In conclusion, aCGH analysis of malignant CMT showed that the clinical and histological heterogeneity of this disease may reflect a wide variation of genomic changes. A large spectrum of genomic imbalances was observed in different histological subtypes of malignant CMT. Our results support that protocadherin 11 X-linked could be involved in canine mammary carcinogenesis, namely in the development of tumors with morphological evidence of mesenchymal differentiation.

Despite the novelty of the majority of the findings of this study, some limitations can also be pointed. The number of analyzed tumors is limited and the number of non-tumor cells within samples was not controlled, which could have reduce the sensitivity for detecting CNAs (van Beers and Nederlof, 2006, Natrajan *et al*., 2010). For future studies, selection of tumoral areas enriched with neoplastic cells and free of stromal, inflammatory and normal surrounding parenchyma should be done by microdissection. Moreover, the aCGH findings deserve validation by independent cell-based method such as FISH or quantitative polymerase chain reaction.

## 8.5 References


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# CHAPTER 9

## CONCLUSIONS

## 9.1 Conclusions and future perspectives

The expansion of knowledge in malignant canine mammary tumors (CMT) biology has still not been reflected on treatment changes of affected animals. Nevertheless, refinements in the management of female dogs with malignant CMT may be markedly influenced by the validation of prognostic factors (Matos *et al*., 2012). In human breast cancer, well defined and validated histopathological predictors, such as histological grade, are routinely used for assisting in prognosis definition and subsequent appropriate stratification of therapy (Rakha *et al.,* 2010).

Traditionally, the histological grading method for malignant CMT has followed human grading systems. Nonetheless, canine-specific morphological and clinical features should be taken into account when classifications systems originally designed to human breast cancer are adapted to malignant CMT (Sassi *et al*., 2010). While in human breast cancer carcinomas of no special type (previously known as ductal carcinoma) comprise the vast majority of cases (Ellis *et al*., 2012), in malignant CMT series the histological variability is much larger. For instance, the presence of myoepithelial proliferative cell population, whose biological role is still a matter of debate, is relatively common in malignant CMT, but a rare finding in human breast cancer (Misdorp 2002; Lakhani *et al*., 2012; Reis-Filho *et al*., 2012; Peña *et al*., 2014).

In human medicine, the current trend is the early detection of sub-clinical breast cancer, reflecting the widely adoption of screening programs (Hoda *et al*., 2014). On the other hand, in veterinary medicine we are still diagnosing very large and clinically advanced tumors, usually detected by the owner. In this vein, we hypothesized that the prognostic importance of the histological markers, including histological grade, will depend on its accuracy to address those morphological and biological specific features of malignant CMT.

Current grading methods for malignant CMT were originally designed to human breast cancer, and were adopted more or less blindly, in the sense that a comprehensive evaluation of several aspects of the method was not performed before its adoption. In fact, such adoption to CMT requires a great deal of detail, particularly in the selection of relevant grading parameters, scoring criteria and in the evaluation of their reproducibility. Moreover, the clinical utility of

quantitative morphological features, estimated by using more objective techniques, such as stereology and morphometry, has never been tested in CMT. This markedly contrasts with human pathology, where quantitative estimations of histopathological parameters have prove to add objectiveness and augment the prognostic power of breast cancer grading (Ladekarl, 1998; Kronqvist *et al*., 2002). In fact, in human breast cancer, different quantitative measurement methods have been reported trying to objectivize the three grading parameters (tubule formation, nuclear pleomorphism and mitotic count) (Ladekarl and Sørensen, 1993a; b; Artacha-Pérula and Roldán-Villalobos, 1997; Kronqvist *et al*., 1998; 2000; 2002).

In the first study of this Thesis (Chapter 2), the value of $\bar{v}_V$ for diagnosis and grading CMT was evaluated. The $\bar{v}_V$ is considered one of the most robust estimates of nuclear 3D size and pleomorphism, being obtained from simple measurements carried out in 2D samples of the tumors, such as the sections used in the routine diagnostic setting (Sørensen, 1992; Ladekarl, 2004). The unbiasedness of the stereological method and the recognized value of $\bar{v}_V$ as a prognosticator in breast cancer were the main reasons to pursue with its estimation in CMT. Our study demonstrated that $\bar{v}_V$ estimations were significantly higher in malignant CMT comparing to benign tumors, with only a small proportion of overlapping $\bar{v}_V$ values between the two types of tumors. This result supported the classification benign *versus* malignant made by the pathologists, as well as the eventual role of $\bar{v}_V$ for grading purposes in case of malignancy. Accordingly, we also compared the $\bar{v}_V$ in carcinomas of each nuclear pleomorphism score (performed following the NHG criteria). Carcinomas with nuclear score 1 or 2 presented similar values of $\bar{v}_{V,}$ which were significantly lower than those obtained in tumors scored 3. This finding resulted in two remarks: firstly, the similarity in the 3D nuclear size and variability could contribute to low reproducibility in the subjective assignment of score 1 or 2 by pathologists; secondly, a simplified binary scoring of nuclear pleomorphism in CMT could be more accurate. The results also suggested that $\bar{v}_V$ could be valuable for identify the most aggressive tumors ($\bar{v}_V$ was significantly higher in cases that presented post-surgical progressive disease).

The study displayed in Chapter 2 was recognized as the first report on the use of stereology in veterinary oncology (Casteleyn *et al*., 2014), and the promising preliminary observations deserve further confirmation in large prospective survival studies. Hopefully, with this quantitative tool it will be possible to determine quantitative thresholds for allocating tumors with different nuclear grade and for predicting their clinical behavior.

Interestingly, the $\bar{v}_V$ values in epithelial cells of the normal mammary parenchyma during the different phase of the estrous cycle showed a particularly high variation. This finding reinforces previous evidences that the normal canine mammary gland undergoes profound structural, cellular and subcellular changes during the estrous cycle (Rehm *et al*., 2007; Santos *et al*., 2010). The pathologist awareness of this variation is important when evaluating and grading mammary lesions. The stereological study of nuclear size pleomorphism in the normal canine mammary gland will be extended, by the inclusion of a large sample of cases that have been previously characterized, both at the histological and immunohistochemical levels (Santos *et al*., 2010). The NHG criteria recommend the use of the normal surrounding parenchyma of a malignant CMT as a reference for scoring nuclear pleomorphism (Elston and Ellis, 1991; 1998). In this vein, it would be important to verify if the level of variability in nuclear volume and pleomorphism (as estimated by the $\bar{v}_V$) in the normal surrounding epithelial cells could be a contributing variable accounting for interobserver scoring discrepancy in nuclear pleomorphism.

Still in Chapter 2, the previously reported evidence that tumor size and vascular /lymph node invasion represented relevant prognostic factors in CMT was reinforced. In this study, a ROC curve analysis was used, since this is the gold standard test for establishing reliable thresholds (Greiner *et al*., 2000). A tumor size of 2.9 cm was recognized as a valuable in order to assess early tumoral aggressiveness, as evidenced by vascular invasion or lymph node metastases at the time of the diagnosis. Hopefully, this cut-off could serve as guide for clinical treatment choices, warning clinicians for the need of a more rigorous and meticulous pre-surgical lymph node evaluation.

Additionaly, the issue regarding the interobserver reproducibility in assigning a score to the grading parameters was introduced and the statistical analysis

revealed only a modest agreement in scoring nuclear pleomorphism. Based on that result, a more complete evaluation of the reproducibility of histological grading in malignant CMT was subsequently performed in Chapter 3.

Grading reproducibility has been well discussed between human pathologists and there is unanimity that high variability in assessments would risk the clinical value of the histological grade (*e.g.*, Dalton *et al*., 2000; Meyer *et al.*, 2005; Longacre *et al.*, 2006). Nevertheless, to the best of our knowledge, this issue had never been addressed among veterinary pathologists. For this first study of interobserver grading reproducibility of malignant CMT, included in Chapter 3, a panel of three observers of the same academic institution was recruited. The inclusion of an experienced medical pathologist permitted a critical judgment over the applicability of the "human-based" grading parameters to malignant CMT. Moreover, a comparison between veterinary and medical grading performances was possible. For these purposes, a series comprised only of simple luminal carcinomas was used, since they have been considered more similar to the common forms of invasive breast carcinomas (Liu *et al*., 2014).

The kappa values were, on average, similar to that reported when NHG is applied to human breast cancer, representing moderate to good agreement. In addition, observers tended to cluster their opinions around two neighboring scores and grades, with discrepancy of more than one point being exceptional. Furthermore, the highest reproducibility was attained when scoring tubule formation, followed by mitotic counts and, finally, nuclear pleomorphism; this was also similar to the majority of human breast studies. The odds for variation in scoring augmented with the increased size of the tumors, but decreased when a consensus grade (performed by 2 observers) was compared with the grade assigned by a third observer. Therefore, a consensus grading should be pursued whenever possible, and particularly in large and heterogeneous tumors. Additionally, based on our data we proposed that the common practice of selecting a single slide (independently from the tumor size) can represent a bias in grading and this procedure should be discouraged.

Certainly, this first report on interobserver reproducibility in grading CMT will encourage other research groups to evaluate this subject in larger studies, with different panel of observers and including all the spectrum of carcinomas.

Nowadays, with the advents of digital pathology imaging, high resolution digital microphotography and whole slide imaging large scale grading reproducibility studies can be designed. It is noteworthy that similar approaches have been used for assessing nuclear grading reproducibility in human breast cancer (*e.g.*, Dunne and Going, 2001), as well as interobserver agreement in mitotic figures recognition (*e.g.*,Tsuda *et al*., 2000; Meyer *et al*., 2005; Al-Janabi *et al*., 2013). The scrutiny of the interobserver agreement level between pathologists from different parts of the world could be an important tool for training and discussion of the scoring criteria.

While addressing the issue of reproducibility of histological grading, the prognostic value of each grading parameter of the NHG in malignant CMT was evaluated (Chapter 4). Such complete assessment has never been performed in veterinary pathology. Interestingly, only nuclear pleomorphism was predictive of survival in CMT, both in univariable and multivariable analysis. However, this occurred only when nuclear pleomorphism was classified as a two-tier system (*i.e.*, score 1 and 2 compared against score 3), which was in line with the quantitative data of $\bar{v}_V$, presented in Chapter 2 (Santos *et al*., 2014). Although being subjective and less reproducible, the finding that nuclear pleomorphism has prognostic value reinforces the need for a strict nuclear grading assessment protocol in CMT. Meanwhile, the association of nuclear pleomorphism and prognosis advises for its inclusion in routine pathological reports of malignant CMT, besides the usually reported overall histological grade.

Considering that the univariable analysis supported that NHG could be a prognostic factor to CMT, we tested, for the first time, its inclusion in a multifactorial prognostic index, based on the well-recognized NPI of human breast cancer. A modification from the original formula was performed in order to make it more suitable for the reality of surgical pathology of CMT. In veterinary oncology, the TNM system for malignant CMT does not consider the number of positive lymph nodes (Rutteman *et al*., 2001). Therefore, the quantitative lymph node stage (values from 1 to 3) of the original NPI does not make much sense in the veterinary scenario, and we opted to incorporate a histological surrogate of the metastatic capacity of the tumor, represented by

the microscopic evidence of vascular invasion and/or the presence of lymph node metastases at the time of the primary diagnosis (Rasotto *et al*., 2012; 2014). It should be noted that the evidence of vascular/lymph node invasion has been already used for prognostic staging purposes in malignant CMT (Gilbertson *et al*., 1983; Kurzman and Gilbertson, 1986; Papparella *et al*., 2002; Sarli *et al*., 2002), but this was the first time that it was included in a multifactorial prognostic index.

Notably, the veterinary-adapted NPI, computed by the modified formula and, analyzed by a ROC curve test, appeared as a promising tool for predicting post-surgical tumor progression. Moreover, in a prognostic perspective it seems valuable to include the NHG in a composite index with tumor size and with a variable related to the spread of the disease. Still, it is important to recall that after proposal of the NPI (Haybittle *et al.,* 1982), its clinical value has been validated in a large cohort by that same group and by studies from other countries (*e.g.*, Todd *et al*., 1984; Sidoni *et al*., 2004; Blamey *et al*., 2007). In this vein, further CMT studies including prospective cohorts are warranted to confirm the prognostic value of the veterinary-adapted NPI and, perhaps, to establish other modifications to the index.

In Chapter 5, we tested a quantitative measurement method by which the level of tumor cellularity could be expressed in numerical terms. The unbiased stereological optical disector counting method was used to estimate the nuclei numerical densities [$N_V$ (nuclei, tumor)] in CMT. This cellularity-related parameter (Ladekarl, 2004) was not significantly associated with the subjective scoring of tubule formation, performed in routine sections of the same tumors. However, the $N_V$ (nuclei, tumor) was negatively correlated with the $\bar{v}_V$ and was associated with post-surgical tumor progression. In this vein, the $N_V$ (nuclei, tumor) seems more important for prognostication than the subjective tubule formation evaluation. At the same time, it should be stressed that the use of the optical disector methodology is laborious and relatively time-consuming (Ladekarl, 2004). Accordingly, in a near future we intend to explore other quantitative unbiased cellularity-related variables, such as the volume fraction of neoplastic nuclei [$V_V$ (nuclei, tumor)], which can be estimated in thin histological sections. Moreover, we plan to evaluate the correlation between the different

quantitative variables and their individual role in scoring malignant CMT according to their biological behavior.

As performed for the other grading parameters, a new quantitative approach for scoring mitotic figures in malignant CMT was developed and tested as prognostic factor in Chapter 6. It should be stressed that the reproducibility of mitotic count could be affected by the non-uniform criteria for mitotic recognition between observers or by the subjective selection of most mitotically active areas (Meyer *et al*., 2005; Baak *et al*., 2009). In that Chapter, mitotic figures were counted in a systematic sampling throughout the tumor, with an additional morphometrical estimation of the area of the neoplastic epithelium in each field of vision. Despite this approach obviated the subjective selection of the areas by the observer, and corrected the counts for the proportion of neoplastic epithelium, no significant improvement in the prognostic value of mitotic count was achieved. It should be noted that the used method was not completely devoided of counting bias. It is well known that 2D estimations of cells and nuclei are closely associated with sampling bias (Ladekarl, 2004). In this particular case, the probability of sampling the mitotic figures could be influenced by the section plane and the particle size (sampling / sectioning usually favors larger particles). Consequently, methodological refinements can be attempted, namely by using modeling of 2D estimates to correct for the sampling bias. Nevertheless, a low expectation exists regarding the prognostic value of mitotic counts in routine stained sections of malignant CMT. At this point, we anticipate that alternative proliferative markers, such as immunolabelling by Ki-67, could be more promising tools, particularly if combined with stereology.

Some evidence has been provided in previous Chapters, as well as in other studies, that NHG has prognostic value in CMT. Nevertheless, at this point, it should be stressed that no efforts have been devised over the development of a new, reliable and canine-specific grading system, which would only include parameters that have proved independent prognostic value. Ideally, the ability to predict clinical outcome of that canine-specific grading system should be compared with that of the other grading system adapted to CMT, as it has been elegantly performed in feline mammary carcinomas (Mills *et al*., 2015). In order

to contribute to this ultimate goal, different morphological features were screened as potential grading parameters in routine sections of CMT, and their association with prognosis was evaluated in Chapter 7. Among the assessed parameters, necrosis (scored by the cut-off of 20%), squamous differentiation (scored by the cut-off of 10%), and abnormal shaped nuclei (scored by the cut-off of 15%) arose as the most promising variables for predicting DFI. Although both DFI and OS were integrated as dependent variables in the regression statistical analyses of this Thesis, it was recognized that DFI is the most objective and more controlled end-point in prognostic studies of CMT (Matos *et al*., 2012).

Using an approach similar to the one used to establish the human NPI (Haybittle *et al*., 1982), a multivariable Cox hazard proportional analysis was performed considering DFI as the dependent variable (Table 1), with the goal of settling the first advances over the design of a new canine-specific prognostic index (CPI).

**Table 1 –** Multivariable survival analyses (Cox proportional hazards regression) of disease-free interval (DFI) as dependent variables in 59 cases of canine mammary malignant tumors.

| Dependent variable | Independent variables | β | SE | Hazard Ratio | *P* | 95% CI |
|---|---|---|---|---|---|---|
| **DFI** | Abnormal nuclei [1] | 2.09 | 1.08 | 8.09 | 0.05 | 0.98- 66.95 |
| | Necrosis [2] | 0.94 | 0.72 | 2.55 | 0.19 | 0.63-10.38 |
| | Squamous differentiation [3] | -0.61 | 0.60 | 0.54 | 0.31 | 0.17-66.95 |
| | Tumor size [4] | 0.37 | 0.54 | 1.44 | 0.50 | 0.50-4.18 |
| | Vascular invasion [5] | 1.13 | 0.59 | 3.09 | 0.06 | 0.97-9.84 |
| | Nuclear pleomorphism [6] | 1.52 | 0.66 | 4.58 | 0.02 | 1.26-16.61 |

Legend: [1]scored by the cut-off 15%; [2]scored by the cut-off 20%; [3] scored by the cut-off 10%; [4]scored by the cut-off 2.9 cm; [5]presence *versus* absence; [6]NHG score 1 plus 2 *versus* 3; β – beta coefficient; CI – confidence interval; SE – standard error.

Using the β coefficients of the first step of the multivariable analysis, with the influence of the tumor size, necrosis and squamous differentiation adjusted, and setting the significance level at *P* = 0.1 (considering the relatively small size of our sample), we arrived to the following formula:

**CPI** = 2 x Abnormal nuclei (1 if less than 15% and 2 if equal or more than 15%) + 1.5 x Nuclear pleomorphism (1 for score 1 or 2 and 2 for score 3 according to NHG) + Vascular invasion (1 if absence and 2 if present)

The values of CPI ranged from 4.5 to 9, being the higher values associated with worst prognosis. In order to establish reliable thresholds to assign each affected female dog to a well defined prognostic group, this formula must be apply prospectively to large cohorts of female dogs with malignant CMT. The validation process of the CPI would also warrant studies from other centers, as was performed with the human NPI (Lee and Ellis, 2008). As a final remark, regarding the findings of the Chapter 7, the scoring reproducibility of the pathologic variables and the role of quantitative estimates to objectivize such putative prognostic parameters will deserve further attention by our research group in the near future.

At this point, it should be emphasized that for grading purposes all slides resulting from at least one largest cross section of the tumor should be used. Notwithstanding, this may represent an extra-effort in term of time, but obviates the possible bias due to the selection of the “most representative” slide in each tumor, which has became a relative common practice. This is probably the best strategy to account with the intrinsic heterogeneity of CMT. Since the tumor size can range from less than 1 cm to more than 10 cm, the selection of one slide per tumor would certainly misrepresent the morphological features of the largest tumors.

Chapter 8 corresponds to one of the first approaches to the genomic profiles of malignant CMT. In this case we jointed our efforts with a research group headed by Professor Breen, well renowned for outstanding contributions in the the understanding of the genetic basis of different canine neoplasias (Alvarez, 2014). This resulted in aCGH analysis of a series of ten malignant CMT. The results highlighted that the majority of malignant CMT of different histological subtypes are associated with chromosomal aberrations. Despite the small scale of the present study, the results pinpointed some chromosomes, such as CFA 9, 22, 27, 34 and X, that may harbor genes potentially relevant for CMT development. In our series we included synchronous tumors from three female

dogs and in two cases the genomic profiles of the synchronous tumors were dissimilar (in the other case the tumors produced balanced profiles and no major DNA copy number aberrations). This preliminary data suggests the independency of synchronous CMT, at least in some instances.

In human medicine, the identification of patterns of DNA copy number alterations harboring important genes for breast carcinogenesis has set the path for searching molecular markers of prognosis and exploring new target therapies (Andre *et al*., 2009). In veterinary medicine, such goals will be pursued, sooner or later, and the work presented in Chapter 8 can be viewed as one of the first steps.

The potential use of aCGH for assessing the aggressiveness of the tumors and, in the cases of synchronous multiple tumors, determine their clonal relation, will certainly contribute to refine surgical treatment of CMT. This would be one of many uses of a high resolution and fast genetic screening technique, as has been demonstrated in human medicine (Weiss *et al*., 2003).

In general, in female dogs with multiple tumors it is recommended to perform a radical mastectomy, instead of multiple nodulectomies or local mastectomies (Sleeckx *et al*., 2011). Proponents of radical mastectomy argue that this approach is faster and avoids the need of a second surgery, by reducing the risk of new tumors (Stratmann *et al*., 2008). Nevertheless, other authors claim that radical mastectomy is a very invasive procedure and may be considered an overtreatment, since approximately half of the cases will have a benign clinical course (Lana *et al*., 2007). Moreover, radical mastectomy does not control the eventual development of metastatic disease from established micrometastases, and no evidence exists that radical mastectomy effectively improves the survival of female dogs with malignant CMT (Misdorp, 2002; Chang *et al*., 2005). Some lessons can be drive from the history of human breast cancer surgery. From the radical mastectomy, proposed by Halsted in the late nineteen century and fairly undisputed for more than fifty years, a new era in surgical procedures and in research began to emerge in late 1950s (Cotlar *et al*., 2003). By that time, randomized studies with large number of patients showed that more conservative surgical approaches (lumpectomy and quadrantectomy) were equally effective, with much less morbidity (Cotlar *et al*., 2003). Moreover, it was then clear that the advances in mammography and the concomitant use of fine

needle aspiration and needle-core biopsy prompted surgeons to an early identification of malignancies (Cotlar *et al*., 2003; Schmitt *et al*., 2012).

In human breast cancer, it was established that fine needle aspirations contain a high percentage of neoplastic cells and yielded sufficient DNA for aCGH analysis (Andre *et al*., 2009). The diagnostic value of aCGH in the preoperative evaluation of female dogs bearing mammary tumors will certainly be highest if the technique could be applied to cytological specimens. As a diagnostic method in CMT, cytology has been associated with low to moderate accuracy (Solano-Galeno, 2010). However, the association of cytology to ancillary techniques, such as stereology and aCGH could be valuable in pre-surgical clinical assessment of female dogs with CMT. It should be recalled that nuclear morphometric analysis in cytological smears of CMT already pointed to significant differences between benign and malignant cases (Simeonov and Simeonova, 2006; 2007).

Gathering all the promising data from this Thesis, we will prospectively evaluate the diagnostic and the prognostic value of the stereological $\bar{v}_V$ in cytological specimens of CMT, as well as the efficiency of DNA extraction from fine needle aspirations performed in the four quadrants of the lesion and its use in aCGH. Hopefully, with the information collected from all these approaches, the treatment of female dogs with mammary tumors may be tailored individually, as is the current trend in human breast cancer care.

## 9.2 References


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