Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

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ABSTRACT

Mast cell tumors (MCTs) are the most common skin neoplasm in dogs, and cytology plays a major role in their diagnosis. It would be valuable to identify reliable prognostic parameters during the cytological examination, as this would allow tailored pre-surgical therapeutic planning. Cellular grading parameters and quantitative features, such as nuclear area (NA), can be determined in cytological smears. The present study assessed the prognostic value of different cytological grading parameters and of the stereological estimation of the NA, in 37 MCT cases for which clinical follow-up data was available. The cytological smears, stained with May Grünwald-Giemsa, were graded by two observers, using the cytological adaptation of the Kiupel system and the Camus system. The slides were then destained and restained with H&E and the cytological grade was assigned again. NA was estimated by the stereology method 2D-nucleator in 100 cells in H&E-restained smears. Cox proportional hazards regression analysis and Kaplan-Meier curves were used to assess the prognostic significance of cytological grading parameters (assigned independently in both stains) and the NA. The cytological adaptation of the Kiupel grading system was superior to the Camus grading system in predicting survival. The presence of ≥7 mitotic figures (in May Grünwald-Giemsa-stained and in H&E-restained smears) and the amount of cytoplasmic granularity were the grading parameters associated with survival. The NA was also related to the clinical outcome. Restaining the smears with H&E did not significantly increase the prognostic value of the cytologic grade or grading criteria, but it was essential to estimate the NA. This study showed that the cytological features granularity and the mitotic activity (especially when mitotic figures in the smear were ≥7), and the stereological estimation of the NA (in H&E-restained smears) are prognostic factors in dogs with cutaneous MCTs and can aid therapeutic planning prior to surgery.

Keywords: cytology; dogs; mast cell tumors; grading; stereology.

RESUMO

Os mastocitomas (MCTs) são os tumores cutâneos mais frequentes nos cães e a citologia é um exame relevante para o seu diagnóstico. A identificação de fatores de prognóstico durante o exame citológico seria útil, visto que permitiria um planeamento terapêutico pré-cirúrgico. Parâmetros celulares de gradação e quantitativos, como a área nuclear (NA) podem ser determinados em citologia. No presente estudo, foi avaliado o valor prognóstico de diferentes parâmetros de gradação citológica e da estimativa estereológica da área nuclear numa série de 37 casos de MCT sujeitos a acompanhamento clínico. O grau citológico foi determinado por dois observadores nos esfregaços corados com May Grünwald-Giemsa, usando uma adaptação do método de grau Kiupel e o sistema de grau Camus. As lâminas foram depois descoradas e recoradas com H&E, tendo o grau sido novamente estabelecido. A NA foi estimada pelo método estereológico *nucleator-2D* em 100 células nos esfregaços recorados com H&E. Testes de regressão Cox e curvas de Kaplan-Meier foram usadas para avaliar o valor prognóstico dos parâmetros de grau citológico (determinado nas duas colorações) e da NA. O sistema de grau Kiupel revelou valor prognóstico superior ao sistema de grau Camus. A presença de ≥7 figuras de mitose (nos esfregaços corados com May Grünwald-Giemsa e recorados com HE) e o nível de granulação citoplasmática foram os parâmetros de gradação citológica associados ao tempo de sobrevivência. A estimativa de NA estava também associada com a sobrevida. Recorar os esfregaços com H&E não melhorou significativamente o valor prognóstico do grau citológico, nem dos seus parâmetros, mas é essencial para a determinação da NA. Este estudo mostrou que os parâmetros citológicos granulação citoplasmática, atividade mitótica (especialmente se as figuras de mitose forem ≥7) e a estimativa estereológica da NA são fatores de prognóstico em cães com MCTs, podendo auxiliar no planeamento terapêutico pré-cirúrgico.

Palavras-chave: citologia; cães; mastocitomas; grau; estereologia.

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ABREVIATIONS

FNA	Fine-needle aspiration
H&E	Hematoxylin and Eosin
HPF	High-power field
MCT	Mast cell tumor
NA	Nuclear area
NAI	Nuclear area determined by morphometry (image analysis)
NAN	Nuclear area determined by stereology (2D-nucleator)

INTRODUCTION

Clinical and Cytological Characteristics

Mast cell tumors (MCTs) are the most common skin neoplasm in dogs, accounting for up to 21% of all cutaneous neoplasms and with a prevalence of 0.27% in the canine population (Bostock, 1986; Rothwell et al., 1987; Shoop et al., 2015; Şmiech et al., 2018). There is no apparent gender or age predisposition for developing MCTs (Hottendorf & Nielsen, 1967; Reynolds et al., 2019; Shoop et al., 2015). Older dogs are the most commonly affected, with a reported average age of 7.5 to 9 years upon the occurrence of this neoplasm (Hottendorf & Nielsen, 1967; Kiupel et al., 2011; Patnaik et al., 1984; Pierini et al., 2019; Şmiech et al., 2018). Nevertheless, a wide range of ages has been documented, from 2-week-old to 19-year-old dogs (Pierini et al., 2019; Rigas et al., 2020). Although MCTs mostly occur in mixed breeds, a predisposition for developing this disease has been found in breeds such as boxers, golden retrievers, Weimaraners, bulldogs shar-peis, pugs, Labrador retrievers, Staffordshire bull terriers, beagles, schnauzers, cocker spaniels, dachshunds, and Rhodesian ridgebacks, among others (Hottendorf & Nielsen, 1967; Mochizuki et al., 2017; Pierini et al., 2019; Shoop et al., 2015).

MCTs may occur in several different organs, but the most common form arises from the skin (Kiupel, 2017; London, 2013). These neoplasms are predominantly found on the trunk, followed by the limbs, and, less frequently, the neck and head (Hottendorf & Nielsen, 1967; Pierini et al., 2019; Rothwell et al., 1987; Simoes et al., 1994; Şmiech et al., 2018). They usually occur as a single nodule, but multiple synchronous masses can also be present (Kiupel, 2017; London, 2013; O'Connell & Thomson, 2013). Because of the diversity in their clinical presentation, MCTs are known as "the great imitators". Their macroscopic appearance ranges from a hairless, raised, erythematous mass to nodular rashes or diffuse edema; their size ranges from a few millimeters to several centimeters; and their consistency ranges from soft, firm, to gelatinous (Bostock, 1986; Hottendorf & Nielsen, 1967; Kiupel, 2016).

During the initial clinical assessment of a canine patient presenting with a suspected cutaneous MCT a fine-needle aspiration (FNA) cytology is recommended (Kiupel, 2017; Kiupel & Camus, 2019; London, 2013). This technique allows a diagnosis in 95% of cases (Baker-Gabb, 2003). Since MCTs exfoliate well, FNA of these tumors typically yields highly cellular samples with easily identifiable mast cells, when the tumor is well-differentiated (DeNicola, 2014; Kiupel, 2017). Usually, the cytology smears contain a

population of round cells with moderate cytoplasm, a variable quantity of small, round, intracytoplasmic magenta granules, and a round, centrally to paracentral nucleus. They are frequently accompanied by eosinophils, reactive fibroblasts, and collagen fibers in varying numbers. Another common finding in these smears is a background with free granules, which can occur as a result of cell destruction during sampling or degranulation due to trauma or tumor microenvironment (DeNicola, 2014; Kiupel, 2017). Although identifying cells as mast cells in less differentiated MCTs can be challenging because of their sparse or absent cytoplasmic granulation, the majority of less differentiated MCTs have a few cytoplasmic granules (DeNicola, 2014; Kiupel, 2017). The presence of discrete granules, as well as a relatively monomorphic population of round cells with a central to paracentral nucleus and a moderate amount of cytoplasm, occasionally forming aggregates, allows for the cytological diagnosis of a MCT in these cases (Sabattini et al., 2018).

Prognostic Factors

The majority of canine cutaneous MCTs are benign, and in these cases, complete surgical excision can be curative (Kiupel et al., 2011; Séguin et al., 2001; Sledge et al., 2016). However, some MCTs spread to local lymph nodes and cause disseminated metastatic disease, requiring multimodal therapy that may include surgery and neoadjuvant or adjuvant therapy (Blackwood et al., 2012; Hottendorf & Nielsen, 1968; M. Kiupel et al., 2011; Simoes et al., 1994). Given the variability in the biological behavior of MCTs and the need for a treatment plan tailored to each tumor's behavior, predicting clinical outcome through the evaluation of prognostic factors is critical (Kiupel, 2017; London, 2013; Sledge et al., 2016).

Information regarding the tumor’s biological behavior is acquired through evaluation and interpretation of a multitude of factors, including patient signalment, clinical signs, tumor size, growth rate, histological grade, clinical stage, c-kit mutations, expression of KIT, and cell proliferation, among others (Kiupel, 2017; London, 2013; Sledge et al., 2016). The analysis of these indicators allows for the prediction of the disease's clinical course, which is then used to plan an appropriate therapeutic approach for each case (Kiupel, 2017; London, 2013; Sledge et al., 2016).

Through clinical exploration, it is possible to pinpoint factors that may have prognostic value, such as patient signalment information, including age and breed, as well as clinical signs and tumor location. Older animals and breeds, such as shar-peis and Weimaraners, have been linked to a higher risk of developing biologically aggressive MCTs (Miller, 1995; Mochizuki et al., 2017; Reynolds et al., 2019; Şmiech et al., 2018).

The presence of paraneoplastic signs, in addition to large, rapidly growing tumors with small satellite nodules and severe ulceration, suggests increased malignancy (Bostock, 1973; Moore et al., 2020; O'Keefe et al., 1987; Shoop et al., 2015). Furthermore, high metastatic rates to regional lymph nodes at the time of diagnosis have been reported in MCTs arising from mucous membranes, particularly in the muzzle (Elliott et al., 2016; Gieger et al., 2003; Hillman et al., 2010). In contrast, younger animals and breeds such as pugs, boxers, and Labrador retrievers have a higher likelihood of a clinically benign course (Mochizuki et al., 2017; Rigas et al., 2020; Śmiech et al., 2019). Well-differentiated MCTs typically present clinically as slow-growing, well-circumscribed alopecic single lesions. However, MCTs with a clinically benign appearance may also behave aggressively (Bostock, 1973; Kiupel, 2017).

Histological grading is the most important prognostic factor in canine cutaneous MCTs (Avallone et al., 2021; Blackwood et al., 2012; M. Kiupel et al., 2011; Sledge et al., 2016). While several histological grading systems have been proposed in the last decades, currently, pathologists either use the Patnaik grading scheme or the more recent Kiupel grading scheme (Avallone et al., 2021). The Patnaik system classified MCTs as Grade I (well-differentiated), II (intermediately differentiated), or III (poorly differentiated) (Patnaik et al., 1984). This grading system is based on the evaluation of cellular and nuclear morphology, surrounding tissue involvement, mitotic activity, stromal reaction, cellularity as well as cellular architecture, and the presence of edema and necrosis. The Patnaik grading system demonstrated a good correlation between survival and histological grade: grade I MCTs are associated with long survival, whereas grade III MCTs are associated with a poor prognosis (Horta et al., 2018; Patnaik et al., 1984; Sabattini et al., 2015; Stefanello et al., 2015; Takeuchi et al., 2013). Despite the observed correlation and validation by various studies, the Patnaik system has been criticized for its subjectivity, resulting in low inter-observer agreement (62.1%), and for the high proportions of MCTs falling into the grade II category (Horta et al., 2018; Northrup et al., 2005a, 2005b ; Sabattini et al., 2015; Simoes et al., 1994). This is considered a drawback because the clinical outcome of grade II MCTs is less predictable, as it has been reported that 5 to 22 % of these tumors exhibit aggressive behavior (Blackwood et al., 2012; Stefanello et al., 2015). The overclassification of MCTs as grade II has been demonstrated by Kiupel et al. (2011), Sabattini et al. (2015), and Shaw et al. (2017), among others, who classified 53%, and 60.6% of the evaluated MCTs as grade II, respectively.

To overcome the limitations of the Patnaik system, Kiupel and colleagues proposed a new, 2-tier histological grading system in 2011. According to this system,

MCTs are divided into low and high grade, based on the evaluation of cellular morphology and mitotic count in 10 high-power fields (HPFs). This grading scheme has higher inter-observer agreement (up to 96.8%) and is associated with overall survival, tumor recurrence, and risk of metastasis (Donnelly et al., 2015; Horta et al., 2018; M. Kiupel et al., 2011; Sabattini et al., 2015; Stefanello et al., 2015; Takeuchi et al., 2013). Therefore, the Kiupel system is considered the most reliable grading system (Horta et al., 2018; Sabattini et al., 2015; Takeuchi et al., 2013). Even so, different studies have reported that around 5 to 16% of dogs with low-grade MCTs died from the disease (Horta et al., 2018; M. Kiupel et al., 2011), 15% presented regional lymph node metastasis at the time of diagnosis, and nearly 20% developed other MCTs (M. Kiupel et al., 2011). Hence, while histological grading is one of the most valuable prognostic tools, it should not be used alone to predict outcome in dogs with MCTs (Horta et al., 2018; M. Kiupel et al., 2011; Moore et al., 2020; Sledge et al., 2016).

A more consistent prognosis can be achieved by combining the histological grade with the clinical stage (Horta et al., 2018; Moore et al., 2020; Stefanello et al., 2015). In most low-grade MCTs, this can be determined through a complete physical examination along with regional cytologic/histologic lymph node assessment (Kiupel, 2017; London, 2013). In high-grade MCTs, additional exams such as thoracic radiography, abdominal ultrasonography, and liver, spleen and bone-marrow FNAs are recommended (Kiupel, 2017; London, 2013). The World Health Organization staging system defines clinical stages as follows: stage 1 includes a solitary mass restricted to the dermis; stage 2 includes a solitary mass restricted to the dermis, as well as regional lymph node metastasis; stage 3 includes multiple dermal or large infiltrative tumors with or without regional lymph node involvement; stage 4 includes any case with distant metastasis (Owen, 1980). This staging system is controversial since it has been reported that dogs with a single mass and regional lymph node involvement (stage 2) had a worst prognosis than dogs exhibiting multiple dermal masses (stage 3) (Dores et al., 2018; Horta et al., 2018). In light of this, an interchange between stages 2 and 3 has been proposed (Horta et al., 2018). Despite the disagreement over the staging system, clinical stage is still regarded as a critical prognostic factor (Horta et al., 2018; Kiupel, M. 2016; Scarpa et al., 2016; Stefanello et al., 2015).

Other relevant prognostic tools are complementary morphologic and molecular methods, namely: (i) PCR for detection of activating c-kit mutations, (ii) immunohistochemistry for analysis of KIT expression patterns, and (iii) evaluation of the proliferation index by using the AgNOR x ki-67 score (Freytag et al., 2021; Sledge et al., 2016; Vascellari et al., 2013; Webster et al., 2008). When combined with the Kiupel

histologic grade, these tests provide the most detailed prognostic assessment of MCTs, and they are especially useful for identifying the subset of low-grade MCTs with aggressive behavior (Kiupel & Camus, 2019; Sledge et al., 2016). As such, they may assist in decision-making regarding the need for additional local therapy in incompletely resected low-grade tumors, and for systemic therapy, in cases with non-metastatic low-grade MCTs (Donnelly et al., 2015; Sledge et al., 2016). PCR for detecting mutations in c-kit and analysis of KIT pattern expressions are also valuable methods for aiding in the choice of pharmacological agents used in systemic therapy (Sledge et al., 2016; Webster et al., 2008).

Following surgical excision, the chance of tumor recurrence can be predicted by assessing the completeness of surgical margins, histopathological grade, and proliferation indices (Sledge et al., 2016). Evaluating MCT margins after surgical removal of the tumor is a routine procedure that is important for assessing the need for additional therapy (Kiupel & Camus, 2019; Scarpa et al., 2012; Sledge et al., 2016). Although tumor margins are clearly defined and easily identifiable in many low-grade MCTs, their assessment may be a challenge in some cases (Kiupel & Camus, 2019; Murphy et al., 2004; Sledge et al., 2016). In more aggressive cases or MCTs surrounded by an inflammatory halo, mast cells can be found in the adjacent tissues, and it is currently impossible to reliably determine whether the cells are neoplastic or non-neoplastic. (Kiupel & Camus, 2019; Murphy et al., 2004; Sledge et al., 2016). Another limitation in tumor margin assessment is the lack of a harmonized method for sectioning the tumor for microscopic evaluation (Kiupel & Camus, 2019). Even though most MCTs are routinely sectioned radially, this method does not allow a complete assessment of tumor margins (Kiupel & Camus, 2019; Milovancev & Russell, 2017; Sledge et al., 2016). For a rigorous margin evaluation, a combination of cross and tangential sectioning is advised (Dores et al., 2018; Kiupel & Camus, 2019; Sledge et al., 2016).

Regardless of the importance of examining tumor margins, their assessment may not necessarily allow to predict MCT recurrence (Dores et al., 2018; Horta et al., 2018; Murphy et al., 2004; Séguin et al., 2001; Weisse et al., 2002). While complete resection of low-grade MCTs is associated with low tumor recurrence rates of 4% (Donnelly et al., 2015), grade II MCTs with clean margins have reported recurrence rates of 5-11% (Séguin et al., 2001; Weisse et al., 2002), and in one study, 36% of totally excised high-grade MCTs recurred, despite the width of the surgical margins (Donnelly et al., 2015). On the other hand, incompletely excised low-grade and grade II MCTs have shown recurrence rates of 10 to 15% (Kiupel, M. 2016(M. Kiupel, 2008); Sledge et al., 2016), and 5 to 23% (Ozaki et al., 2007; Séguin et al., 2006; Weisse et al., 2002), respectively.

Moreover, regardless of tumor margins, low-grade MCTs with a low AgNOR x ki-67 score have a 10% chance of local recurrence (Kiupel & Camus, 2019). These findings suggest that histological tumor grade and proliferation indices are more effective than tumor margin assessment in predicting local relapse (Kiupel, 2017; Sledge et al., 2016).

Cytological Grading

Histological grading is the most commonly used tool for MCT prognostication, as previously stated (Avallone et al., 2021; Blackwood et al., 2012; Kiupel & Camus, 2019; Sledge et al., 2016). However, grade assessment follows surgical excision, and predicting MCTs' biological behavior earlier in the clinical course would be of great interest, as it would allow an individualized, tailored, therapeutical approach (Camus et al., 2016; Hergt et al., 2016; Paes et al., 2022; Pedraza et al., 2011; Scarpa et al., 2016). Considering that the Kiupel histological grading system is based on the evaluation of cellular features and that cytology allows detailed observation of cell morphology, the possibility of creating a cytological grading system based on the assessment of cellular features appeared to be promising.

Scarpa et al. (2014) and Hergt et al. (2016) attempted to propose an adaptation of the Kiupel grading system for cytology by applying the criteria assessed in the 2-tiered histological grading system to cytological specimens in two different retrospective studies. MCTs were classified as high-grade in the presence of at least one of the following criteria: three or more multinucleated cells, three or more bizarre nuclei, seven or more mitotic figures, and karyomegaly. When comparing cytological and histological grades, Scarpa et al. (2014) and Hergt et al. (2016) found that in 94% of cases, cytological evaluation correctly predicted grade. Furthermore, sensitivity and specificity of both studies was similar, with the former reporting values of 85% and 97% and the latter 87% and 97%, respectively.

Despite their similar findings, each research group adopted a different methodology. Scarpa and colleagues assessed approximately 1000 cells in May Grünwald-Giemsa-stained smears, while Hergt et al. (2016) evaluated 10 HPF in H&E-stained smears. In the Scarpa et al. (2014) study, 2 out of 13 histologically high-grade MCTs were considered low-grade by cytology. The authors hypothesized that this could be due to the masking of the atypical nuclear features by the cytoplasmic granules. In the Hergt et al. (2016) study, staining the smears with H&E allowed clear visualization of nuclear characteristics, which resulted in a slight increase in sensitivity. The grading parameters were found to be more prevalent in histological samples than in the matched cytological slides in both studies. Furthermore, the authors pointed out that tumor

sampling areas, low cellularity in smears compared to histology, and smear quality could limit the ability to detect the Kiupel system's high-grade features in cytology. Indeed, Scarpa et al. (2014) reported that the number of cellular features detected by cytology in histologically low-grade MCTs and histologically high-grade MCTs differed significantly.

The authors consistently found lower numbers of mitosis and inconsistent numbers of bizarre nuclei in cytological samples, resulting in low sensitivity values and poor agreement with histology in these specific nuclear criteria. The presence of multinucleation in cytological smears was found to have a fair to consistent correlation with histology. Furthermore, the cytological parameter karyomegaly was found to have the highest sensitivity and the best concordance with histology. However, in both studies, the presence of karyomegaly led to the misclassification of histologically low-grade MCTs as cytologically high-grade MCTs. Although these results were promising, the use of the Kiupel criteria in cytology did not allow a sufficiently accurate prediction of tumor grade in either method. The findings suggested that additional cytological parameters should be investigated in order to develop of a novel cytological grading system.

A new cytological grading system was proposed by Camus et al. (2016) in a prospective study. In this study, histological specimens were graded, and the respective adequately cellular FNA smears were reviewed for assessment of the following parameters: cell granularity, nuclear pleomorphism, collagen fibrils, mitotic figures, binucleated and multinucleated cells, and anysokariosis. The correlation between each parameter and survival time was determined, and those significantly associated with shorter survival times were used to create grading algorithms. The algorithm showing the highest correlation to histological grade was proposed as the new cytological grading system. According to that system, a MCT was considered to be high-grade if granularity was poor or if two or more of the remaining criteria were present (binucleation or multinucleation, bizarre nuclei, mitotic figures, and anysokariosis). This grading scheme rendered a sensitivity of 88%, a specificity of 94%, and an overall accuracy of 94%, when compared to histological grade. The correlation between cellular features and 2-year survival was highest for granularity and lowest for the presence of collagen fibers and nuclear pleomorphism. A poor correlation between nuclear pleomorphism and histology was also reported, supporting the previous evidence found by Scarpa and Hergt. Moreover, Camus et al. (2016) observed that in cytological high-grade MCTs the probability of survival significantly decreased, whilst the probability of developing new tumors increased. The use of the Camus system revealed a propensity for overestimating high-grade MCTs, which resulted in 32% of false-positive high-grade tumors. Facing this limitation, the authors argued that although the low positive predictive value was not ideal,

a cytological false-positive high-grade was preferable to a false-negative one, since this would reduce the likelihood of tumors demanding aggressive treatment being overlooked. However, the serious consequences of a worst-case scenario should not be neglected, as a cytological diagnosis of a high-grade MCT could lead to unnecessary limb amputation or even euthanasia.

Nowadays there is evidence that it would be useful to explore other cytological parameters for a more accurate prediction of tumor behavior (Marcos et al. 2022; Paes et al, 2022). Recently, Paes et al. (2022) proposed a new grading system that included fibroblasts and collagen fibrils in addition to the criteria already recognized by the previous grading schemes. In this study, cytological evaluation of the pre-established criteria was performed, and tumor microenvironment features were assessed, including cellularity, proportion of clustered cells, concentration of fibroblasts and/or collagen fibrils, and also eosinophils and/or neutrophils. These variables were correlated with the survival rate, and the features showing the strongest correlations were selected as potential grading criteria. Then, the cytological features were correlated with the histological Kiupel grade in order to create the system proposed by Paes et all. (2022). According to this new grading system, a MCT is classified as high-grade if it is poorly granular, or if at least two of the subsequent variables are present: (i) multinucleated cells, (ii) mitotic figures, (iii) karyomegaly, and (iv) low/absent concentrations of fibroblasts and/or collagen fibrils. Paes et al. (2022) found substantial agreement between the proposed grading system and the Camus grading system. Both cytological grading systems and the histological Kiupel system were associated with the 1-year survival data. Compared to the histological grade, the overall accuracy of the new cytological system was 77%. Furthermore, 45% of false-positive high-grade MCTs, and 10% of false-negative low-grade MCTs were reported. Although the two previous values were higher than those reported by Camus et al. (2016), the authors claimed that the proposed grading system was superior in predicting survival. The mortality rates in dogs graded by this new system suggested that cytological grade may have a prognostic value superior to that of histopathologic grade. Nonetheless, further studies are needed to assess the prognostic value and reproducibility of this newly proposed grading system.

Quantitative Methods

Although several cytological grading systems are being scrutinized, their performance is still debatable, raising concerns about their routine application. In this context, the use of quantitative methods in cytological samples may play an important role in MCT prognosistication (Marcos et al., 2022; Neto et al., 2010; Strefezzi et al., 2003; Strefezzi et

al., 2009). Quantitative methods, such as morphometry and stereology, allow the establishment of numerical parameters, enabling an objective and reproducible assessment of cellular characteristics (Mandarim-de-Lacerda, 2003; Mandarim-de-Lacerda et al., 2010). In morphometric methods, cells are measured either directly, with rulers or caliper micrometers, or indirectly, through image analysis software that converts pixels into micrometers. Meanwhile, stereological methods, which are based on mathematical principles, are able to determine geometrical parameters including volume, surface area, length, or the number of cells. These parameters are obtained through the application of geometrical probes directly over cells in a 2-dimensional plane (either a tissue section or a cytological sample), allowing an estimation of their 3-dimensional features (Mandarim-de-Lacerda, 2003; Mandarim-de-Lacerda et al., 2010; Marcos et al., 2012).

With the purpose of increasing the ability to predict MCT behavior, Strefezzi et al. (2003) used computerized morphometric methods in cytological smears to determine which morphometric nuclear parameters could be associated with histological grade. These authors observed a correlation between the Patnaik histological grade and nuclear parameters, such as area, perimeter, and mean diameter. The relationship between the nuclear area (NA) and histological grade was further supported by a later study by Neto et al. (2010). Moreover, the results of Strefezzi et al. (2003) confirmed the tendency of nuclear size to increase with malignancy in MCTs, which had been described by several authors, although in an imprecise or subjective manner (Bostock, 1973; Hottendorf & Nielsen, 1967; Patnaik et al., 1984). Further research to assess the prognostic value of cytological nuclear morphometry in MCTs, found an association between the NA and patient survival along with a significant decrease in patient survival time as the NA increased (Strefezzi et al., 2009). These findings not only corroborated the link between nuclear morphometric parameters and histological grade demonstrated previously, but also revealed that mast cell NA was an independent prognostic factor.

Large-scale application of morphometry implies a previous assessment of its reproducibility, as inter-observer variability is irreconcilable with the principles of using quantitative methods. To assess the reproducibility of morphometric methods in MCTs, nuclear measurements were performed by two independent observers, during two different time periods, in cytological specimens stained either with Diff-Quick or H&E (Barbosa et al., 2014). No statistically significant differences were reported between the measurements performed by the two observers, or the same observer in different time periods.

Regardless of the prognostic value of the NA in MCTs demonstrated in previous studies, there are limitations to the use of morphometry. Firstly, morphometrical analysis is a time-consuming procedure as it usually requires manual outlining of the nuclear limits (Marcos et al., 2022; Strefezzi et al., 2003). Secondly, in highly granular MCTs, the nuclear limits may not be clearly visible if routine cytological stains are used. Considering that the presence of partially distinguishable or indistinguishable nuclear limits can lead to inaccurate tracing or total exclusion from quantification, respectively, it can be assumed, at least theoretically, that using routine staining for quantitative methods can result in bias (Marcos et al., 2022).

To improve the efficiency and accuracy of using the NA as a prognostic indicator in MCTs, stereological methods were applied for the first time in MCT cytological samples by Marcos et. al. (2022). In this study, morphometric and stereological approaches were compared for assessing the NA in H&E-restained cytological samples of MCTs. Additionally, histological grade was correlated with the NAs determined by each method, which allowed the establishment of NA cut-off values for distinguishing low and high-grade MCTs. The evaluated cytological slides were originally stained with Hemacolor (Merck) and then destained and restained with H&E, for clear visualization of the nuclear borders. In the morphometric analysis, the NA (NAI) was determined by the software Image J (https://imagej.net) and the nuclei were manually outlined. In the stereological analysis, the 2D-nucleator tool was used and the NA (NAN) was determined by the software CAST-Grid v.1.60, coupled to a microscope and camera system. The results showed that there was a significant correlation between the mean NAN and mean NAI and that the quantification methods were highly comparable, regarding the NA values. However, they differed regarding the time needed for each method, since determining the NAN was more time-efficient (NAN required 40 minutes per smear and NAI took 60 minutes per smear).

Regarding the 2-tiered Kiupel histological grading system, both the NAI and NAN differed between low and high-grade MCTs. As for the Patnaik 3-tiered grading system, the NAN differed between grades I and III, and also between grades II and III, whereas NAI only differed between grades II and III. The results revealed that using the NAN allowed a 79% chance of distinguishing high-grade from low-grade MCTs. Furthermore, if NAN is $<50.1\mu m^2$, the MCT is definitely low-grade; if NAN falls in the range $50.1\mu m^2 \leq NAN < 62.8\mu m^2$ there is a 50% chance that the MCT will be high-grade; whereas if NAN is $>62.8\mu m^2$, the MCT is definitely high-grade. The cut-off of $62.8\mu m^2$ could be particularly useful for treatment decisions in cases where more extensive or radical surgery is being considered. This study demonstrated that the NA assessed by stereology in cytologic smears helped in the identification of morphologically aggressive MCTs.

Although the results suggested the potential of this quantitative parameter to predict the outcome in MCTs, further studies were needed to assess its prognostic value.

AIM OF THE STUDY

Cytological evaluation is a well-established technique for the diagnosis of MCTs, as it is a simple, rapid, non-invasive, and low-cost procedure. Identifying reliable cytological features for MCT prognostication would be of great value, allowing for a pre-surgical tailored therapeutical plan without the need for additional costs or exams. In histology, tissue samples are subjected to processing techniques, namely fixation, paraffin-embedding, and sectioning. These techniques alter cell morphology, since tissues suffer some level of shrinkage, and the orientation of section planes influences the perceived nuclear size (Peleteiro MC, 2011). In contrast, in cytological smears, the cells are flattened on the slide rather than sectioned, and the complete projection of each cell is depicted (Gil, 1994; Strefezzi et al., 2003).

Cytological parameters for assessing MCT grade have been examined in several studies (Camus et al., 2016; Hergt et al., 2016; Neto et al., 2010; Scarpa et al., 2016). However, to the best of our knowledge, the cellular grading features in the same cytological slides, stained with Romanovsky and restained with H&E, have never been compared. Furthermore, the prognostic value of the cytological adaptation of the Kiupel grading system has never been evaluated.

In previous studies, the NA of MCTs assessed by morphometry in cytological smears has been correlated with the histological grade and survival (Strefezzi et al., 2003; Strefezzi et al., 2009). A correlation has also been found between histological grade and the NA of MCTs assessed by stereology in cytological slides (Marcos et al., 2022). These findings suggest that nuclear stereology may be predictive of survival. In the recent study by Marcos et al. (2022), cytological slides, originally stained with Hemacolor (Merck), were screened for estimating the percentage of mast cells with indistinct nuclear limits, which was determined to be 43.6%±25.5 (mean ± standard deviation, SD). The high percentage of non-quantifiable nuclei demonstrates that assessing NA in Romanovsky-stained slides inevitably leads to biased results. For this reason, determining NA in H&E-restained smears would be preferable, as the nuclear limits are visible with this staining technique. Evaluation with H&E restaining may require destaining and restaining of the slides, which can influence the NA. However, this seems unlikely, since no apparent differences were observed in a study that compared the same fields in slides that underwent a destaining/restaining process (Marcos et al., 2017).

The aim of the present study was to explore the role of qualitative and quantitative cytological features in predicting MCT behavior. To accomplish this aim, the main questions addressed were: (i) Which cytological grading system and parameters have stronger prognostic value? (ii) Does the evaluation of the same cytological grading parameters in H&E-restained smears increase their prognostic value? (iii) Can NA assessed by 2D-nucleator be used as a prognostic indicator?

MATERIALS AND METHODS

Archived MCT cases were retrospectively selected from the repositories of the cytology service of the Dipartimento di Medicina Veterinaria e Scienze Animali (DIVAS), Università degli Studi di Milano. The samples had been collected by FNA, air-dried, processed routinely, and stained with May Grünwald-Giemsa. Cytology slides were reviewed by a pathologist for the assessment of cellularity and cell preservation. Only cases with clinical follow-up information and a representative slide with adequate cell preservation and more than 100 intact, spread-out mast cells were enrolled. The selected cytology slides were scanned for archival purposes (Olympus VS110 slide scanner, Olympus, Japan). Clinical (sex, age, breed, clinical stage) and pathologic data (histologic grade according to Kiupel system, surgical margins) were retrospectively retrieved from the archives.

Cytological Grading

May Grünwald-Giemsa-stained smears were reviewed by two observers through a dual-head microscope. The cytological grading criteria of the Camus system and the Kiupel system adapted by Scarpa et al. (2014) and Hergt et al. (2016) were used and MCTs were graded accordingly to each system. The parameters evaluated in the Camus grading system were the following: cell granularity (well granulated, poorly granulated, or mixed granulation) (Figure 1.), mitotic figures (absent or present), binucleation or multinucleation (absent or present), nuclear pleomorphism (absent if nuclear shapes range from round to oval, and present if non-rounded nuclei were noted), and anisokaryosis (absent or present if nuclear size varied in more than 50%) (Figure 2.). According to this grading system, a MCT is considered high-grade if cell granularity is poor or if at least two of the remaining criteria are present. On the other hand, the cytological Kiupel grading parameters assessed were the following: ≥3 multinucleated cells, ≥3 bizarre nuclei, ≥7 mitotic figures, and karyomegaly, which was considered present if nuclear sizes varied at least 2-fold in most observed fields (Figure 2.). Based on this system, a MCT is classified as high-grade in the presence of at least one of the previous parameters.

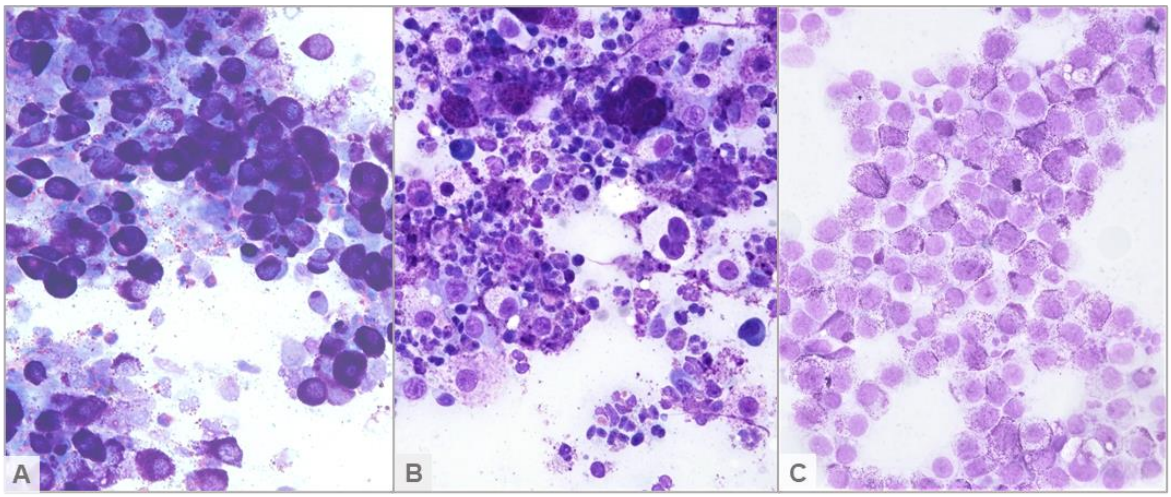

Figure 1. Differences in granularity in MCT cytological smears stained with May Grünwald-Giemsa. (A) High granularity. (B) Mixed granularity. (C) Poor granularity.

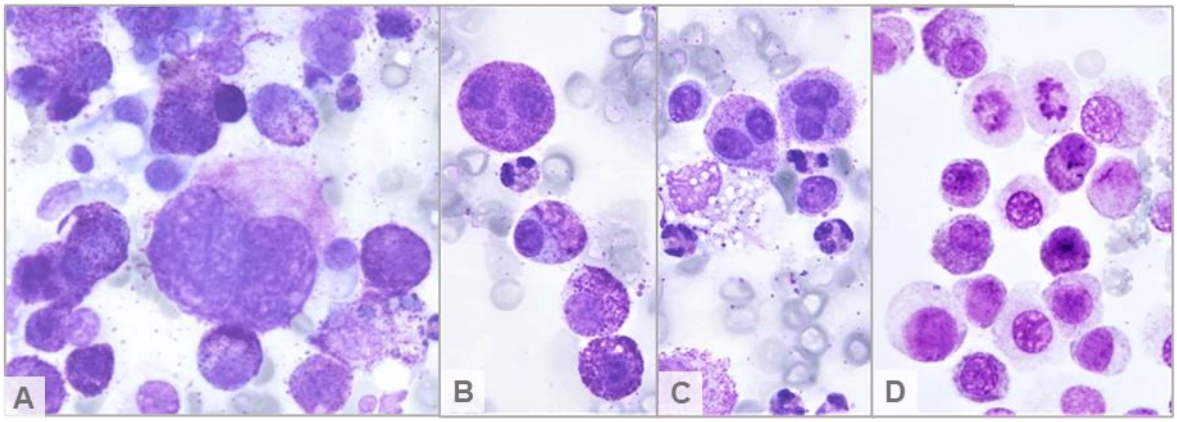

Figure 2. Atypical nuclear features used as cytological grading parameters in MCTs. (A) Aberrant binucleated cell. (B) Multinucleated mast cell. (C) Binucleated and multinucleated mast cells. (D) Mitotic figures and karyomegaly.

After cytological grade classification by each system, the slides were destained and restained with H&E (Gill's hematoxylin). According to previous reports, the latter has a lower affinity for the mast cell granules and highlights the nuclear features of the neoplastic mast cells (Marcos et al., 2017; Marcos et al., 2022). For the destaining/restaining procedure, routinely stained slides were immersed in xylol for one to five days for complete removal of the coverslip. Then, the smears were rehydrated with absolute alcohol 99% for 1 minute, followed by alcohol 95% for 1 minute, and 70% alcohol for 1 minute. The smears were then destained with 1% acid alcohol, for a period of time determined by the cellular density of the smear, as assessed through microscopic observation. Following destaining, the slides were rinsed in running tap water for 5

minutes. Finally, the smears were restained using Gill's hematoxylin for 30 minutes, rinsed in running tap water for 5 minutes, and stained with eosin for 5 minutes.

All the cytologic grade grading parameters of the Camus and Kiupel adaptation systems were assessed once more by the same two observers. During the initial analysis with May Grünwald-Giemsa staining, the presence and abundance of other cells such as fibroblasts, eosinophils, and macrophages were documented to avoid misjudging them as mast cells after restaining with H&E.

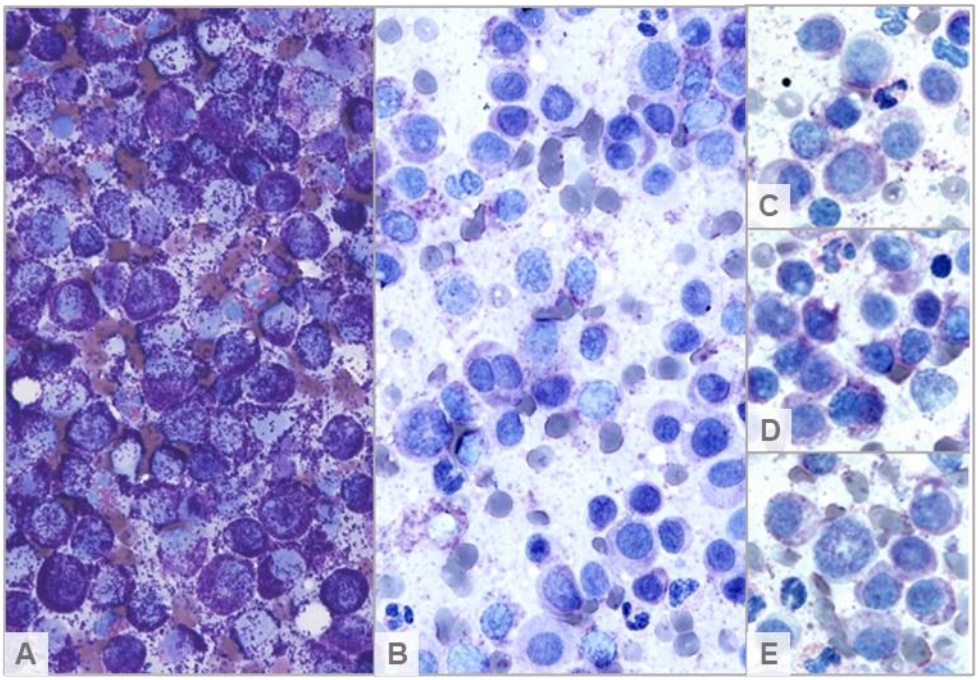

Figure 1. Demonstration of the efficacy of destaining and restaining the May Grünwald-Giemsa-stained specimens with H&E. (A) Cytological smear of a highly granular MCT stained with May Grünwald-Giemsa in which no atypical morphological features are visible. (B) May Grünwald-Giemsa-stained specimens restained with H&E. The low affinity of this staining for the cytoplasmic granules revealed several atypical nuclear features, namely binucleation, mitotic figures and anisokaryosis. (C) Anisokariosis. (D) Binucleation. (E) Mitotic figure.

Determination of Nuclear Area

The method selected for NA determination was the 2D-nucleator and the assessment was performed with a microscope (Olympus BX50, Japan) coupled to a camera system (SONY), a motorized stage (Prior Scientific), and a computer equipped with stereology software (CAST-Grid v.1.60, Denmark).

The slides were evaluated under the 100x oil-immersion objective, and the microscopic fields were projected onto the computer monitor achieving a final magnification of 4050x. The first field was randomly selected, and from there on, the motorized stage was methodically moved until measurements in 100 different cells were completed. Only intact, well-spread-out mast cell nuclei were measured. The nuclei of poorly preserved or clustered mast cells as well as other cell types were excluded from the analysis. Additionally, when assessing the NA of binucleated or multinucleated mast cells, each nucleus was measured and recorded separately from the mononuclear measurements.

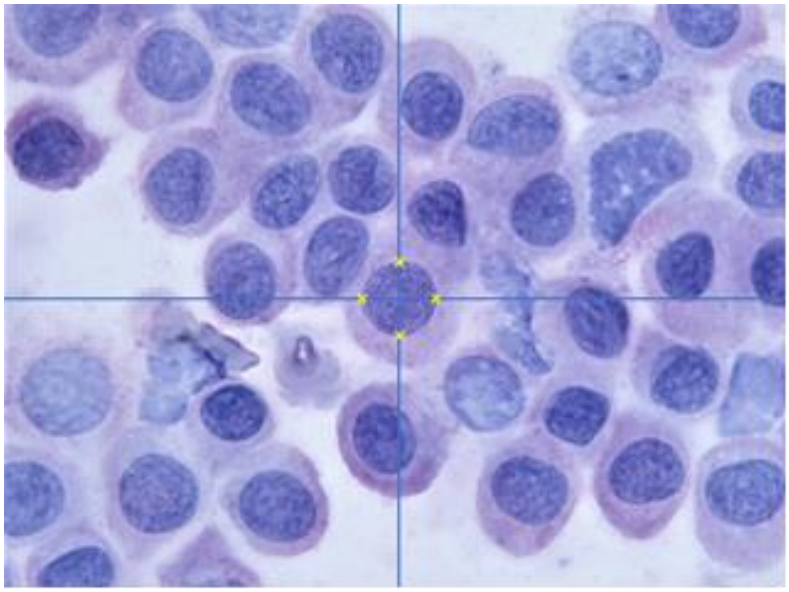

Figure 3. Determination of the NA in the H&E-restained slides with the 2D-nucleator method.

The 2D-nucleator methodology entailed using a computer mouse to select a nucleolus or, if the nucleolus was not visible, the center of the nucleus. After clicking on the nucleolus/center of the nucleus, the software generated two perpendicular lines, whose intersections with the nuclear limits were selected by the operator using the computer mouse (Figure 3.). Afterwards, the software calculated the average distance between the nuclear limits and the nucleolus/center of the nuclei (l) and determined the NA by applying the formula $NA = \pi l^2$ (Gundersen et al., 1981; Marcos et al., 2022).

Statistical Analysis

Medical records were reviewed and referring veterinarians consulted to retrieve the follow-up information. The histological grade, surgical margin status, clinical stage, overall survival, and cause of death were obtained.

The disease-specific overall survival was calculated from the time of diagnosis to the time of death (by natural causes or euthanasia). All animals that died or were euthanized for reasons unrelated to the tumor, were lost to follow-up, or were still alive at the time of the current study were censored.

Survival statistical analyses were performed using R, version 4.1.2. (R Core Team, 2021). Kaplan-Meier curves were created for each variable to display differences between the survival time. A Cox proportional univariable analysis was performed to estimate hazard ratios and assess the statistical relationship between survival and each variable (cytological grade, cytological parameters, mean NA, mean NA≥62.8μm^2, clinical-stage, Kiupel histological grade, surgical margins, gender, and age). The variable NA≥62.8μm^2 was included to assess the prognostic value of using the high-grade NA cutoff established by Marcos et al (2022). The statistically significant variables were included in the multivariable Cox regression analysis. Due to sample size of this case series, the significance level of 0.1 was used to select variables from the univariable analysis. A $p<0.05$ was considered to define statistical significance.

RESULTS

Considering the inclusion criteria, 39 MCT specimens from 37 dogs (20 female and 17 male) were selected for this study. Their ages ranged from 3 to 12 years, with an average of 8.5 years. Mixed breeds were the most represented (n=9), followed by boxers (n=5), Labrador retrievers (n=3), Boston terriers (n=2), beagles (n=2), French bulldogs (n=2), and a total of 14 other breeds were considered but not enumerated due to a sample size lower than 2. Histopathological evaluation identified 30 low-grade and 8 high-grade MCTs. The distribution of the cases according to the clinical stage, number of tumors and completeness of the surgical margins is represented in Supplemental Table A.

The cytologic grade classification according to the Kiupel system adapted by Scarpa et al. (2014) and Hergt et al. (2016) and the Camus system in May Grünwald-Giemsa-stained and H&E-restained specimens is depicted in supplemental Table B. When the parameters based on the Kiupel grading system were applied to the May Grünwald-Giemsa-stained slides, 10 MCTs (10/39; 25.6%) were considered high-grade. Restaining with H&E allowed re-classification of 1 tumor as low-grade, as well as identification of 10 more specimens that met the high-grade criteria, making a total of 19 high-grade MCTs (19/39; 48.7%). On the other hand, according to the Camus grading system, 21 MCTs (21/39; 53.8%) were classified as high-grade in the May Grünwald-Giemsa-stained smears. In the reassessment with H&E restaining, 1 case was re-

classified as low-grade, and 6 additional high-grade MCTs were detected, amounting to a total of 26 (26/39; 66.7%) high-grade MCTs.

When comparing the May Grünwald-Giemsa-stained slides with the H&E-restained slides, the parameters of the cytological Kiupel grading system that varied the most were karyomegaly, multinucleation, and bizarre nuclei (Supplemental Table C). Restaining with H&E led to an increase in the presence of these criteria of 15.4%, 12.8%, and 12.8%, respectively. On the other hand, comparisons between the two staining methods in the slides graded by the Camus system revealed that the parameters that differed the most were nuclear pleomorphism and binucleation. The identification of these two parameters increased by 15.4% following H&E re-staining. It was also noted that, in this system, re-staining with H&E decreased detection of anysokariosis by 10.3%. This was the single parameter whose presence was decreased by H&E restaining in both grading systems.

When comparing the two grading systems in the May Grünwald-Giemsa-stained specimens, there was agreement on assigning a high-grade classification in 10 MCTs, (Supplemental Figure E-A). In the y Grünwald-Giemsa-stained slides, all the samples classified as high-grade by the cytological adaptation of the Kiupel grading scheme were also considered high-grade by the Camus grading system. However, 11 MCTs classified as high-grade by the Camus grading system did not meet the high-grade criteria of the Kiupel grading system adapted by Scarpa et al. (2014) and Hergt et al. (2016). After restaining with H&E, grade classification agreement was found in 17 high-grade MCTs (Supplemental Figure E-B). Nevertheless, 9 cases only met the high-grade criteria of the Camus grading system, while 2 cases were considered high-grade exclusively by the cytological adaptation of the Kiupel grading system.

In the study group, 29 dogs were censored: 23 died or were euthanized for reasons unrelated to the tumor, 3 were lost to follow-up and 3 were still alive at the time of the current study. The censored group had a mean follow-up period of 737 days, ranging from 100 to 1587 days. There were 8 MCT-related deaths in those cases mean survival was of 335 days, ranging from 29 to 1100 days. As for the stereological analysis, an average of 104 nuclei were measured in each case, with a mean NA of 57.55 ± 11.68 μm^2.

The variables with a statistically significant association with overall survival in the univariable Cox regression analysis were the following: (1) the Kiupel cytological grade adaptation in May Grünwald-Giemsa staining (Coef=2.03, Hazard=7.63, p=0.0155); (2) the presence of ≥ 7 mitotic figures in May Grünwald-Giemsa staining (Coef=2.62, Hazard=13.72, p=0.0015) and (3) in H&E restaining (Coef=1.47, Hazard=14.14, p= 0.003); (4) the amount of cytoplasmic granules (Coef=1.67, Hazard=5.32, p=0.009); (5) the mean NA (Coef=0.07, Hazard=1.07, p=0.0154). Regarding the latter parameter, the

cut-off NA of ≥ 62.8 μm^2 was also associated with the survival (Coef=1.56, Hazard=4.76, *p*=0.0339). The presence of mitotic figures, as described by the Camus system in H&E restained slides, was close to the level of significance (Coef=1.5, Hazard=4.49, *p*=0.053), while the same parameter in the May Grünwald-Giemsa slides was not associated with survival.

The Kaplan-Meier plots for the variables significantly associated with survival in the univariable Cox regression analysis are presented in Figures 1. and 2. The univariable regression analysis did not converge when parameters such as binucleation in H&E restaining, anisokaryosis in H&E restaining, the Camus cytological grade in H&E restaining, and the Kiupel histological grade were included in the model. The distribution of the disease related-death events according to these grading and histologic parameters is presented in Table 1. The cytological adaptation of the Kiupel system showed superior prognostic value than the Camus grading system in May Grünwald-Giemsa staining. A dog with a high-grade MCT classified by the cytological Kiupel system had 7.63 (*p*= 0.0155) higher risk of dying from the disease, whereas a dog with a high-grade MCT classified by the Camus system had a non-significant trend (*p*= 0.054) towards a high risk of disease-related death.

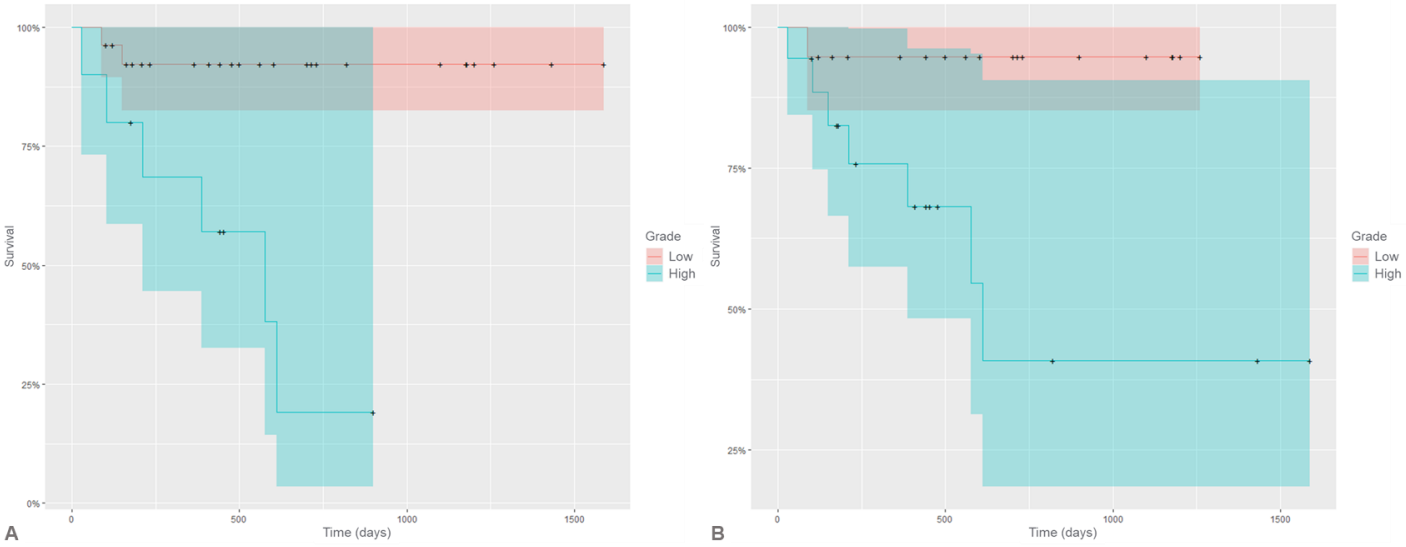

Figure 1. Kaplan-Meier plots comparing overall survival with time (days) in 37 dogs with MCTs. Censoring is indicated by the vertical marks (A) Cytological adaptation of the Kiupel grading system in May Grünwald-Giemsa staining. (B) Cytological adaptation of the Kiupel grading system in H&E restaining.

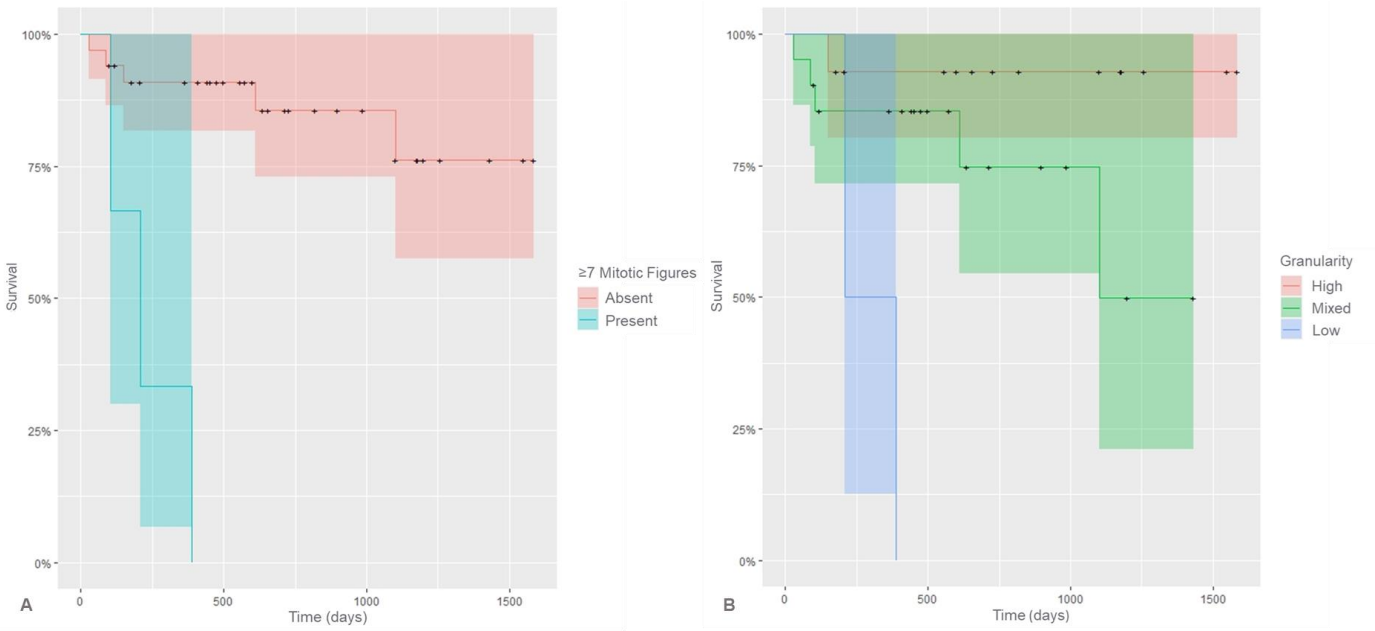

Figure 2. Kaplan-Meier plots comparing overall survival with time (days) in 37 dogs with MCTs. Censoring is indicated by the vertical marks (A) Cytological grading parameter “Presence of ≥ 7 mitotic figures in May Grünwald-Giemsa staining”. (B) Cytological parameter “Granularity”.

Table 1. Distribution of the variables that did not converge in the univariable Cox regression analysis in the dogs that died from MCT-related disease MCT.

Variables	**Dogs that died from the MCT (n=8)**
Binucleation in H&E- restaining	8
Anisokaryosis in H&E- restaining	8
Camus system grade in H&E- restaining	8
Kiupel Histological high grade	7

In the cytological Kiupel grading system, the only parameter significantly associated with survival was the presence of ≥7 mitotic figures in both the May Grünwald-Giemsa-stained smears and the H&E restained smears. As for the Camus system, only the amount of cytoplasmic granules in the May Grünwald-Giemsa-stained smears was significantly correlated with survival. Regarding the NA, the results highlighted that for each increment of 1 μm^2 relative to the mean NA, the risk of disease-related death increased by 1.07.

The results from the multivariable Cox regression analysis did not show a significant statistical relationship between the variables and overall survival. Convergence was not achieved when the parameters "presence of ≥7 mitotic figures in May Grünwald-Giemsa staining" and "presence of ≥7 mitotic figures in H&E staining" were included in the models, so they were removed from the multivariable analysis.

DISCUSSION

In this study, a series of MCT cases with clinical follow-up information were thoroughly reviewed in order to assess the prognostic value of different cytological features. Since it is unknown whether these parameters are better evaluated in Romanowsky or H&E-stained slides, which in theory would allow better assessment of nuclear features, cytological grade parameters were evaluated first in Romanovsky-stained slides and then in H&E-restained slides. The NA was then assessed by 2D-nucleator in the H&E-restained slides.

According to our results, the cytological adaptation of the Kiupel grading system was superior to the Camus grading system in predicting survival. The grading parameters most associated with survival were the presence of ≥7 mitotic figures either in May Grünwald-Giemsa-stained smears or in H&E-restained slides, and cell granularity. The presence of mitotic figures as classified by the Camus system in H&E-restained smears also showed a trend towards an association with a worst prognosis, while in May Grünwald-Giemsa-stained smears the same parameter was not associated with survival. Overall, the prognostic value of the grading systems or their criteria was not significantly increased by destaining and restaining the smears with H&E. Furthermore, this procedure jeopardizes the recognition of granularity, which is assessed in the Camus system. Still, destaining and restaining was essential for unmasking the nuclear limits of the neoplastic mast cells, thus allowing an unbiased NA quantification by stereology. The NA determined by the 2D-nucleator was significantly correlated with survival, confirming that this parameter could be used as a prognostic factor in MCTs.

While the Camus grade has been shown to be associated with survival in two studies, the prognostic value of the cytological adaptation of the Kiupel grading system had never been assessed, to the best of our knowledge (Camus et al., 2016; Paes et al., 2022). According to the findings of this study, the cytological adaptation of the Kiupel grading system outperformed the Camus grading system in predicting survival. This superior prognostic value could be attributed to the quantitative evaluation of the cytological Kiupel system parameters, which allowed a more objective assessment. The Camus criteria, on the other hand, are more subjective and high-grade classification can easily be inaccurately assigned. According to the Camus system, the presence of binucleated cells and pleomorphic nuclei, for example, is sufficient for a MCT to be classified as high-grade. Since this system does not have numerical thresholds, the presence of more than two cells for each of these parameters can be judged by the observer as sufficient to consider their overall presence. Furthermore, nuclear

pleomorphism and binucleation have been reported to have a low correlation with survival (Camus et al., 2016; Paes et al. 2022). These two subjective grading parameters were not associated with survival in the current study, and they appeared to decrease the prognostic significance of the Camus grading system. In order to use the Kiupel system cutoff values, Scarpa et al. (2014) determined that at least 1000 cells should be evaluated; however, that many cells were not present in all smears in the current study. In light of this, our findings suggest that evaluating 1000 for the application of this grading system may not be necessary.

The prognostic value of cytological parameters has been evaluated in two previous studies, and their results are partially in agreement with the current findings (Camus et al., 2016; Paes et al., 2022). In those studies, the Camus system parameter for assessing mitotic figures, which categorizes them as present or absent, was found to be significantly associated with survival. Accordingly, the relationship between the presence of mitotic figures in H&E-restained smears and survival in the current study was close to the level of statistical significance. However, the association with survival was stronger when the mitotic activity was evaluated according to the Kiupel grading system, which considers the presence of ≥7 mitotic figures. Because of the variation in cellularity between cytological specimens, it has been suggested that MCT grading criteria should not have numerical thresholds in cytology (Kiupel & Camus, 2019; Scarpa et al., 2016). This was considered a drawback to using the Kiupel grading criteria directly in cytological smears (Kiupel & Camus, 2019). In the two studies that applied the Kiupel criteria to MCT cytological specimens, the number of mitoses observed in the cytological specimens was consistently lower than in the histological specimens (Hergt et al., 2016; Scarpa et al., 2016). Furthermore, the presence of ≥7 mitotic figures was one of the parameters with the lowest correlation to histological grade. In the present study, it was not possible to compare the mitotic activity in matched cytology and histology specimens (the histologic grade was retrospectively retrieved from the archives), but the presence of ≥7 mitotic figures in smears was the grading parameter with the strongest prognostic value. The current findings also suggest that an objective evaluation of the presence of mitotic figures improves the prognostic value of this parameter. While the cutoff value may not be achieved in all cases, pending on the cellularity and preservation of the cytology smears (Hergt et al. 2016; Scarpa et al., 2014), when ≥7 mitotic figures are present, a poor prognosis can be expected. More specifically, a dog with a MCT that has ≥7 mitotic figures in cytology has a 13.7 higher risk of dying due to the MCT. The role of mitotic count as a prognostic factor in MCTs is widely recognized. In fact, both the Patnaik and Kiupel grading systems consider the number of mitoses as a criterion, and several authors

have reported a correlation between overall survival and the mitotic index in histological samples. The importance of mitotic activity in MCTs is further supported by the current study.

According to Camus et al. (2016) and Paes et al. (2022), granularity was the grading criteria with the strongest association with survival, and cases with poor granularity resulted in lower survival rates. The findings of the present study strengthened the growing body of evidence that this cytological parameter is important for predicting survival. The amount of granules in the cytoplasm is a rapid appraisal of the degree of mast cell differentiation, with undifferentiated mast cells presenting sparse granulation. Cytoplasmic granularity is also a criterion in traditional histological grading systems, which were based on cellular differentiation (Bostock, 1973; Patnaik et al., 1984). The application of these systems has revealed that mast cell differentiation was predictive of tumor behavior, with well-differentiated MCTs having a low metastatic potential and undifferentiated MCTs having a higher metastatic rate.

Binucleation and, unexpectedly, multinucleation, were not significantly associated with survival in this study, despite being frequently observed in our case series. This result seems to contradict the conclusions reported by Camus et al. (2016) and Paes et al. (2022). However, it should be stressed that in the latter study, the statistical relationship between survival and binucleation was weak, resulting in its exclusion from the grading system proposed by those authors (Paes et al. 2022).

Non-quantitative and subjective nuclear size parameters were frequently found in the cytological samples of this case series, but they were not found to be associated with survival in the current study. In the study by Paes et al. (2022) a statistically significant relationship between survival and karyomegaly was observed, but not between survival and anisokaryosis. According to the cytological adaptation of the Kiupel grading system, karyomegaly was considered to be present if there was a variation in nuclear size of at least twofold in the majority of the observed fields, and a good correlation between this parameter and histopathology has been reported (Hergt et al., 2016; Scarpa et al., 2016). Despite this, karyomegaly was the parameter responsible for all the cytologically misclassified high-grade MCTs that were later revealed to be low-grade in histopathology in those studies. The authors justified this result as a consequence of the difficulty in comparing cells in poor-quality smears with sparse, ruptured or shrunken mast cells. On the other hand, Camus et al. (2016) observed a correlation between survival and anisokaryosis. The presence of anisokaryosis was defined by the Camus grading system as ">50% variation in nuclear size". However, the proportion of cells in which that variation should be observed and the nuclear sizes to be compared were not specified by those

authors. This lack of information may raise the questions: should anisokaryosis be considered present if a >50% variation in nuclear size is found only in two cells?; is the >50% variation relative to the mean nuclear size, or should all mast cell nuclei be compared with each other? Furthermore, as illustrated in Supplemental Figure F., a 50% increase in nuclear size is difficult to assess visually by the naked eye. This could lead to variation in the observer assessment of this subjective criterion, which jeopardizes the prognostic value of this grading parameter.

Nuclear pleomorphism was the cytological parameter that was least predictive of survival in the study by Camus et al. (2016). As a result, the statistical relationship between survival and nuclear pleomorphism or bizarre nucleation was not evaluated by Paes et al. (2022) and those criteria were not included in their newly proposed grading system. In the present study, there was also no significant association between survival and nuclear pleomorphism or bizarre nucleation. The latter parameter was defined by the presence of highly atypical nuclei with prominent indentations, segmentation, and irregular shape (Hergt et al., 2016; Scarpa et al., 2014), while nuclear pleomorphism was defined by Camus et al. (2016) as non-rounded nuclei. These criteria were also subjective, as Scarpa et al. (2016) pointed out when reporting low agreement between cytological and histological specimens on bizarre nucleation. Moreover, Camus et al. (2016) suggested that because cells are spread during cytological preparation, features observed in tissue samples such as lobulation and indentation may not be preserved in cytology smears. Destaining and restaining the May Grünwald-Giemsa-stained slides revealed atypical nuclear features in several specimens. However, atypical nuclear features, even in the H&E-restained slides, were not useful for MCT prognostication when analyzed separately. Thereby, atypical nuclear morphological parameters appear to be unreliable for predicting outcome in cytology smears.

The NA assessed by morphometry in cytological samples was associated with histological grade and survival, making it a promising prognostic tool in MCTs (Strefezzi et al. 2003). In the present study, the prognostic value of NA assessed by 2D-nucleator was confirmed, as mean NA was significantly associated with survival. The results show that for each increment of 1 μm^2 relative to the mean NA, the risk of dying from MCT-related disease increases by 1.07. The cutoff value of 62.8μm^2 established by Marcos et al. (2022) was also correlated with survival, but further large prospective studies are needed to establish NA thresholds with high sensitivity and specificity to identify clinically aggressive MCTs.

The small sample size and low number of MCT-related deaths were the main limitations of the present study. Non-convergence occurred in the regression analysis

when some of the covariates were included due to the low number of MCT-related death events. As a result, the estimated risk was unacceptably high (data not shown), but the precision of this determination was not in accordance with Cox proportional hazards assumptions. The lack of a statistical relationship between the variables in the multivariate analysis may also be due to the small sample group and low number of events, as it has been established that at least ten events per variable in each model are required for an accurate estimation of regression coefficients (Peduzzi et al., 1996). Furthermore, the current statistical survival analysis was not stratified according to the different treatment modalities. This should be performed when more cases are enrolled in the present study.

Cytological examination does not distinguish between cutaneous and subcutaneous MCT, which can be viewed as a drawback in the cytological assessment of prognosis in MCTs, since these tumor subtypes have been reported to have different biological behaviors (Kiupel & Camus, 2019; Scarpa et al., 2016; Thompson et al., 2011). Nevertheless, the presence of multinucleated cells and ≥4 mitotic figures per 10 HPF has been associated with a decrease in survival in subcutaneous MCTs (Thompson et al., 2011). Histopathology revealed that one tumor in our study was subcutaneous, and it belonged to one of the dogs that died from the disease. In this case, the mean NA was higher than the mean NA of the study group. While the smear was highly granular and only binucleation and anisokaryosis were observed in May Grünwald-Giemsa staining, H&E-restaining revealed ≥7 mitotic figures. To further evaluate the prognostic value of cytological parameters in subcutaneous MCTs, studies including exclusively this tumor subtype would be required. However, establishing these parameters could allow cytological evaluation to help in the prediction of the outcome in these two biologically different MCTs. This information, combined with the clinical evaluation of the mass, which, in the case of subcutaneous MCTs, tend to present as soft masses resembling lipomas (Kiupel, 2016), would allow the referring veterinarian to design a therapeutical plan according to the suspected subtype of tumor.

Based on the findings of this study, mast cell granulation and the presence of mitotic figures are reliable cytological parameters for predicting outcome in routinely stained MCT smears. Since the overall goal of the study was to maximize the prognostic potential of cytological evaluation in MCTs, it is recommended that the criteria that were found to be predictive of survival be included in routine cytological examination reports. It is important to note that mitotic figures may only be observed occasionally, and their absence does not rule out aggressive MCT behavior. As such, cytologic grading parameters should be used along with the NA assessment in order to provide useful prognostic information to clinicians. While stereology software may not be readily

accessible, which may be viewed as a drawback in stereological approaches, manual methods or free software can be used instead. As suggested by Marcos et al 2022, the NA can be estimated either by overlaying an acetate with a grid of points on a computer screen or by using free web tools including the STEPanizer19 or ImageJ stereology plugins (Mironov, 2017; Tschanz et al., 2011). Larger studies should be conducted to further explore the prognostic value of NA determined by stereological methods, establish NA cut-off values based on survival, and evaluate the reproducibility of these methods. Nevertheless, this study found that the NA assessed by stereological tools is predictive of outcome in canine cutaneous MCTs and could be a valuable resource in tailored treatment planning prior to surgery in these patients.

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SUPPLEMENTAL MATERIAL A

Supplemental Table A. *Characteristics of the* study population: World Health Organization clinical stage, number of tumors, surgical margin completeness, and histological grade.

	Stage (WHO)[1]				**Number of MCTs**	
	I	II	III	IV	1	2
n	20	4	10	1	34	2
	Tumor Margins				**Histological Grade**	
	Complete		Incomplete[2]		Low	High
MCTs	24		14		30	8

[1]No staging information was available for 1 dog; [2] Infiltration by neoplastic cells in at least one margin.

SUPPLEMENTAL MATERIAL B

Supplemental Table B. Distribution of cytological grade classification according to the Kiupel system adapted by Scarpa et al. (2014) and Hergt et al. (2016) and the Camus system in May Grünwald-Giemsa-stained and H&E-restained specimens.

	Grade			
	Kiupel System		Camus System	
Specimen	Low	High	Low	High
May Grünwald-Giemsa-stained	29	10	18	21
H&E-restained	20	19	13	26

SUPPLEMENTAL MATERIAL C

Supplemental Table C. Distribution of the presence of cytologic criteria from the adaptation of the Kiupel grading system for cytology in May Grünwald-Giemsa-stained and H&E-restained specimens.

	Kiupel Cytological Criteria							
	≥7 Mitotic Figures		≥ 3 Multinucleated cells		≥3 Bizarre nuclei		Karyomegaly	
Specimen	Absent	Present	Absent	Present	Absent	Present	Absent	Present
May Grünwald-Giemsa-stained	36	3	35	4	38	1	33	6
H&E-restained	33	6	30	9	33	6	27	12

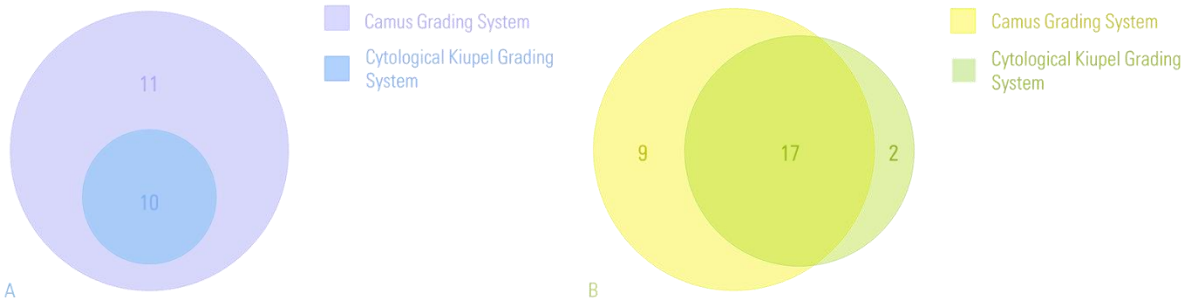
SUPPLEMENTAL MATERIAL D

Supplemental Table D. Distribution of the presence of cytological criteria from the Camus Grading system in May Grünwald-Giemsa-stained and H&E-restained specimens.

	Camus Cytological Criteria												
	Granularity			Mitotic Figures		Binucleated Cells		Multinucleated Cells		Nuclear Pleomorphism		Anisokaryosis	
Specimen	H	M	L	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.
May Grünwald-Giemsa-stained	16	21	2	33	6	11	28	33	6	38	1	9	30
H&E-restained				29	10	5	34	29	10	32	7	13	26

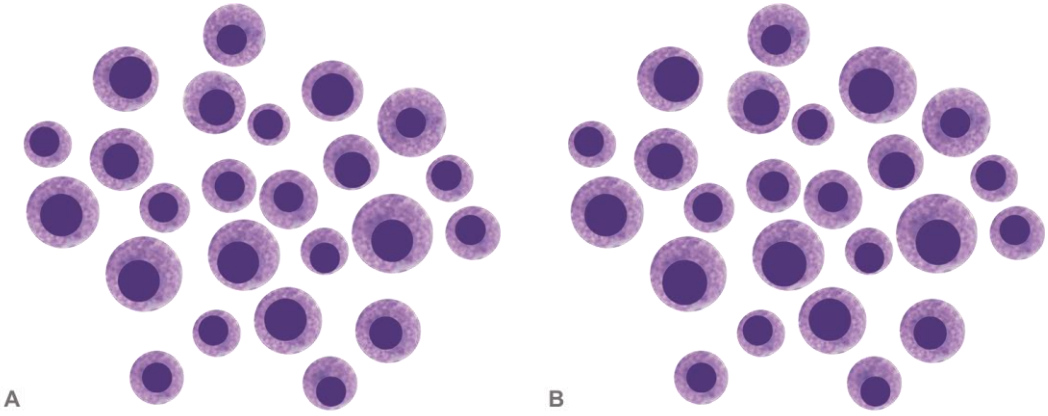
H- High; M- Mixed; L-Low; Abs.- Absent; Pres.- Present.

SUPPLEMENTAL MATERIAL E



Supplemental Figure E. Diagrams illustrating the agreement in high-grade classification between the two grading systems in the May Grünwald-Giemsa-stained and H&E-restained slides. (A) Euler diagram representing the agreement on the high-grade classification in the May Grünwald-Giemsa-stained specimens. This diagram also shows that all the samples classified as high-grade by the cytological adaptation of the Kiupel grading scheme were also considered high-grade by the Camus grading system. (B) Venn diagram representing the agreement on the high-grade classification in the H&E-restained slides.

SUPPLEMENTAL MATERIAL F



Supplemental Figure F. Demonstration of the challenges of visually assessing anisokaryosis according to the Camus grading system's definition. Although the images are similar, variation in nuclear size is less than 50% in image **A**, indicating that anisokaryosis is not present. In image **B**, however, the variation in nuclear size is greater than 50%, which implies anisokaryosis is present.