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Use of the optical dissector in canine mammary simple and complex carcinomas

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Running title: Numerical density in canine mammary tumors
Summary
Grading of canine mammary carcinomas (CMC) is associated to 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 \([N_v (\text{nuclei, tumor})]\) 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 \(N_v (\text{nuclei, tumor})\) was \(1.6 \times 10^6 \pm 0.5 \times 10^6\) 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 densities. The \(N_v (\text{nuclei, tumor})\) and the correlated parameters mirror to some extension 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.

Keywords: canine mammary tumors; disector; grade; prognosis; stereology
Introduction

The level of knowledge in canine mammary carcinomas (CMC) has increased considerably in recent years, with various putative prognostic factors been pointed (1). However, it is still recognized that the marked clinical and morphological heterogeneity, including the possibility of multiple synchronous CMC of different subtypes could make the assessment of the prognosis difficult (2). Moreover, the different methodological approaches and end-points used in prognostic studies of CMC puzzled the identification of definitive prognostic factors (2).

Despite the development of sophisticated “omics” technologies in oncology, tumor morphology continues to be a powerful mode of providing clinical and prognostic informative data (3). Still, it is recognized that the histopathological assessment of tumor features is not entirely objective and this can jeopardize the biological conclusions, namely in terms of prognosis (4). Such subjectivity may be overcome by quantitative morphological parameters assessed by suitable morphometric or stereological methods (5, 6). These methods are substantially different: while morphometry describes quantitatively what is seen in conventional sections [under 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 three-dimensional (3D) information inherent of all biological tissues (5-7). Stereology can be used in histological sections of tumors, allowing 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 (4, 8).

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 (4, 8-10). In CMC, the use of stereology is still very incipient (11), but it already started to solve issues related with the subjective assessment of nuclear pleomorphism in grading of CMC (12).

CMC are classified according to the cell populations presented within the tumor, as simple (one neoplastic cell population, epithelial or myoepithelial of origin) or complex (when epithelial and myoepithelial cells coexist) (13). In simple carcinomas, the architectural arrangement of the neoplastic epithelial cells, e.g., the presence of tubulopapillary structures or solid sheets is included in the histological classification, with some special subtypes such as squamous cell or mucinous carcinomas being characterized by specific morphological features (13).

It has been suggested that highly cellular CMC, *i.e.* solid subtypes, are associated with a poorer prognosis compared with tubulopapillary tumors (13, 14). However, cellularity assessed by pathologists tends to be qualitative and may be 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 (7, 15). 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 (5, 6, 16).

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 subtype, vascular/lymph node invasion and histological grading 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.
Materials and Methods

Selection of cases and histological analysis

Forty four spontaneous CMC treated at UPvet (Veterinary Hospital of the University of Porto) were retrospectively selected, blinded to clinical and other pathological data. The female dogs were submitted to surgical resection of the tumors with the owner’s consent. For twenty seven cases follow-up data were collected prospectively over two years following a protocol detailed elsewhere (2). The histological diagnosis and grading was reviewed by two pathologists (MS and PD P) using the criteria of the World Health Organization classification (17) and the Nottingham histological grade (NHG) (18). For this, routine 5 µm sections resulting from the largest cross slab of the tumor were retrieved and screened. For every case, the tumor size and the histological evidence of vascular invasion and/or regional lymph node metastases were recorded. As to tumor size, it was categorized according to World Health Organization (WHO) criteria (T1< 3 cm, T2=3-5 cm and T3> 5 cm), as previously described (19).

Sectioning and stereological analysis

For 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. 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. All 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, Tokyo, Japan) equipped with a 100x oil-immersion lens (Olympus
Uplan NA = 1.35, Tokyo, Japan) 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, Fulbourn, 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 (Olympus CAST-Grid, version 1.5, Albertslund, 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 ($h = 16$ µm), a software generated counting frame was superimposed, having a defined area of 253 µm$^2$ and inclusion and forbidden lines (Fig. 1), to prevent the edge effect counting bias (20).

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 an upper guard height (4 µm) and a lower one (from 20 µm downward) (5). Spindle-shaped nuclei were excluded from the counts.

The $N_V$ (nuclei, tumor) was estimated using the formula (21):

$$N_V (\text{nuclei, tumor}) = \frac{\Sigma Q^*}{[h x a(\text{frame}) x \Sigma P]}$$

where $\Sigma Q^*$ corresponded to the sum of neoplastic nuclei counted in the sampled fields, and $a(\text{frame}), h$ and $\Sigma P$ were, respectively, the area of the counting frame, disector height and the 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 (16):

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

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 (16):

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

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 $N_V$.

Shrinkage estimation

It would be reasonable to assume that the shrinkage in x-y would be alike in all the included cases, as they were handled by the same surgical team and submitted to similar processing protocols. Despite this, estimation of the shrinkage in thick sections of each case was performed. For this, blood vessels were randomly photographed and the erythrocyte diameter was measured in 30 cells (measurements were restricted to erythrocytes appearing as clear circles). It should be stressed that: 1) animals had no hematological abnormality in their pre-surgical evaluation; and 2) a diameter of 7.0 µm was considered for normal canine erythrocytes (22).

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 $N_V$ (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; and 4) histological subtypes, were tested with one-way ANOVA, followed by Tukey post-hoc tests. The association degree between the $N_V$ (nuclei, tumor) and the volume-weighted mean nuclear volume [previously assessed by point sampled intercepts (12)] 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).
Results
Thirty out of 44 tumors were diagnosed as simple carcinomas (11 tubulopapillary, 16 solid, 2 squamous cell and 1 mucinous) and 14 were complex carcinomas. At the time of diagnosis, 12 cases (27%) presented vascular/regional lymph node invasion. With regard to NHG, 9, 15 and 20 cases were grade I, II and III, respectively. Follow-up data were available for 27 female dogs and during this period 30% (8/27) presented progression of the disease (defined as recurrence and/or metastases de novo). Of the remaining, 56% (15/27) were alive and clinically disease-free at 24 months after the surgery, whilst 14% (4/27) were censored for being lost to follow-up or for non-malignancy-related death. The clinicopathological parameters are summarized in Table 1. The optical dissector procedure was straightforward. Sections had a mean thickness of 28.9 ± 2.8 µm and around 6 cells nuclei were computed per dissector. In average, 259 nuclei per tumor were counted and the $N_V$ (nuclei, tumor) was estimated as $1.6 \times 10^6 \pm 0.5 \times 10^6$ nuclei mm$^{-3}$ (Fig. 2). The mean CE of the $N_V$ (nuclei, tumor) estimations was 0.07 (ranged from 0.04 to 0.11), meaning 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 $N_V$ (nuclei, tumor) was significantly higher in simple carcinomas ($1.7 \times 10^6 \pm 0.5 \times 10^6$ nuclei mm$^{-3}$) comparing with complex carcinomas ($1.3 \times 10^6 \pm 0.2 \times 10^6$ nuclei mm$^{-3}$) (t-test, $P=0.002$). No statistical difference was observed when solid carcinomas were compared with any other subtypes. The $N_V$ (nuclei, tumor) was $1.3 \times 10^6$, $1.7 \times 10^6$ and $1.6 \times 10^6$ nuclei mm$^{-3}$ in grade I, II, III tumors, respectively, without statistically significant differences. With regard to NHG parameters, the $N_V$ (nuclei, tumor) did not differ with the tubule formation score, 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, $P=0.021$). Similarly, a statistically significant increase in numerical nuclear density existed from tumors scored 1 or 2 to those scored 3 in mitotic 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 the three WHO size category. However, when tumors larger than 5 cm were compared with smaller tumors, the first ones presented a significant higher $N_V$ (nuclei, tumor) ($t$-test, $P=0.030$).

The $N_V$ (nuclei, tumor) was weak-to-moderately correlated with the volume-weighted mean nuclear volume ($r=-0.34; P=0.027$) — *i.e.* the $N_V$ (nuclei, tumor) tended to be lower in tumors presenting higher nuclear pleomorphism.

As to vascular/lymph node invasion status, the $N_V$ (nuclei, tumor) was similar in tumors with and without evidence of invasion. In the same line, no significant association between the $N_V$ (nuclei, tumor) and the post-surgical disease progression was detected. However, the eight cases that showed post-surgical disease progression during the follow-up period presented a lower $N_V$ (nuclei, tumor) ($1.4 \times 10^6$ nuclei mm$^{-3}$) when compared with cases without evidence of metastases and/or recurrence ($1.8 \times 10^6$ nuclei mm$^{-3}$) ($t$-test, $P=0.047$).

The estimated shrinkage in x-y was 35.8% ± 2.3%, with no significant differences between cases.
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 (9, 23-28). Stereological methods not only achieve such quantification, but have additional advantages of unbiasedness and reproducibility (5, 6). These have been applied for long in breast pathology (8, 27), but their use in the veterinary oncology is still incipient (11).

Herein, the optical dissector was used to assess the $N_V$ (nuclei, tumor) in CMC. Notably, the mean value for CMC ($1.6 \times 10^6$ nuclei mm$^{-3}$) was higher (but in the same order of magnitude) than that reported for human breast cancer ($0.4 \times 10^6$ nuclei mm$^{-3}$) (10). Interspecies differences may underlie such discrepancy, along with eventual technical discrepancies, particularly in the definition of the reference space (for example, we excluded stromal areas). Still, our data suggest that CMC present a higher numerical density of nuclei than human breast carcinomas. Despite the differences in figures between our and human studies, some observations in breast cancers were mirrored to some extension in CMC. For instance, 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 in our study and in the two existing breast cancer estimations [respectively, (10) and (27)].

Another interesting finding in both species is that cancers with worst survival outcomes presented a lower $N_V$ (nuclei, tumor) (10). 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 (13). 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 appear more distant (e.g. either 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 it was previously described that the volume-weighted mean nuclear volume was significantly higher in more aggressive tumors (12), and herein a negative correlation between the nuclear volume parameter and the \( N_V \) (nuclei, tumor) existed.

Herein the \( N_V \) (nuclei, tumor) did not differ between solid and tubulopapillary carcinomas. This supports that the presence of luminal structures in routine sections is not directly correlated with cellularity at 3D level. According to the present data, both solid and tubulopapillary carcinomas are heterogeneous regarding the 3D densities of nuclei, which is in accordance to previous studies describing variability in those subtypes of tumors using immunohistochemistry (e.g. 29). 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 (13). 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).

Paraffin shrinkage during tissue processing can influence the reference space and, therefore, lead to overestimations of the \( N_V \) (5, 30). In this study, the shrinkage was similar to that reported for thick paraffin sections (30, 31). In this case, the overall \( N_V \) (nuclei, tumor) corrected for shrinkage would be \( 1.17 \times 10^6 \pm 0.5 \times 10^6 \) nuclei mm\(^{-3}\).

Theoretically, problems arise by comparing estimations of tissues with different amounts of shrinkage. This is unlikely to have influenced our results, not only because
all the cases were handled and processed similarly, but also because no significant
differences in the diameter of erythrocytes between cases were noted. In fact, it should
be stressed that the possibility of bias related to tissue handling when stereology is
applied to routine diagnostic material should never cloud the advantages of stereology
over traditional 2D techniques (32). These latter are not only affected by shrinkage, but
are also severely influenced (in an uncontrolled extent) by the shape, orientation and
size of the particles being counted (6, 16, 30).

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 (16), and the error due to
the methodology was low. For future studies and for practical purposes, the 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).

Spontaneous CMC have been pointed as a suitable model for human breast cancer,
based on similarities in epidemiological data, risk factors, molecular characteristics, and
clinical course of the disease (e.g. 33, 34). The subtypes of simple CMC are more
similar, in terms of the histological features, to the most frequent human breast
carcinomas. The quantitative data presented herein strengthened the similarity of those
canine tumors with human breast carcinomas.

Conclusion

We showed in CMC that an unbiased and reproducible estimation of a cellularity-related
parameter — expressed as $N_V$ (nuclei, tumor) — can be obtained by stereological
methods. The mean $N_V$ (nuclei, tumor) was lower in complex carcinomas, in smaller
tumors, and in those with low mitotic activity and high nuclear pleomorphism. No
association with vascular/lymph node invasion was observed, but nuclear numerical
density was lower in cases that progressed during follow-up. This association is a
promising finding, suggesting that the $N_v$ (nuclei, tumor) have potential to be used to
assess survival outcome in CMC. For this, further and larger studies are required.

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Table 1: Numerical nuclear density and relevant clinicopathological parameters of the 44 canine mammary carcinomas used in this study.
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.
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.
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 dissector (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, and so few nuclei are counted in the dissector.