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Use of the optical disector 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 (nuclei, tumor)] is a cellularity-associated parameter that can be estimated by the optical disector. Herein, it was estimated in 44 CMC and its association with clinicopathologic factors — such as tumor size, histological subtype and grade, vascular/lymph node invasion, nuclear pleomorphism and survival — was evaluated. Considering all the cases, the mean N_V (nuclei, tumor) was $1.6x10^6$ ± $0.5x10^6$nuclei mm^{-3}. Lower values were attained in complex carcinomas, comparing to simple carcinomas, in tumors smaller than 5 cm, with low mitotic activity and in those with high nuclear pleomorphism. No statistically significant association with grade or vascular/lymph node invasion was observed, but tumors with disease progression had lower nuclear densities. The N_V (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 tubulopapillar 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 PDP) 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, Fulbourm, 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):

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

where ΣQ^- corresponded to the sum of neoplastic nuclei counted in the sampled fields, and a(frame), h and ΣP 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):

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

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

The CE of the N_V estimations was then compared with the observed relative variance among cases, OCV^2, according to the formula (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).

178 **Results**

179 Thirty out of 44 tumors were diagnosed as simple carcinomas (11 tubulopapillary, 16
180 solid, 2 squamous cell and 1 mucinous) and 14 were complex carcinomas. At the time
181 of diagnosis, 12 cases (27%) presented vascular/regional lymph node invasion. With
182 regard to NHG, 9, 15 and 20 cases were grade I, II and III, respectively. Follow-up data
183 were available for 27 female dogs and during this period 30% (8/27) presented
184 progression of the disease (defined as recurrence and/or metastases *de novo*). Of the
185 remaining, 56% (15/27) were alive and clinically disease-free at 24 months after the
186 surgery, whilst 14% (4/27) were censored for being lost to follow-up or for non-
187 malignancy-related death. The clinicopathological parameters are summarized in Table 1.
188 The optical disector procedure was straightforward. Sections had a mean thickness of
189 28.9 ± 2.8 µm and around 6 cells nuclei were computed *per* disector. In average, 259
190 nuclei *per* tumor were counted and the N_V (nuclei, tumor) was estimated as $1.6x10^6$ ±
191 $0.5x10^6$ nuclei mm^{-3} (Fig.2). The mean CE of the N_V (nuclei, tumor) estimations was
192 0.07 (ranged from 0.04 to 0.11), meaning that the estimation methodology was
193 responsible for 5% of the total observed variance. Therefore, the biological variability
194 was by far the most important component of the observed variability of the N_V (nuclei,
195 tumor) estimations.
196 The N_V (nuclei, tumor) was significantly higher in simple carcinomas ($1.7x10^6$ ±
197 $0.5x10^6$ nuclei mm^{-3}) comparing with complex carcinomas ($1.3x10^6$ ± $0.2x10^6$ nuclei
198 mm^{-3}) (t-test, P =0.002). No statistical difference was observed when solid carcinomas
199 were compared with any other subtypes. The N_V (nuclei, tumor) was $1.3x10^6$, $1.7x10^6$
200 and $1.6x10^6$ nuclei mm^{-3} in grade I, II, III tumors, respectively, without statistically
201 significant differences. With regard to NHG parameters, the N_V (nuclei, tumor) did not
202 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) ($1.4x10^6$ nuclei mm^{-3}) when compared with cases without evidence of metastases and/or recurrence ($1.8x10^6$ nuclei mm^{-3}) (*t*-test, P =0.047).

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 disector was used to assess the N_V (nuclei, tumor) in CMC. Notably, the mean value for CMC ($1.6x10^6$ nuclei mm^{-3}) was higher (but in the same order of magnitude) than that reported for human breast cancer ($0.4x10^6$ nuclei mm^{-3}) (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 $1.17x10^6 \pm 0.5x10^6$ 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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	Simple carcinomas (*n=30*)			Complex carcinomas (*n=14*)
	Tubulopapillary (*n=11*)	Solid (*n=16*)	Others (*n=3*)	
N_V (nuclei, tumor) (mean, μm)	$1.8x10^6$	$1.7x10^6$	$1.6x10^6$	$1.3x10^6$
Tumor size <3 cm	10	5	1	9
Tumor size 3-5 cm	0	4	1	2
Tumor size > 5 cm	1	7	1	3
Histological grade I	2	0	0	7
Histological grade II	7	3	1	4
Histological grade III	2	13	2	3
Vascular/lymph node invasion	2	8	0	2
Disease progression (recurrence and/or metastasis)*	1	5	0	2

*Follow up data was available for 22 cases with simple carcinomas and 5 cases with complex carcinomas

Table 1: Numerical nuclear density and relevant clinicopathological parameters of the 44 canine mammary carcinomas used in this study.

Figure legends

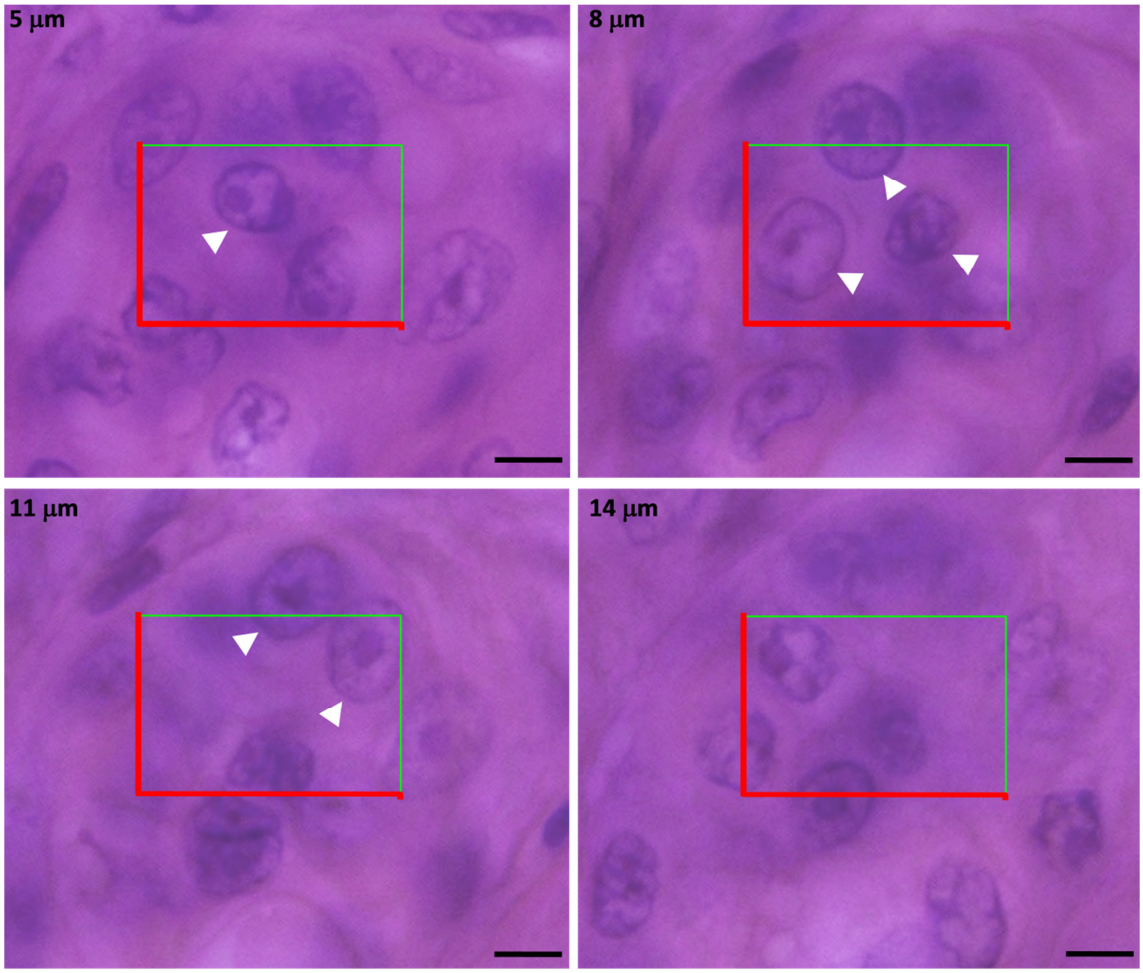

Fig. 1: Series of light micrographs from a thick section (30 µm) of a canine mammary carcinoma that form an optical disector (the depth of each optical plane is indicated in the upper left corner). Nuclei of neoplastic cells are counted if they are seen within the counting frame or touching the inclusion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

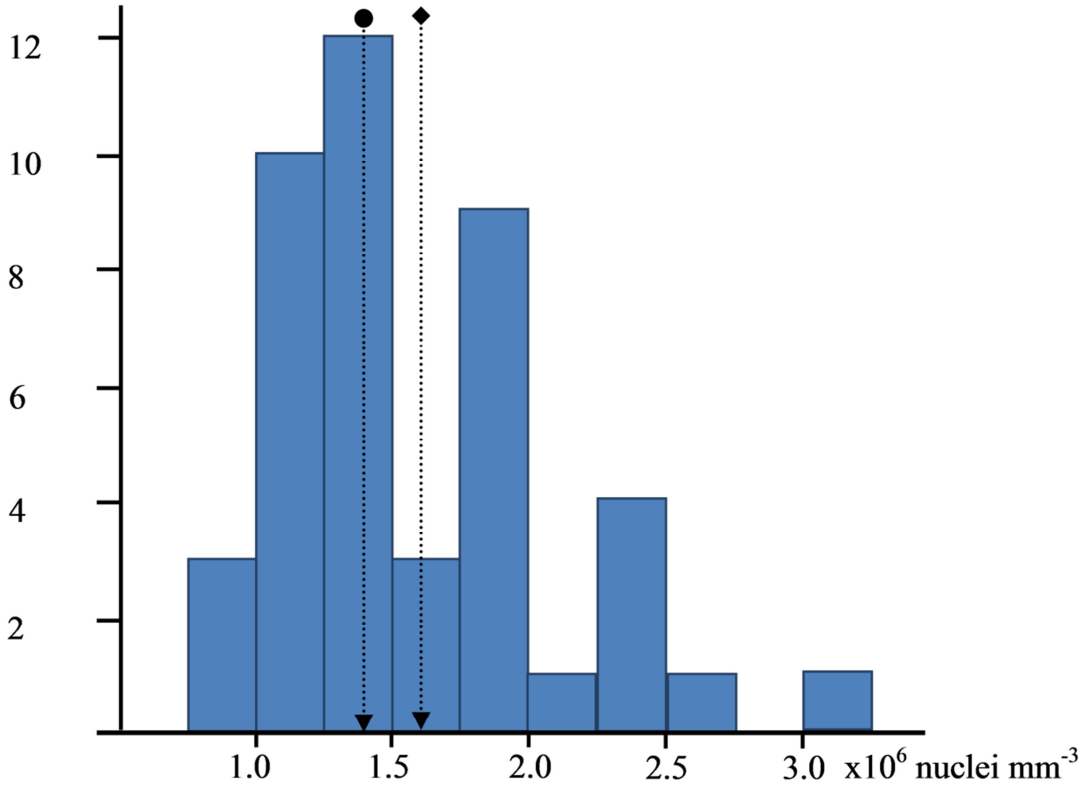

Fig. 2: Histogram of the mean N_V (nuclei, tumor) values in the 44 canine mammary carcinomas; lozenge-arrow: mean value; circle-arrow: median value.

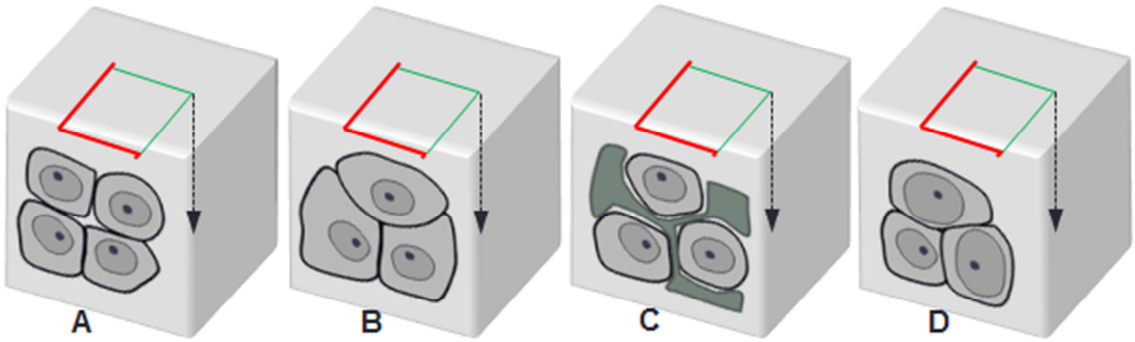

Fig. 3: Potential (theoretical) explanations for the changes in the N_V (nuclei, tumor). For the sake of illustration consider a reference space (gray cube) holding particles that are counted through the optical disector (A). From B to D the N_V (nuclei, tumor) decreases through different mechanisms.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