# MIB-1 Labelling Indices According to Clinico-pathological Variables in Canine Mammary Tumours: A Multivariate Study

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**Abstract.** *The relationship between MIB-1 labelling indices (LI), as detected by immunohistochemical methods, and other clinico-pathological characteristics was studied in a series of 77 malignant mammary tumours surgically removed from 47 female dogs. The immunostaining was assessed on the basis of the estimated percentage of positive cells in the areas of highest labelling. Multivariate logistic regression demonstrated no influence of breed, age, previous pregnancies, previous progestin administration, histological type or location of the tumour on MIB-1 LI. MIB-1 LI was significantly related to the size of the tumour, necrosis, invasive growth and histological grade, but not with ulceration, lymph node metastasis, skin fixation or E-cadherin expression. The significant relationship between MIB-1 LI and other known factors of poor prognosis suggests that a high LI may have prognostic value in canine malignant mammary tumours.*

The accumulation of data regarding the histological appearance of canine mammary tumours led, in 1999, to a new method of classification of these neoplasms, based on the correlation between descriptive morphology and its prognostic value (1). This system refrains from using other methods of classification, namely cellular kinetic markers, although its authors admit that these may have prognostic value.

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Ki-67 is a non-histone nuclear protein expressed in all cell cycle stages except $G_0$ (2) that can be detected by the MIB-1 antibody on formalin-fixed, paraffin-embedded tissues (3-5). Although the MIB-1 labelling index (LI) is highly correlated to the growth fraction (4), it might overestimate the growth rate of tumours, when compared to *in vivo* bromodeoxuridine labelling, considered the standard in the analysis of proliferative activity (6).

The expression of Ki-67 was studied in canine mammary tumours and proved to be inversely associated with oestrogen-receptor alpha scores (7) and positively correlated with metastasis, low disease-free survival rate and death from neoplasia (8, 9). Other studies, however, failed to correlate Ki-67 immunohistological scores with the clinical outcome (10). A large number of variables, such as differences in several steps of tissue processing, methods of immunohistochemical evaluation and interpretation of proliferation measurements (11), might explain the discordance.

In this study, the correlation between the MIB-1 LI and other clinical and histological characteristics of 77 canine malignant mammary tumours was examined in order to determine whether it could be regarded as a prognostic factor. In refining the possible influence of clinical stage, cytokeratin staining-based evaluation of regional lymph nodes (12) was considered instead of the routine haematoxylin and eosin (H&E) staining-based staging (13). The MIB-1 LI of tumours was defined as the percentage of immunostained cells in the areas of highest positivity.

## Materials and Methods

*Tissue samples.* Seventy-seven malignant mammary tumours and corresponding local and regional lymph nodes were surgically

removed from 47 female dogs, aged from 5 to 13 years (mean: 10.1 years), of various pure or mixed breeds (one Basset Hound; three Boxers; seven Poodles; one Portuguese Water Dog; five Cocker Spaniels; one Fox Terrier; one Siberian Husky; one German Shepherd; two Pekinese; two Pointers; and 23 mixed breed). The dogs were characterized regarding weight (large: >23 Kg; medium: 10 to 23 Kg; small: <10 Kg); age (recorded in months); previous pregnancies and previous progestin administration.

Before removal, each tumour was observed clinically and palpated and the following data were recorded: location in the mammary chain, dimensions, skin ulceration and cutaneous and underlying tissue fixation. For statistical purposes, the location was divided into three groups: thoracic glands (M1 and M2); cranial abdominal glands (M3); and caudal abdominal and inguinal glands (M4 and M5). For statistical study, the tumours were also grouped as either smaller or larger than 3 cm in diameter.

Small tumours (<1 cm) were entirely included, while sequential segments 5 mm apart were cut from larger tumours to provide tissue blocks. After dehydration and embedment in paraffin wax, sections (3 μm) were cut from each block. One section was stained with H&E and selected sections, representative of the tumour type and free of necrosis, haemorrhage and inflammatory cell infiltrates, were used for immunohistochemistry (IHC).

*Histological examination of the tumours.* The tumours were classified independently by two pathologists on sections stained with H&E, using the diagnostic criteria proposed by the World Health Organization Classification of Tumours in Domestic Animals (1). The same sections were used for the determination of the histological grade according to the Nottingham method for human breast tumours (14). In accordance with the mentioned method, mitotic counts were determined as the number of mitoses per 10 high-power fields (HPF) in the areas where higher MIB-1 LI were determined. The presence of intra-tumoral necrosis was also registered. The tumours were further assessed regarding mode of growth and were classified as expansive, infiltrative, or with evident vessel invasion.

*Histological examination of lymph nodes.* In all cases where lymph nodes were submitted, longitudinal central sections were used for H&E staining and adjacent sections were immunostained using the modified avidin-biotin-peroxidase complex (ABC) method (15). The following primary antibodies were used: anti-pancytokeratin antibody AE1/AE3 (Zymed Laboratories, USA), diluted 1:50; and anti-cytokeratin 14 (clone LL002 – Serotec Laboratories, UK), diluted 1:10.

Evaluation of the IHC-stained slides was performed without knowledge of the H&E results. Tumours were classified as positive for lymph node metastasis whenever tumour cells were identified in at least one local or regional node, regardless of the staining method or the size of the metastases.

In five dogs, where more than one malignant tumour was present and lymph nodes were considered positive, statistical analysis was performed by attributing the nodal status to the tumour with more aggressive characteristics, based on its size, mode of growth and histological grade.

*Immunohistochemistry of primary tumours.*

*E-cadherin:* Tumour sections adjacent to those used for H&E staining were immunostained and evaluated for E-cadherin expression, as described elsewhere (16). For statistical purposes, tumours were classified according to the estimated percentage of cells with membranous expression of E-cadherin and grouped as: less than 50% (severe loss of expression); 50 to 75% (moderate loss of expression); and more than 75% positive cells (no loss of expression).

*MIB-1:* Tumour sections adjacent to those used for H&E staining were immunostained using the modified ABC method (15). Briefly, the formalin-fixed paraffin sections were dewaxed, rehydrated and then submitted to proteolytic digestion by immersion in 10% target retrieval solution (Dako, Denmark) and were kept in a water bath at 100˚C for 20 min. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck, Frankfurt, Germany) in methanol (Merck) for 10 min. The sections were then incubated in a moist chamber for 20 min with normal rabbit serum (Dako) diluted 1:5 in TBS/BSA 10% (Sigma, USA) to eliminate non-specific staining. Excess serum was removed and the sections were incubated with monoclonal antibody MIB-1 (Dako) diluted 1:50 in TBS/BSA 5% (Sigma), overnight at 4˚C. This was followed by incubation for 30 min with a 1:200 biotin-labelled anti-mouse secondary antibody (Dako) and with ABC (Dako), for an additional 30 min, using diaminobenzidine as chromogen. Careful rinses were done with PBS between each step.

For negative control purposes, the primary antibody was replaced by a mouse IgG1 antibody-clone Dak-Go1 (Dako). Sections from normal canine mammary tissue were used as positive tissue controls. The staining was nuclear and considered positive regardless of the intensity. The area of highest labelling was searched, avoiding areas of necrosis or inflammatory cell infiltration. To determine the MIB-1 labelling index, 1000 tumour cells were counted with the help of a microscopic grid, at high magnification and the index was expressed as a percentage.

*Statistical analysis.*

*Factors influencing MIB-1 LI:* After assessment and certification of a normal distribution of the sample, an analysis of covariance, using the SAS procedure GLM, was performed considering MIB-1 LI as the dependent variable and breed, age, previous pregnancies, previous progestin administration, histological tumour type, histological grade and location as explanatory variables. Differences and statistical significance were determined where appropriate, using contrasts between adjusted means (least square means).

An analysis of risk was also performed by categorizing the MIB-1 LI according to tertiles, in low (less than 27% positive cells), medium (27 to 43% positive cells) and high (more than 43% positive cells) and by running a multivariate logistic regression, using the SAS procedure LOGISTIC (17). The explanatory variables corresponded to those used in the previous analysis.

*Factors influenced by MIB-1 LI:* To understand the independent influence of the MIB-1 LI in other clinico-pathological characteristics of the tumours, the following parameters were also studied as dependent variables: ulceration, lymph node metastasis, presence of necrosis, skin fixation, largest diameter, mode of growth, E-cadherin expression and histological grade. A selection of explanatory variables was performed based on Pearson's correlation coefficients between all variables that showed a significant ($p<0.05$) correlation (SAS procedure CORR, 1989). Each dependent variable was then analysed by applying a multivariate logistic regression model, using the backward system of independent variable selection and using $p<0.1$ as the criterion for inclusion. Regardless of the correlation results, MIB-1 LI was included in all sets of explanatory variables.

## Results

The studied tumours included: 20 solid carcinomas, 18 complex carcinomas, 12 carcinosarcomas, four carcinomas in benign tumours, 17 tubulopapillary carcinomas, four mucinous carcinomas and two micropapillary carcinomas. The distribution of tumours according to the studied variables are found in Table I.

The areas of highest labelling were, in the majority of cases, in the periphery of the tumours but, in some cases, the focal areas of highest immunolabelling were detected in central areas. MIB-1 immunostaining was nuclear, although variable in intensity and all mitotic cells exhibited chromosomal MIB-1 reactivity. The mean MIB-1 LI was 38.7 % (range 8.6 to 84.2%).

*Factors influencing MIB-1 LI.* In the analysis of covariance, from all the explanatory variables (breed, age, previous pregnancies, previous progestin administration, histological tumour type, histological grade and location), only the histological grade was significantly correlated with the MIB-1 LI. Thus, grade III tumours showed higher LI than grade I (least square mean 51.3% for grade III and 26.9% for grade I, $p<0.0001$) and grade II tumours (least square mean 51.3% for grade III and 34.4% for grade II, $p=0.0002$). No significant differences were found between grade I and II tumours.

Multivariate logistic regression, after categorization of MIB-1, confirmed the absence of a significant correlation between the MIB-1 LI and all explanatory variables, except histological grade, and showed significant differences only between grade I and grade III tumours (OR=0.07; CI=0.02-0.27; $p=0.0009$).

*Factors influenced by MIB-1 LI.* The results of the multivariate logistic regression are found in Table II. Factors statistically influenced by the MIB-1 LI included the presence of necrosis ($p=0.039$), largest diameter ($p=0.013$), infiltrative growth ($p=0.022$) and histological grade ($p=0.0005$). In the multivariate analysis, MIB-1 LI demonstrated an absence of significant correlation with ulceration, lymph node metastasis, skin fixation and E-cadherin expression.

Table I. *Distribution of specimens in each studied category.*

| Clinico-pathological variables | No. |
|---|---|
| Breed | |
| Large (>23 Kg) | 14 |
| Medium (10-23 Kg) | 39 |
| Small (<10 Kg) | 24 |
| Previous pregnancies | |
| None | 60 |
| One | 7 |
| Two | 10 |
| Progestin administration | |
| No | 66 |
| Yes | 11 |
| Location | |
| Thoracic | 14 |
| Cranial abdominal | 17 |
| Caudal abdominal/inguinal | 46 |
| Largest diameter | |
| <3 cm | 56 |
| >3 cm | 21 |
| Ulceration | |
| No | 68 |
| Yes | 9 |
| Skin fixation | |
| No | 60 |
| Yes | 17 |
| Underlying tissue fixation | |
| No | 71 |
| Yes | 6 |
| Necrosis | |
| No | 32 |
| Yes | 45 |
| Lymph node metastasis * | |
| No | 57 |
| Yes | 12 |
| Histological grade | |
| I | 15 |
| II | 36 |
| III | 26 |
| Mode of growth | |
| Expansive | 26 |
| Infiltrative | 41 |
| Vessel invasion | 10 |

*No lymph nodes were submitted with eight tumours.

## Discussion

Proliferation markers are indicative of disturbances in proliferation, the cell-cycle time, differentiation and senescence (11). It has been hypothesised that Ki-67 may be a marker of conditions other than growth rate, probably due to prolonged expression during G0 and increased time to traverse G1 or G2 (6).

Comparison between the studies of proliferation markers by immunohistochemistry is hampered by the large number of procedure variables that may influence the interpretation of the results, such as fixation, antigen retrieval, choice of the area to be examined, number of cells evaluated, methods of assessment, use of computerized image analysis, *etc.* It was demonstrated, in human breast invasive carcinomas, that there are differences in the MIB-1 LI between the periphery and the centre of some tumours and that peripheral and mean LI correlated with more prognostic variables than central LI (18). These facts highlight the need for a methodological standardization of the index determination in order to evaluate the real prognostic significance of MIB-1 LI.

Table II. *Multivariance analysis of clinco-pathological characteristics of malignant tumours.*

| Studied variable | Constant | Selected variable | | Coefficient±SE | Odds ratio (CI 95% ) | *P* |
|---|---|---|---|---|---|---|
| Ulceration * | 1.343 | Tissue fixation | | 1.56±0.67 | 22.42 (1.60-314.94) | 0.0211 |
| | | Histological grade | | | | |
| | | (III *vs.* I +II) | | 1.45±0.66 | 18.21 (1.39-238.73) | 0.0271 |
| | | Diameter (> 3 cm *vs.* < 3 cm) | | 0.99±0.51 | 7.25 (0.98-53.55) | 0.0522 |
| Lymph node metastasis | –6.934 | Age | | 0.04±0.02 | 1.04 (0.99-1.09) | 0.0627 |
| | | Growth** | infiltrative | –0.48±0.61 | 2.38 (0.22-26.20) | 0.4314 |
| | | | vessel invasion | 1.82±0.72 | 23.80 (1.54-368.65) | 0.0116 |
| | | E-cadherin† | <50% | 1.33±0.60 | 17.99 (1.67-194.21) | 0.0263 |
| | | | 50-75% | 0.24±0.66 | 6.06 (0.47-78.03) | 0.717 |
| Necrosis | –0.893 | Skin fixation | | 1.06±0.46 | 8.36 (1.36-51.27) | 0.0218 |
| | | MIB-1 LI | | 0.03±0.02 | 1.04 (1.002-1.07) | 0.0385 |
| | | Progestagen administration (no *vs.* yes) | | 0.93±0.46 | 6.38 (1.06-38.45) | 0.0433 |
| Skin fixation | –0.338 | Diameter (>3 cm *vs.* < 3 cm) | | 1.20±0.36 | 11.11 (2.733-45.140) | 0.0008 |
| | | Tissue fixation | | 1.26±0.65 | 12.42 (0.99-156.12) | 0.0511 |
| | | Necrosis | | 0.73±0.44 | 4.28 (0.76-24.03) | 0.0991 |
| Larger diameter (>3 cm) | –7.577 | MIB-1 LI | | 0.05±0.02 | 1.05 (1.01-1.09) | 0.0131 |
| | | Age | | 0.04±0.02 | 1.04 (1.003-1.08) | 0.031 |
| | | Skin fixation | | 0.88±0.40 | 5.79 (1.23-27.27) | 0.0263 |
| Infiltrative growth** | a=–3.129 | MIB-1 LI | | 0.03±0.01 | 1.03 (1.004-1.05) | 0.0219 |
| | b=–0.115 | E-cadherin† | <50% | 0.29±0.38 | 3.00 (0.94-9.54) | 0.4479 |
| | | | 50-75% | 0.53±0.39 | 3.82 (1.15-12.65) | 0.1751 |
| E-cadherin expression | a=–0.399 | Diameter (>3 cm *vs.* <3 cm) | | 0.48±0.26 | 2.60 (0.95-7.11) | 0.0627 |
| | b=0.637 | Growth◊ | expansive | 0.81±0.38 | 6.23 (1.35-28.68) | 0.0296 |
| | | | infiltrative | 0.20±0.32 | 3.36 (0.85-13.38) | 0.5375 |
| Histological grade | a=–1.877 | MIB-1 LI | | 0.05±0.02 | 1.05 (1.02-1.09) | 0.0005 |
| | b=0.775 | Ulceration | | 1.18±0.59 | 10.69 (1.06-107.52) | 0.0443 |

*All ulcerated tumours were skin-fixated.
**Baseline: expansive.
†Baseline: >75%.
◊Baseline: vessel invasion.

In previous canine and feline mammary tumour studies, between five (10) and 20 tumour fields (19) were used to count 500 (10) or 1000 cells (7-9, 19-21) on representative areas of the tumour. In some studies, only epithelial cells were considered (10), while in others the highest and lowest stained areas were included (19).

In our study, 1000 cells were counted only in the area of highest labelling. This method is, in our opinion, less subject to differences in the selected areas between tumours and between observers, eases the work of the observer and allows a more rigorous comparison between large tumours, where various non-overlapping fields may be found, and smaller ones, where it is difficult to obtain such variety.

From a prognostic point of view, it was important to understand if this methodology yielded different results from those previously published, if it was related to other known prognostic factors and, as a consequence, whether the methodology has a prognostic potential.

Tumour size is a recognized prognosticator of canine mammary malignant tumours (8, 22-24). More than a reflection of the speed of tumour growth, size is also the result of owner attention and care of the animal, as well as the surgeon's individual decision on when to recommend surgical excision. We found a significant influence of MIB-1 LI on the size of malignant tumours. The fact that animals included in this study were from a limited number of Veterinary Hospitals and Clinics, in a demographically uniform urban area, allows for the assumption that the level of owner care and veterinary services are relatively uniform, hence the positive correlation between size and the MIB-1 LI in malignant tumours. Our results contradict those previously reported where MIB-1 LI was not related to the size of a group of 93 tumours and dysplasias (8). Different methods of LI determination, tumour size grouping and statistical methodologies may explain these differences. The results of our study suggest that, instead of the subjective assessment of growth rate as the time needed from first detection of a tumour to obtain a specific size, the MIB-1 LI may more accurately determine the growth rate, although aberrant overexpression of MIB-1 in malignant disease, not related *per se* to increased rate of proliferation (6), needs future consideration.

In both statistical methods, a significant correlation between histological grade and MIB-1 LI was evidenced, with grade I tumours showing lower indices than grade III ($p=0.0009$). Previous reports of feline (21) and human mammary tumours (18, 25, 26), using the same criteria for the grading of tumours, reported similar results regarding the association between histological grade and MIB-1 LI. Peña *et al.* (8) could not find an association between histological malignant grade or nuclear grade and MIB-1 LI, but different criteria for the grouping of tumours in each grade were used, including vascular or lymphatic invasion, rendering a comparison between the two studies difficult.

A step-wise increase in MIB-1 LI was observed in parallel with increased mitotic counts (mitotic score 1:mean=25, SD=10.5; score 2:mean=39.2, SD=15.8; score 3:mean=48.9, SD=20.6). A similar relationship was found in previous studies in human breast carcinomas (25, 27, 28). This fact leads to the assumption that mitotic counts may be the most influential factor in the relationship between MIB-1 LI and histological grade. However, the association between MIB-1 LI and histological grade was not step-wise (no significant differences between grades I and II) here, so some influence can be attributed to the other characteristics of the histological grade (tubular formation and cellular pleomorphism).

MIB-1 LI proved to be significantly related to infiltrative growth. It is difficult to compare these results with those reported by Peña *et al.* (8) since, in that study, vessel invasion was included in the histological malignant grade together with differentiation. However, our results are in accordance with other canine (9) and human (18) mammary cancer studies.

A significant influence of MIB-1 LI on the presence of tumour necrosis was determined. To our knowledge, there are no previous reports of this association. It is possible that, in the presence of rapid proliferation, the building of a vascular network to support the tumour mass is not possible and ischaemic necrosis occurs. Comparative studies between MIB-1 LI and vessel proliferation markers in malignant tumours are needed to confirm this hypothesis. It is interesting to note that previous progestin administration also emerged as a significant factor in tumour necrosis. Hence, tumours from dogs that had never received progestins had a significantly higher probability of evidencing necrosis than those from dogs that had been treated with progestins. *In vitro* studies demonstrated that human breast cancer cells treated with progesterone expressed higher levels of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor (29, 30). In a mouse model study, medroxyprogesterone acetate promoted angiogenesis and inhibited apoptosis of induced mammary cell tumours (31). These observations suggest that the negative correlation between progestin treatment and tumour necrosis in the present dog tumours might be mediated by a progestin-dependent increase in angiogenesis in these tumours.

The MIB-1 LI was not significantly related to lymph node metastasis. Rather, it appeared that invasive behaviour and loss of adhesion molecules, such as E-cadherin, play a more important role in the development of metastatic capacity. This result may be explained by the fact that lymph node metastasis requires specific biological characteristics of tumour cells as well as interactions between the tumour and patient factors (25) other than the rate of proliferation as assessed by Ki-67 nuclear expression. Our results contradict previously published data on canine mammary tumours (8), but are similar to those of human breast cancer series (27, 28, 32). The use of cytokeratin immunostaining for the identification of lymph node metastasis from epithelial tumours allowed a more accurate estimation of the relationship between MIB-1 LI and the metastatic capacity of the malignant tumours (12) that may explain the differences with previous studies.

Neither ulceration nor skin fixation were correlated with MIB-1 LI. It seems plausible that both factors are more influenced by tumor-stroma interactions independent of the tumour proliferation rate.

The significant factors related to ulceration were tissue fixation and high histological grade (grade III compared to grades I and II taken together). Our results are in accordance with those previously reported (8).

Loss of E-cadherin expression was also as an independent phenomenon from MIB-1 LI. Thus, proliferation, involving the expression of Ki-67 protein and loss of cell adhesion, assumed as loss of E-cadherin expression, appear as independent processes in the evolution of malignant mammary tumours.

In conclusion, the significant relationship between the MIB-1 LI and other known factors of poor prognosis of canine malignant mammary tumours, such as tumour size (8, 22-24), invasive growth (9, 34), histological grade and necrosis, suggests that the MIB-1 LI, defined as the percentage of immunostained cells in the areas of highest positivity, may have prognostic value in canine malignant mammary tumours. Future survival and disease-free interval studies are needed to confirm this hypothesis.

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