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1 Analysis of Ki67 density maps reveals variation of spatial proliferation patterns in
2 different canine neoplasms

3 Running title: spatial proliferation in canine neoplasms

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26 Abstract

27 Mitotic count (MC) is a well-established prognostic factor in many canine
28 malignancies. While standardization efforts have improved inter-pathologist
29 agreement regarding the morphology of mitotic figures and the size of the counting
30 area, the selection of the tumor region for MC assessment remains to be standardized.
31 This study aimed to evaluate the spatial distribution of the most proliferative areas in
32 selected canine tumor types, using Ki67 immunohistochemistry, to identify optimal
33 candidate regions for MC assessment. Tumor types analyzed included melanomas,
34 cutaneous mast cell tumors (cMCT), canine mammary carcinomas (CMC), and soft
35 tissue sarcomas (STS). Using image analysis, Ki67 density maps were generated from
36 digital slides and classified according to their distribution pattern (focal/multifocal or
37 diffuse) and location within the tumor (central, peripheral, or scattered). A total of 202
38 cases were included: 43 melanomas, 30 cMCTs, 42 CMCs, and 87 cSTSs. The vast
39 majority of tumors (92.6%) exhibited a multifocal hotspot distribution. Peripheral
40 hotspot localization was predominant in 55% of cases, particularly in cMCTs (73.3%)
41 and melanomas (76.7%). In contrast, cSTSs more frequently showed a scattered
42 hotspot pattern (60.9%) ($\chi^2 = 41.9$; $p < 0.001$). CMCs had a higher proportion of
43 centrally located hotspots (16.7%). These findings suggest that pathologists should
44 focus on peripheral tumor regions when assessing MC in cMCTs and melanomas.
45 Both central and peripheral regions should be considered in CMCs, while a more
46 extensive, comprehensive evaluation may be required in STSs. The observed
47 association between tumor histotype and proliferation pattern likely reflects inherent
48 biological differences among the tumor types studied.

49 **Keywords**

50 Mitotic count, Ki67, image analysis, density map, hotspot.

51 **Introduction**

52 Mitotic count (MC) is one of the most commonly used prognostic factors in veterinary
53 tumor pathology. The association between a high number of mitotic figures (MF) and
54 the aggressive behavior of several neoplasms is widely acknowledged.¹⁻⁷

55 In veterinary medicine, MC is also included in the histological grading systems of
56 canine mast cell tumors (cMCT),^{8,9} mammary carcinomas,^{10,11} canine melanomas,¹²
57 and canine soft tissue sarcomas (cSTS).^{13,14}

58 The importance of standardizing histological parameters such as MC, has become
59 increasingly apparent.¹⁵ A major challenge in this standardization lies in the limited
60 reproducibility, primarily due to the lack of clearly defined and reproducible
61 assessment methods.^{5,16,17} As a result, the evaluation of several parameters, including
62 MC, often relies on subjective approaches, which hinders consistent comparisons
63 across studies.¹⁸

64 Considerable efforts are underway to address these limitations, with proposed
65 guidelines and protocols outlining best practices for measuring specific histological
66 parameters across various tumor types (<https://vcqp.org/guidelines-and-protocols/>).¹⁸

67 In the context of mitotic count (MC) assessment, standardization should address the
68 following key elements:

69 (1) The histologic morphology of mitotic figures (MF): Only figures from prometaphase
70 to telophase should be counted, focusing on chromosomal aggregates lacking a
71 nuclear membrane;¹⁸

72 (2) The size of the counting area: MC should be assessed in a total field area of 2.37
73 mm², which varies depending on the field number (FN) of the ocular and the
74 magnification of the objective lens;^{5,17}

75 (3) The selection of tumor regions for MC assessment: Counting should be performed
76 in areas exhibiting the highest proliferative activity.^{19–22}

77 In many tumor types, the MC is evaluated in regions of maximal mitotic density within
78 tumors.^{5,23–26} However, identifying these regions is not straightforward, and there are
79 no clear indications regarding the choice of site with higher proliferative activity across
80 different tumor types.

81 Some studies suggest that the tumor periphery is the preferred site for mitotic count
82 (MC) assessment, as it often represents the invasive front and may exhibit higher
83 proliferative activity.^{5,19} Additionally, tissue fixation is typically more effective at the
84 tumor edges.^{5,19,24} A study on human breast carcinoma reported that the tumor
85 periphery contained a higher density of Ki67-positive hotspots, and the proportion of
86 Ki67-positive nuclei in these regions correlated with patient prognosis.²⁷ However,
87 other studies in human breast carcinoma have found that assessing Ki67 hotspots—
88 regardless of their location within the tumor—yields stronger prognostic value than
89 location-based assessment alone.²² In veterinary medicine, Bertram and colleagues
90 demonstrated that in canine mast cell tumors (cMCT), the MC varied significantly
91 across different tumor regions. Notably, pathologists were often unable to accurately
92 identify the area with the highest MC when evaluating whole-slide images.¹⁹

93 Although MC has long been a mainstay of tumor assessment, until recently, there has
94 been no standardization of any element of this parameter in veterinary pathology.
95 Performing the MC is considered laborious and is characterized by inter-pathologist
96 variation.^{28–30}

97 Standardizing methods should increase reproducibility, decrease subjectivity, and
98 improve the predictability of this commonly used prognostic marker. Automated image

99 analysis systems represent promising tools for selecting the area with the highest
100 mitotic activity within large tumor sections more accurately.¹⁹

101 The aim of this study is to evaluate the spatial distribution of the most proliferative
102 regions in different canine tumor types, in order to identify the most appropriate
103 areas for MC assessment, with the aid of image analysis software.

104 **Methods**

105 Case selection

106 Tumor types for which the MC is considered a prognostic parameter or is integrated
107 in the grading system were included. Cutaneous canine mast cell tumors (cMCT),
108 canine mammary carcinomas (CMC), oral and cutaneous canine melanomas
109 (malignant melanomas) and canine soft tissue sarcomas (cSTS) were selected from
110 the archives of three institutions. Complex and mixed canine mammary carcinomas
111 were excluded to avoid the confounding effect of myoepithelial or mesenchymal cell
112 proliferation. cSTS cases with a specific diagnosis were enrolled, including
113 perivascular wall tumors, myxosarcomas, fibrosarcomas, liposarcomas, and
114 leiomyosarcomas. cSTS diagnoses were performed by two board certified pathologist
115 experienced in the diagnosis of cSTS and were based on histological features
116 according the most recent classification system³¹ and, when necessary, with the
117 application of the appropriate immunohistochemical protocols. Cases with uncertain
118 diagnosis were not included.

119 Only cases with recognizable tissue orientation and with at least half of a transversal
120 section of the neoplasm available for immunohistochemistry were included. The
121 diagnoses were confirmed by re-assessment of the original hematoxylin and eosin
122 stained sections by two board certified pathologists.

123 Immunohistochemical labeling

124 All cases underwent immunohistochemistry for Ki67. Sections 3 to 4 μm thick were
125 obtained from selected paraffin blocks to assess at least half of the transversal section
126 of the neoplasm. Pigmented melanomas underwent de-pigmentation before
127 immunohistochemical staining by incubation for 27 minutes in potassium
128 permanganate (0.25 g/100 mL; Merck, Germany), followed by rinsing in distilled water
129 and immersion for 20/30 seconds – depending on the required level of depigmentation
130 – in oxalic acid (5 g/100 mL; DDK Italia, Italy). Sections were air-dried, dewaxed, and
131 rehydrated. Endogenous peroxidase was blocked by immersion in 3% H_2O_2 in
132 methanol for 30 minutes. Heat-induced antigen retrieval was used (pH 6.0 citrate
133 buffer, in a pressure cooker at 110°C for 20 minutes).

134 After cooling, sections were preincubated with a blocking solution (10% normal goat
135 serum with phosphate-buffered saline) for 30 minutes. Anti-Ki67 primary antibody
136 (mouse monoclonal, clone MIB-1, dilution 1:1500, Dako, Glostrup, Denmark) was
137 applied and incubated overnight at 4 °C.

138 Sections were then incubated for 30 minutes at room temperature with anti-mouse
139 biotin-conjugated secondary antibody (dilution 1:200, Dako, Glostrup, Denmark). The
140 reaction was amplified by the avidin-biotin method (ABC kit elite; Vector, Burlingame,
141 CA) and visualized by incubating with 3,30-diaminobenzidine for 2 minutes. Harris'
142 hematoxylin was used as counterstaining. Finally, the sections were dehydrated, and
143 cover-slipped. Sections of the canine small intestine were used as positive control.
144 Negative controls included slides incubated with a nonspecific antibody or the
145 omission of the primary antibody.

146

147 Immunohistochemical evaluation and image analysis

148 The immunohistochemical slides were scanned using Grundium Ocus ® 20 (Tampere,
149 Finland) at 20X magnification (0.25 µm/pixel) to obtain a whole slide image (WSI). The
150 digital images were analysed using the open-source DIA software QuPath v0.5.0.3.
151 Analysis was performed starting with manual selection of the tumor section area
152 (Figure 1A) and positive cell detection tool (Analyze → Cell analysis → Positive cell
153 detection) (Figure 1B). Neoplastic tissue was selected while non-neoplastic tissue
154 adjacent to or entrapped within the neoplasm, necrotic areas, granulation tissue and
155 clearly recognizable inflammatory aggregates were avoided.
156 Afterward, a density map was created to define areas of higher proliferation and
157 demonstrate their predominant distribution within the section (Figure 1C). For each
158 image, the selection parameters were adjusted iteratively to find the optimal
159 combination, resulting in an accurate selection of Ki67-positive cells (Figure 1D, E).
160 Each density map was subsequently subjected to visual analysis by experienced
161 pathologists to categorize it into a specific group, classified based on:

- 162 - Hotspots distribution: focal/multifocal (one or multiple hot spots were visible) or
163 diffuse (relatively uniform positive cell density within the tumor);
- 164 - Hotspot location: central (at the center of the neoplasm), peripheral (at the
165 external regions of the neoplasm), or scattered (when both at central and
166 peripheral regions of the neoplasm).

167 Examples of the most common spatial arrangements of Ki67 hot spots are depicted in
168 Figure 2. The dataset explores the relationships between two diagnostic variables and
169 the host spots' spatial arrangements. The first diagnostic variable, "Diagnosis 1",
170 identifies broad tumor categories, including CMC, cSTS, melanoma, and cMCT. The
171 second diagnostic variable, "Diagnosis 2," provides a more detailed classification and
172 was applied to soft tissue sarcomas (including perivascular wall tumors,

173 myxosarcomas, fibrosarcomas, liposarcomas, and leiomyosarcomas) and melanomas
174 (oral or cutaneous).

175 The analysis was performed using the chi-square test to assess the association
176 between distribution and location (alone and in combination) with diagnosis 1 and
177 diagnosis 2 (when available). This test was chosen to compare the observed
178 frequencies with the expected ones under the null hypothesis, determining statistical
179 significance. A Kruskal-Wallis 1-way analysis of variance was used to assess the
180 association of distribution and location of hot spots with MC, and Ki67 labelling index
181 (expressed as number of positive cells/mm²). Post-hoc pairwise analysis was
182 performed with Dwass-Steel-Critchlow-Fligner test. Results were considered
183 significant for p-values < 0.05. Statistical analyses were performed using Jamovi
184 (version 2.4.12.0).

185 **Results**

186 A total of 202 cases were collected: 43 melanomas (22 oral and 21 cutaneous), 30
187 cMCT (21 low grade and 9 high grade), 42 CMC (14 grade 1, 20 grade 2, and 8 grade
188 3), and 87 STS (49 grade 1, 32 grade 2 and 9 grade 3). STS included 22 perivascular
189 wall tumors, 15 myxosarcomas, 16 leiomyosarcomas, 22 liposarcomas, and 12
190 fibrosarcomas.

191 Hot spot distribution was diffuse in 10 cases (5%), focal in 5 (2.5%), and multifocal in
192 187 (92.6%), and was associated with diagnosis 1 (chi-square = 25.5; p<0.001).

193 Specifically, distribution was diffuse in 2/30 (6.7%) cMCT, 5/42 (11.9%) CMC, 2/43
194 (4.7%) melanomas and 1/87 (1.1%) cSTS; focal in 4/30 (13.3%) cMCT, 1/42 (2.4%)
195 CMC, 0/43 melanomas and 0/87 cSTS; and multifocal in 24/30 (80%) cMCT, 36/42
196 (85.7%) CMC, 41/43 (95.3%) melanomas and 86/87 (98.9%) cSTS (Figure 3A).

197 The hot spot location was central in 12 cases (5.9%), peripheral in 111 (55%), and
198 scattered in 79 (39.1%), and was associated with diagnosis 1 (chi-square = 41.9;
199 $p < 0.001$).

200 Specifically, the location was central in 2/30 (6.7%) cMCT, 7/42 (16.7%) CMC, 0/43
201 melanomas, and 3/87 cSTS; peripheral in 22/30 (73.3%) cMCT, 25/42 (59.5%) CMC,
202 33/43 (76.7%) melanomas and 31/87 (35.6%) cSTS; and scattered in 6/30 (20%)
203 cMCT, 10/42 (23.8%) CMC, 10/43 (23.3%) melanomas and 53/87 (60.9%) cSTS
204 (Figure 3B).

205 Considering the hot spot patterns derived from the combination of distribution and
206 location, the peripheral-multifocal pattern was found in 100/202 cases (49.5%),
207 scattered-multifocal in 79/202 (39.1%), central-multifocal in 8/202 (4.0%), peripheral-
208 diffuse in 6/202 (3.0%), peripheral-focal in 5/202 (2.5%), and central diffuse in 4/202
209 (2.0%), correlating with diagnosis 1 (chi-square test, $p < 0.001$).

210 Specifically, in cMCT the pattern was peripheral multifocal in 17/30 (56.7%), scattered
211 multifocal in 6/30 (20.0%), peripheral focal in 4/30 (13.3%), central diffuse in 1/30
212 (3.3%), central multifocal in 1/30 (3.3%), and peripheral diffuse in 1/30 (3.3%).

213 In CMC, it was peripheral multifocal in 21/42 (50.0%), scattered multifocal in 10/42
214 (23.8%), central multifocal in 5/42 (11.9%), peripheral diffuse in 3/42 (7.1%), central
215 diffuse in 2/42 (4.8%), and peripheral focal in 1/42 (2.4%).

216 In melanomas, the patterns were peripheral multifocal in 31/43 (72.1%), scattered
217 multifocal in 10/43 (23.3%), peripheral diffuse in 2/43 (4.7%), and no cases were
218 recorded for the other patterns.

219 In STS, the pattern was as follows: scattered multifocal in 53/87 (60.9%), peripheral
220 multifocal in 31/87 (35.6%), central multifocal in 2/87 (2.3%), central diffuse in 1/87
221 (1.1%), and no cases were observed for the remaining patterns.

222 No statically significant association of the host spot distribution, location and combined
223 pattern with diagnosis 2 (STS histotype and cutaneous versus oral melanoma) was
224 found.

225 In 10 out of 21 cases of cutaneous melanoma and 13 out of 22 cases of oral
226 melanoma, we were able to assign a third pattern based on the superficial or deep
227 localization of the hotspots. It was visually observed that, in 7 out of 10 (70%)
228 cutaneous melanomas, the hotspots were located on the superficial side, while in 8
229 out of 13 (62%) cases of oral melanomas, the hotspots were found on the deep side
230 of the neoplasm. Similarly, in 10 MCTs cases, 5 had superficial hotspots, and 5 had
231 deep hotspots.

232 Finally, in CMC, the localization of the third pattern was assigned in 10 cases, and in
233 4 of these the distribution mirrored the lobular arrangement of the carcinoma. Detailed
234 results of distribution, localization, Ki67 labeling index, MC, grade and area assessed
235 are reported in Supplementary table 1.

236 A statistically significant association was found between Ki67 labeling index and both
237 location and distribution, being higher in case with scattered location compared with
238 peripheral ($W=3.33$, $p=0.49$), and in cases with multifocal distribution compared with
239 a diffuse one ($W=4.66$, $p=0.003$).

240 **Discussion**

241 MC is one of the most commonly used histological prognostic factors in veterinary
242 tumor pathology.⁵ However, the current method of MC assessment presents significant
243 limitations due to intra-tumoral variability and observer subjectivity. Considerable
244 efforts have been dedicated to overcoming these limitations, resulting in the
245 establishment of a standard area for MC and the morphology of mitotic figures.
246 Nevertheless, there are no guidelines on selecting the tumor region where the count

247 should be performed.⁵ These limitations compromise the reliability of MC as a
248 prognostic factor.

249 The introduction of computer-assisted diagnosis systems offers promising solutions to
250 address the current challenges of MC.³² Nevertheless, until most laboratories have
251 entirely switched to digital microscopy,³² this approach will not be widely applied.
252 Furthermore, a limited number of prognostic studies on canine tumors have used
253 digital microscopy and associated the digital counts with outcome.^{21,33}

254 This study aimed to investigate the regional distribution of proliferative hotspots for
255 different canine tumor types, providing guidelines for pathologists when assessing the
256 MC, following the general rule that MC should be evaluated in areas of highest
257 proliferation.¹

258 The multifocal distribution was the predominant location of hot spots for all tumor types
259 included in the study. In STS and melanoma, multifocality was observed in all cases
260 except for one and two cases, respectively. In contrast, a diffuse or focal distribution
261 was found in more than 10% of CMC and cMCT cases, respectively.

262 We interpreted the multifocality as a consequence of tumor heterogeneity, further
263 confirming the need for more clearly defined indications for selecting the tumor region
264 for MC assessment. This heterogeneity, together with other factors, may be at the root
265 of the low inter-pathologist agreement on MC results. The significant number of CMC
266 with a diffuse distribution may indicate a lower heterogeneity of this tumor type, which
267 may derive from the inclusion of simple carcinoma only.

268 Regarding the hot spot location, the peripheral one was the most common in all tumor
269 types examined, except for STS. In the past, it has been postulated that the peripheral
270 portion of the tumor, representing the invasive front, was the most proliferative.^{34–36}

271 This appears to be especially true in cMCT and canine melanoma, in which the

272 peripheral distribution represented approximately 75% of the cases. This suggests
273 that, in these tumor types, pathologists should focus mainly at the periphery, when
274 assessing the MC.

275 Interestingly, despite the higher frequency of the peripheral pattern, CMC included a
276 large number of cases characterized by central hotspots. This result may indicate that,
277 when a low MC is found, or when mitotic figures are difficult to find at the periphery of
278 a CMC, it could be useful to assess the tumor center. Anyway, this indication cannot
279 be extrapolated to all CMC types, as only simple carcinomas were included in this
280 study. Furthermore, the central proliferation in CMC could represent a biologically
281 distinct variant.

282 On the other hand, in cSTS Ki67 scattered distribution was the most frequent, followed
283 by the peripheral one, in both cases associated with a multifocal distribution. This data
284 indicates a high intra-tumoral heterogeneity of proliferative activity of cSTS cells and
285 suggests that sections should be carefully scrutinized for the detection of regions with
286 high mitotic activity.³¹

287 Furthermore, the different spatial distribution of Ki67 hotspots may also partially reflect
288 tumor biology. In melanomas and cMCT, proliferative activity is concentrated at the
289 tumor edges, suggesting these areas are the most representative of accurate mitotic
290 diagnosis. Peripheral proliferation in these tumors can be indicative of active margins,
291 which may contribute to greater infiltrative potential. In cSTS, the predominant
292 scattered proliferation could reflect a less focused distribution, potentially associated
293 with their less aggressive tumor behavior. This hypothesis should be confirmed by
294 assessing the spatial distribution of hotspots in a caseload with available clinical
295 follow-ups.

296 Furthermore, the Ki67 labeling index was significantly higher in cases with a multifocal
297 distribution compared with those with a diffuse pattern, and to a lesser extent, in cases
298 with a scattered location compared with a peripheral one. This may suggest that while
299 a baseline proliferative activity is uniformly present throughout the tumor, peaks of
300 proliferative activity occur in areas where more aggressive neoplastic cell clones
301 undergo selection.

302 Finally, in some cases, a low correspondence was observed between MC and the Ki67
303 labeling index. A low MC in tumors with a high Ki67 index is likely due to the count
304 being performed in an area that did not correspond to the most proliferative region.
305 Conversely, a high MC in tumors with a low Ki67 index may result from the count being
306 carried out in a localized hotspot within a tumor otherwise characterized by few
307 proliferative foci and an overall low proliferation rate, considering that the Ki67 index
308 was calculated across the entire section. Both scenarios highlight the need for more
309 detailed guidelines to standardize the selection of tumor regions for MC assessment.

310 One limitation of this study is the use of an anti-Ki67 antibody, which detects cells at
311 any phase of the cell cycle except for G₀, thus labeling not only cells in mitosis but
312 also those in G₁, G₂, and S phase.³⁷ As a consequence, it is likely that there is no
313 exact correspondence between Ki67 hotspots and mitotic figures hotspots.
314 Nevertheless, we chose to use this marker since these results can also be applied to
315 the selection of regions for assessing the Ki67 labeling index. This additional
316 prognostic parameter is often required by oncologists for canine melanoma and cMCT,
317 necessitating the identification of highly proliferative regions.^{38,39} A second limitation is
318 the exclusion of complex and mixed mammary tumors. This was opted because the
319 separation of the myoepithelial and non-epithelial components would have required a
320 different and more complex image analysis protocol. Finally, we selected cases having

321 a small diameter, so we could examine at least half of a transversal section. This
322 criterion might have introduced a bias related to a possible different distribution of
323 proliferative hotspots in larger lesions.

324 Further studies are advisable to confirm our results, to expand the caseload to other
325 tumor types, including subcutaneous cMCT and other mammary tumor types, and to
326 compare the distribution patterns with the biological behavior of the tumors.

327 In conclusion, the peripheral proliferation observed in melanomas and cMCT, as well
328 as the scattered pattern typical of cSTS, represent distinctive features that may assist
329 in selecting hotspots and could have significant diagnostic and prognostic implications.

330 The association between histological diagnosis and the pattern of proliferation found
331 in this study likely reflects the biological differences among the tumor types studied.

332 A better understanding of proliferative hotspot distribution can contribute to a more
333 targeted prognostic stratification in canine tumors.

334

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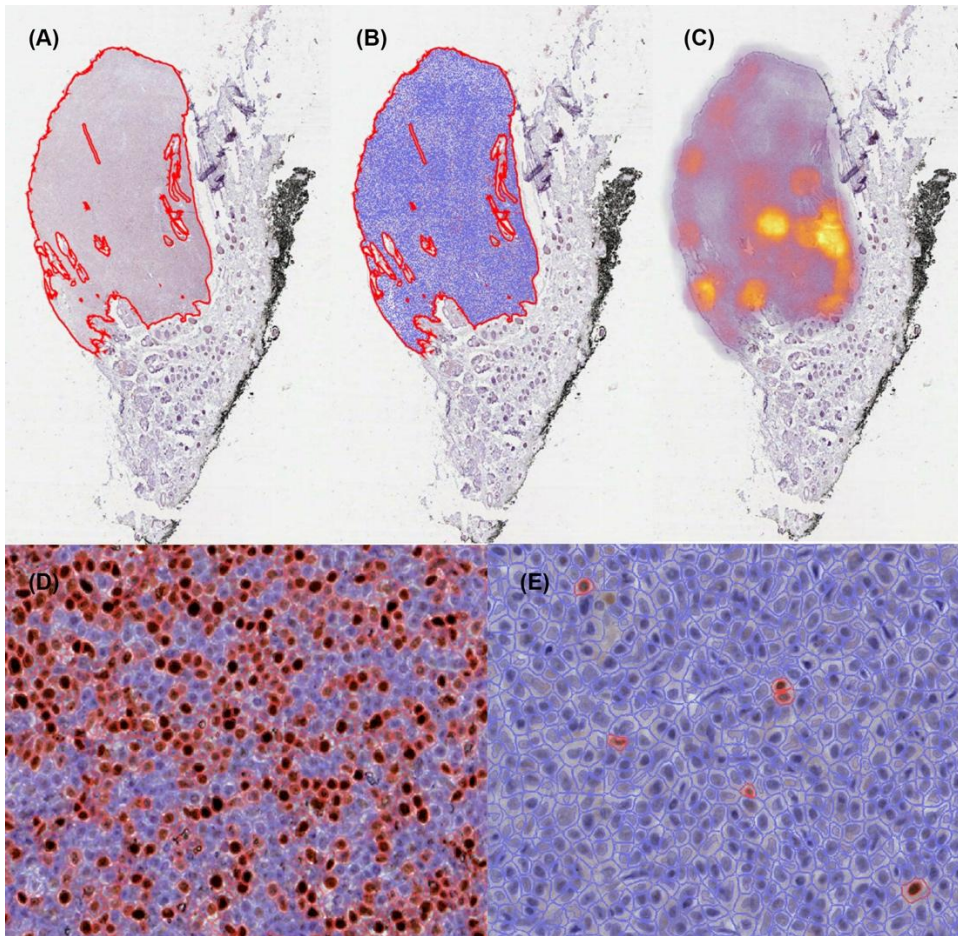
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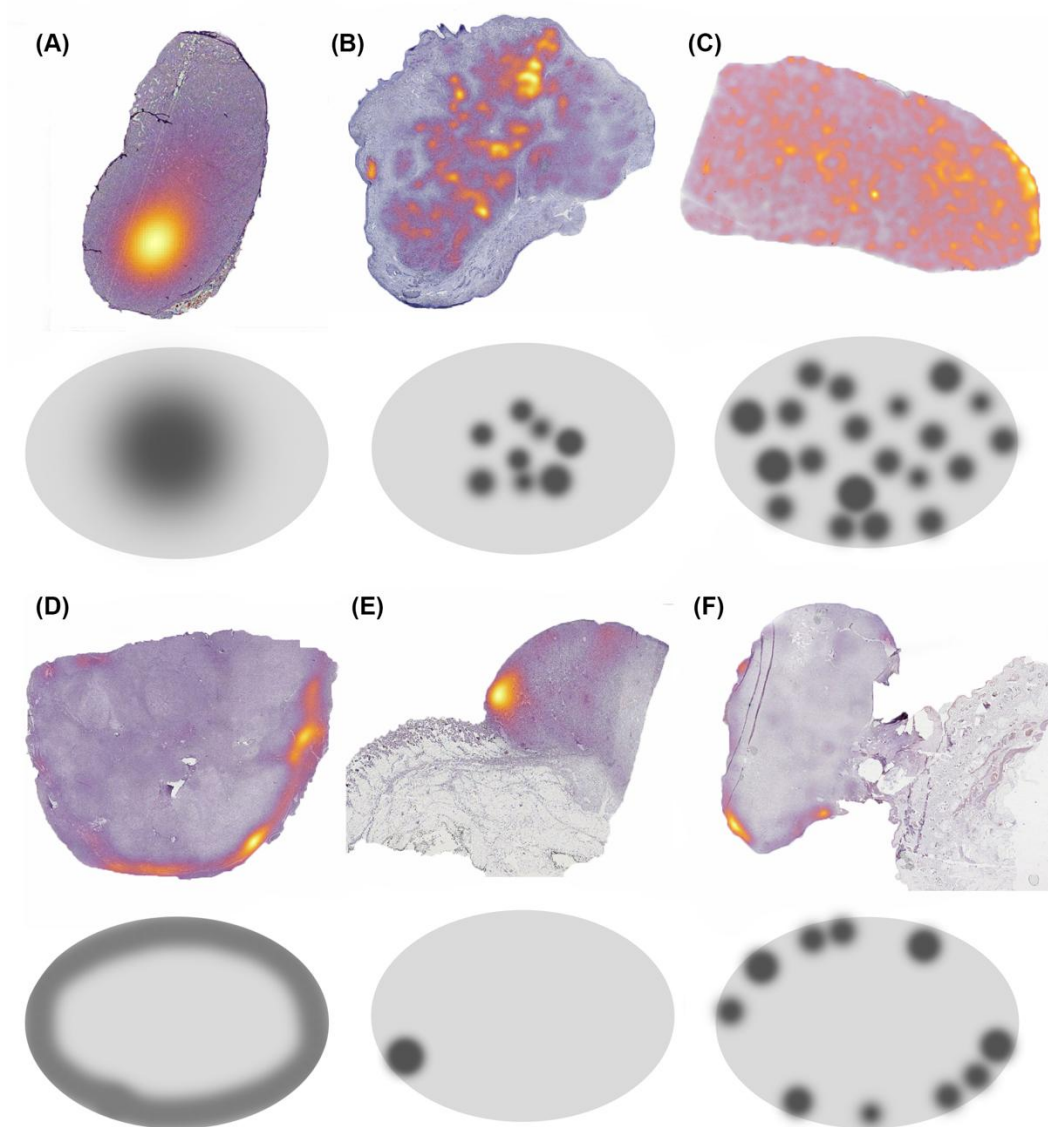
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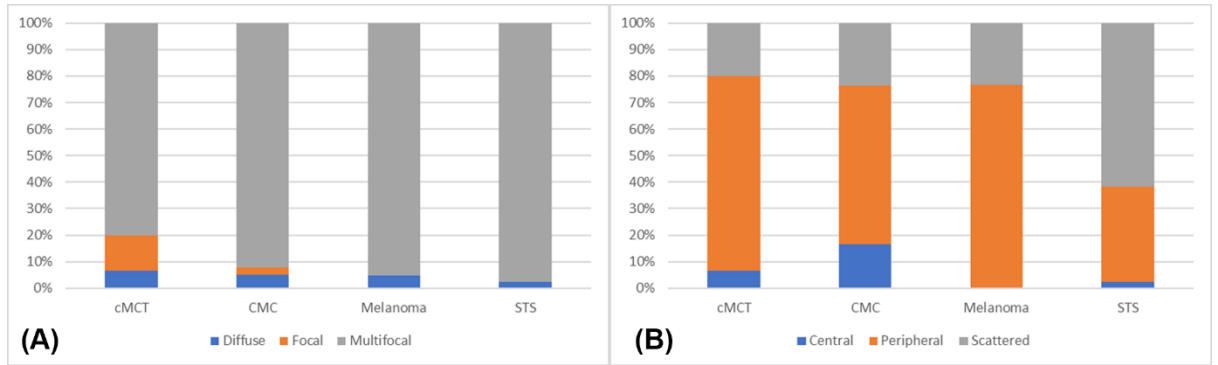
461 Figure 1: Examples of image analysis on whole slide image of Ki67
 462 immunohistochemistry. A) Selection of the region of interest (ROI) (red line):
 463 non-neoplastic tissue, holes and artifacts are excluded; B) Positive cell detection
 464 performed on the ROI: in blue are negative cells, in red are positive cells; C)
 465 Density maps showing the regions of the tumor with higher positive cell density
 466 (yellow shade); D) Positive cell detection in a canine mast cell tumor with high
 467 proliferative activity, and E) in a canine soft tissue sarcoma with low proliferative
 468 activity. In blue are negative cells, in red are positive cells



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470 Figure 2: Examples of the most frequent patterns of density maps and
 471 corresponding example pictograms. A) Central diffuse pattern in a canine
 472 mammary carcinoma; B) Central multifocal pattern in a canine mammary
 473 carcinoma; C) Scattered multifocal pattern in a canine liposarcoma; D)
 474 Peripheral diffuse pattern in a canine perivascular wall tumor; E) Peripheral focal
 475 pattern in a cutaneous mast cell tumor; F) Peripheral multifocal pattern in a
 476 canine cutaneous melanoma.

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Figure 3: Bar plots depicting the percentage of cases presenting A) diffuse, focal or multifocal distribution and B) central, peripheral and scattered location. Mast cell tumors have a higher percentage of focal distribution and soft tissue sarcomas have a higher percentage of scattered location. cMCT: cutaneous mast cell tumors

CMC: canine mammary carcinoma

STS: soft tissue sarcoma