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International Guidelines for Veterinary Tumor Pathology: A Call to Action

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## 1 **International Guidelines for Veterinary Tumor Pathology: A Call to Action**

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## 67 **Introduction**

68           Reproducibility is the foundation of experimental science but irreproducibility of  
69 published oncological studies is a crisis in human oncology and certainly also a problem  
70 in veterinary oncology. In many instances the lack of reproducibility stems from  
71 inadequate description of published methods.(Begley and Ellis 2012, Oransky Ivan  
72 2017, Stark 2018, Wen, Wang et al. 2018) Efforts to address this crisis have been  
73 implemented in human medicine, including attempted reproduction of published studies  
74 and formulation of checklists for use by journal editors and reviewers to ensure inclusion  
75 and transparency of detailed methods and materials in publications.(Oransky Ivan 2017,  
76 Editorial 2018, Schott, Tatiarsky et al. 2018, Stark 2018, Wen, Wang et al. 2018) Less  
77 than 10% of observational studies are able to be replicated and incredibly, less than  
78 20% of preclinical trials can be replicated.(Begley and Ellis 2012) If that is the state of  
79 oncology studies in human medicine, how do we compare in veterinary oncology? How  
80 far have we advanced in the last 40 years? Attempts to validate existing studies and or  
81 grading schemes are almost nonexistent. Grading schemes and the methods employed  
82 require appropriate validation before they should be adopted and used to provide  
83 prognoses or direct clinical therapy. Yet our philosophy seems to be that once a system  
84 or method is created, it is put in use and remains in use regardless of whether the  
85 system has been validated or not. We do not know how that system or method will  
86 perform when different pathologists use it and when it is applied to new patients.  
87 Consensus statements that support use of studies are not validation. Authors should  
88 feel complimented when colleagues attempt to reproduce their methods and study  
89 designs. Results will not replicate exactly, but our methods must. Validation of new  
90 grading systems is impossible if the original methods cannot be duplicated by other  
91 investigators.

## 92 **Reproducibility**

93           One of the major reasons that published studies are not able to be reproduced is  
94 the lack of sufficient details of the methods used to assess basic histological parameters  
95 including mitotic figure (MF) recognition, mitotic count (MC), lymphovascular invasion,  
96 tumor necrosis and margin evaluation.(Meuten, Munday et al. 2018, Schott, Tatiarsky et

97 al. 2018) Currently, the assessment of these parameters requires pathologists to make  
98 subjective evaluations without clearly defined methods. Due to the inconsistency of  
99 these qualitative evaluations, there is weak or poor concordance between pathologists.  
100 This may result in negativity of the parameters or rejection of the grading system. The  
101 materials and methods section of manuscripts should contain descriptions of each  
102 method in sufficient detail to allow others to reproduce the study and validate the  
103 results. Citing that the methods described in a prior published study were followed is  
104 acceptable protocol, providing that any modifications used are described in detail.  
105 Failure of studies to be replicated can be due to poorly described methods, not following  
106 methods, and other confounders. Interobserver variation between pathologists reported  
107 in manuscripts is often ascribed to a method that is inadequate or too subjective.  
108 However, investigators may not have considered that the original methods were flawed  
109 or inadequately described, such that pathologists could not consistently follow the  
110 method. Stating that MF were counted in 10 consecutive high-power fields (hpf) at 400X  
111 is insufficient detail for others to reproduce the method, compare results and validate  
112 the data. Since the area within one hpf can vary by greater than 200% because of  
113 different microscope configurations, then of course there will be interobserver variation  
114 in MC if the microscopes used by study pathologists were not configured in the same  
115 way. Not only MC, but all parameters that were enumerated histologically (e.g.  
116 pleomorphism) with a microscope or with whole slide images (WSIs) have the potential  
117 for error and variability of results if the area enumerated is not defined in  $\text{mm}^2$ . Until  
118 methods are detailed such that others can reproduce them, we will have inconsistent  
119 and contradictory data in the literature. Even with standardized methods, there will be  
120 variability that needs to be reported and practical considerations that cannot be  
121 standardized.

122

123 Standardizing methodology is one step toward consistent results, but it does not  
124 guarantee consistency and certainly not usefulness. The methods must be followed,  
125 done carefully, using the same materials (e.g. antibody), and methods applied to  
126 reference populations and diseased groups with known and well defined outcomes.  
127 Accredited clinical pathology labs use standardized methods, calibration of instruments

128 and quality control measures to quantitate substances and report the reproducibility,  
129 sensitivity, specificity, validation, and reliability, such as positive and negative predictive  
130 values of test results. Similar principles need to be applied to anatomic pathology.  
131 Quantitation of morphologic structures by semi- or fully automated means will need to  
132 be validated and applied with similar rigid standards.(Boyce, Dorph-Petersen et al.  
133 2010) When the methods are reproducible, they can be applied to cases with known  
134 outcomes. It would be helpful if new methods to quantitate structures were compared to  
135 the existing more subjective means to enumerate structures in HE stained slides.(Puri,  
136 Hoover et al. 2019, Bertram, Aubreville et al. 2020) Then colleagues can compare  
137 results to determine if they wish to adopt the new method. Technology will continue to  
138 spur development of new methods that can be applied to diagnostic cases. Many  
139 owners will pay for new techniques at any cost, but other owners will decline based on  
140 practical considerations such as cost, age of pet, or emotional value of the pet to their  
141 family. How to balance best care with practicality of animal ownership is not simple.  
142 Researchers can help address this by comparing new methodologies with those that  
143 can be performed without additional costs, specialized equipment, or expertise.  
144 Development of interlaboratory proficiency programs to promote standardization of  
145 tumor grading system results and performance of ancillary testing, such as  
146 immunohistochemistry, is sorely needed. Although new methods may initially be  
147 restricted to the institutions in which they were developed, standardization and  
148 proficiency are critical as these techniques are validated in other laboratories and  
149 become routinely used for tumor diagnosis. Centers that develop novel tests (e.g.  
150 computational pathology or CPATH), artificial intelligence, molecular, genetic) should  
151 have a goal that the methods can be applied uniformly and are described in sufficient  
152 detail that other labs can perform and validate the tests. Newly developed, specialized  
153 assays should be compared to current methodology and to patient outcomes to assess  
154 their utility and ideally seek FDA approval.(Boyce, Dorph-Petersen et al. 2010, Puri,  
155 Hoover et al. 2019, Bertram, Aubreville et al. 2020)

## 156 **Outcome assessment**



157 In addition to a lack of standardized assessment of histological criteria,  
158 reproducibility in animal studies is also limited by a lack of standardized guidelines for  
159 outcome assessments of animal patients.(Meuten, Munday et al. 2018, Schott,  
160 Tatiarsky et al. 2018) Euthanasia unrelated to tumor progression appears to be a  
161 significant confounder. Reported patient survival times are impacted by euthanasia  
162 which may be elected due to personal decisions, varying judgements regarding quality  
163 of life, owner income or other factors which do not reflect tumor behavior. Furthermore,  
164 the Start time (T=0) needs to be clearly and consistently defined in presented survival  
165 analyses.(Nguyen) How often has an assigned grade or reporting that a tumor is in a  
166 lymph node resulted in euthanasia and the patient may have lived significantly longer?  
167 Survival time statistics in veterinary oncology are influenced by many factors outside of  
168 tumor and host biology. Metastasis needs to be subdivided into confirmed or suspected.  
169 Evidence for metastases determined by imaging should be labelled as suspected or  
170 *metastases as determined by imaging* when reported in journals. Histopathology is  
171 required to confirm that metastases are present and are of the same tumor type.  
172 Multiple aggressive tumors can occur in the same patient and are well recognized in  
173 breeds such as Golden retriever, Rottweiler and Bernese mountain dogs.(Cullen and  
174 Breen 2016) Oncology studies no longer routinely include results of autopsy, the  
175 perceived value of which seems to have hit a nadir. Owner/client permission to perform  
176 autopsies should be pursued with sympathy and empathy but as vigorously as other  
177 tests. Autopsy findings greatly increase the objectivity of results such as metastases  
178 and recurrence and therefore confidence of study results. Veterinary oncology studies  
179 need institutions and labs to pool their resources so that large numbers of cases can be  
180 collected. If results of these studies are correlated with accurate patient outcomes, the  
181 archived materials are a precious resource. The materials from these studies (slides,  
182 blocks, images, statistical data) could be shared with others such that new methods can  
183 be applied to case series with known outcomes. This was done by Bergin et al.(Bergin,  
184 Smedley et al. 2011) in a study of canine oral and lip melanocytic neoplasms. In  
185 addition to a set of oral/lip melanocytic neoplasms from the authors' own diagnostic  
186 laboratory, this study used archived blocks from two previous studies(Spangler and  
187 Kass 2006, Esplin 2008) in order to validate the histologic parameters described in

188 those studies (nuclear atypia, mitotic count, and pigmentation) using the same methods  
189 and to compare them to a new parameter, Ki67 index. This can serve as an example of  
190 the value in validating prior reports, which adds confidence to conclusions and provides  
191 a new method. Archived images of tumors could also be used to test inter-pathologist  
192 variation on diagnoses, MC, necrosis, and other basic parameters from laboratories  
193 worldwide. Outcome assessment determines if a test predicts treatment or prognosis  
194 and may differ between tertiary and primary care patients. Standardization of outcome  
195 assessment data is as critical as the standardization of techniques involved in tumor  
196 assessment. These two components of oncology must be linked as it is useless to  
197 create a new tumor grading system without knowledge of patient outcomes

### 198 **Appendices and Protocols**

199 In 2011 Veterinary Pathology published a series of recommendations and  
200 reviews about tumors in animals and how they should be evaluated. The manuscripts in  
201 that issue are excellent. They exceed the goals of the present manuscript but like "all"  
202 veterinary pathology publications there is no provision to update information, which is  
203 now a decade old. Much of the information consisted of literature reviews, and  
204 descriptions of the multiple methods to perform a parameter, without prioritizing or  
205 choosing one. The present manuscript aspires to be a continuum of the information  
206 published in 2011 but with a focus on establishing standardized histopathology methods  
207 to evaluate tumors. These methods are guidelines that will help accrue similar data  
208 such that studies can be cross compared and validated. A website will be established to  
209 publish guidelines for standard methods of tumor evaluations with the purpose of  
210 advancing veterinary pathology and oncology. This will require modifying the contents  
211 when publications have substantial data driven results that warrant updating these  
212 guidelines. These changes will be dated, and references cited. The present system of  
213 waiting for publication of a book or a fascicle is outdated. Updates are also needed as  
214 *errors* are possible (authors are humans) in the present appendices and protocols, and  
215 it is possible that some important references were missed. The authors hope that  
216 readers will bring such errors to our attention by contacting one or more of the  
217 *communication authors*. Unlike an error or omission in a manuscript or book that

218 remains in print, and results in our names indelibly associated with the words *retraction*  
219 or *correction*, the website can be quickly and easily updated. Journals and books will  
220 remain vital to our professions as they provide the means to publish peer reviewed  
221 research and to describe in detail an entire topic. The website will attempt to remain  
222 focused, and current, more of a CliffNotes' version of a topic designed to aid  
223 pathologists, editors and researchers in the standard parameters used to evaluate  
224 tumors and checklists of information that should be gathered about specific tumor types.

225 *Appendices* are guidelines to be used for identification of MF, perform MC,  
226 assess lymphovascular invasion (LVI), margin evaluation, percent tumor necrosis,  
227 CPATH, lymph nodes and outcome assessments. These parameters have not been  
228 standardized for animal tumors. The methods are detailed for MF, MC, LVI and margin  
229 assessments while others are newly developed methods (CPATH) or need clarification.  
230 Tumor necrosis is used in grading systems for some tumors, yet the method to  
231 determine percent necrosis in tumors from pets has never been described or not in  
232 sufficient detail such that others can reproduce the method (see Appendix 4). At the end  
233 of each appendix is a section titled "Future Considerations", which provides a list of  
234 possible ways to improve that method. *Protocols* are designed to gather complete data  
235 sets for the evaluation of commonly graded canine neoplasms. Protocols are provided  
236 for Soft Tissue Tumors/Soft Tissue Sarcomas (STT/STS), and are in process for canine  
237 mast cell tumor, and canine melanoma (cutaneous and oral). Protocols for other tumors  
238 can be developed and are needed, including mammary, splenic, osteosarcoma,  
239 hematopoietic and lymphoid tumors as well as cytologic protocols. If we do not  
240 standardize the methods used to identify tumors, we will continue to have conflicting  
241 data in the literature. Protocols and Appendices can be used as guides for reviewers  
242 and editors of manuscripts to ensure all required data was included and standard  
243 methods were followed. Journals serve as a gatekeeper for scientifically sound data,  
244 and they should also not refrain from publishing negative results. Investigators can use  
245 protocols as a checklist to ensure complete data sets are included for study participants.  
246 The protocols are modeled after the College of American Pathologists with an emphasis  
247 on gathering uniform data on specific tumor types. What are the consequences of not  
248 following an appendix or protocol? Nothing, no accreditation or certification or plaques

249 of accomplishment will be awarded or rescinded. The methods described herein are  
250 intended to be “best practices” that will add consistency and reproducibility to our  
251 methods with an eye to our clients: clinicians, oncologists, patients, and the public.  
252 Appendices and Protocols extend beyond “best practices” as they provide brief  
253 literature reviews, areas of weaknesses and list suggested fields of investigation for  
254 future studies to improve a method. The guidelines described are based upon review of  
255 literature and authors’ expertise, and are intended to bring consistency and  
256 reproducibility to the evaluation of tumors in animals. These guidelines have not been  
257 certified, accredited or reviewed by any standards-creating body and represent the  
258 authors’ own interpretation and application of the data reviewed. Application of these  
259 guidelines may vary with different laboratories and personnel, and each pathologist  
260 should consider whether these guidelines are appropriate based on the equipment,  
261 tissues or other materials available. Whether a governing body will aid in further  
262 development in updating these guidelines will depend upon the success of the website  
263 and how widely it is used.

#### 264 **Future Collaboration**

265 The website being constructed will address some of these needs, but additional  
266 personnel will be needed to maintain the site, develop different protocols, generate new  
267 data, and validate studies. The initiative of a website with living appendices and tumor  
268 protocols will be successful if others use this information in their diagnostic, research,  
269 and publication efforts and if the appendices and protocols are updated in a timely  
270 manner as new information becomes available. A key benefit of standardization of  
271 tumor evaluation is the ability to evaluate data accrued from studies of many  
272 investigators at various institutions world-wide. This will permit analysis of larger data  
273 sets and increase the statistical power of the observations. The eventual goal would be  
274 to develop veterinary pathology industry standards with international input and  
275 acceptance. The goal is to accrue data on the important parameters that should be  
276 evaluated for a specific tumor type so that, over time, large data sets with comparable  
277 information about specific tumor types can be evaluated to provide accurate prognostic  
278 information that improves patient care. This will take multi-institutional participation and

279 specialists from different disciplines. The driving force will likely come from younger  
280 generations. Future appendices might include molecular profiles, genetic tests, and  
281 checklists for surgical pathology reports. Protocols are unlimited, think of a tumor, write  
282 a protocol using these as templates. Edits and updates are encouraged: contact the  
283 communication authors of an appendix or protocol. Submission of additional tumor  
284 protocols is welcomed and can be accomplished by contacting the administrators of the  
285 website. Confirming the need for standardized parameters to evaluate animal tumors  
286 met with near unanimity. Agreement for the guidelines of each parameter is not always  
287 unanimous. To compare data between labs, and ultimately improve patient care, we  
288 need to apply the same methods to basic parameters used to evaluate tumors. Using  
289 unstandardized methods that can cause variation in results is not scientifically sound.  
290 Drawing conclusions for clinical cases based on methods that are not standardized is  
291 misleading.

292 Completed Appendices and Protocols are in the supplemental section of this  
293 manuscript and they will be posted on the website and updated as needed  
294 ([www.vetcancerguidelinesandprotocols.cldavis.org](http://www.vetcancerguidelinesandprotocols.cldavis.org)). The following are excerpts and  
295 summaries of each appendix or protocol, not the completed documents. Readers  
296 interested in a parameter should read the details in completed documents provided in  
297 the supplemental section of this manuscript and on the website.

## 298 **SUMMARY**

299 The goal of this project is to help advance veterinary oncology and pathology by  
300 promoting standardization of tumor assessment and patient outcomes. Guidelines are  
301 proposed to increase the uniformity and consistency of methods used to evaluate  
302 tumors along with suggestions for future consideration to help improve their  
303 discrimination and utility. Scientific journals, editors and reviewers can ensure progress  
304 in the goals of tumor assessment standardization and study reproducibility by  
305 establishing certain requirements of manuscripts being reviewed. Oncology studies  
306 which include histopathologic and gross features of tumors should have a pathologist as  
307 a co-author and journals should require this. Data obtained from record review without  
308 knowledge of the diagnostic or grading criteria limits conclusions and confidence in the

309 study. Review of gross description and histologic slides or images by an authoring  
310 pathologist or multiple pathologists is needed to ensure accuracy and uniformity of the  
311 pathology data and that current methods and terminology are used. The appendices are  
312 designed to help accomplish this. Certain appendices are completed: MF recognition,  
313 MC, necrosis, LVI, margin assessment and synoptic reporting, while others are in  
314 progress. The key steps to performing each method are condensed into checklists  
315 within the appropriate appendix. These checklists should integrate well with synoptic  
316 reporting (see Appendix 8). There are also discussion and notes to clarify certain steps.  
317 The checklists for margin evaluation are subdivided by responsible persons, the list that  
318 a pathologist should report are short and practical. LVI can be evaluated in HE sections  
319 and methods to confirm and differentiate LVI from pseudo-vascular invasion (see  
320 Appendix 3). Future investigations need to determine the importance of identifying if the  
321 tumor thrombus is in a lymphatic or blood vessel, and if the distinction has practical  
322 importance it will need to be determined how capable pathologists are of distinguishing  
323 each type of vessel with HE stained sections. Some authors would like to see necrosis  
324 abandoned as a parameter but that will require additional investigations. Suggestions to  
325 improve how necrosis is determined are provided in Appendix 4. CPATH will aid new  
326 investigations and synoptic reporting will provide a means to summarize and readily  
327 retrieve information. Outcome assessments are central to improvement of prognostic  
328 parameters but are under the umbrella of oncologists. However, histopathology is  
329 needed to confirm it is the same tumor in a recurrence or metastasis.

330 Until there are data driven results that can be standardized and proven prognostically  
331 useful, tumor assessment will need to include a wide range of parameters. Some  
332 practices, such as reporting margins of benign tumors or mitotic counts in tumors in  
333 which significance is not established will be left to the discretion of the pathologist and  
334 clinician. Clinicians faced with decisions on patient therapy rely extensively on  
335 pathologists' assessments. The prognostic significance of various factors changes over  
336 time necessitating clarity in communication of pathological findings, giving clinicians the  
337 information needed. The website is a window for clinicians to see pathologists'  
338 perspective of tumor assessment. Fascial planes to the surgeon are not the same as to  
339 the pathologist, a high power field is not a standard unit of area, if surgical margins are

340 not inked by the clinician, there is no accuracy to HTFD and there are other examples to  
341 illustrate our different perspectives. We need to do our best for the clients, owner, and  
342 pet, but we also need to explain and defend our discipline. The latter will be easier if  
343 veterinarians entering our profession understand our roles, and the limitations of our  
344 techniques.

345 The appendices and protocols require updating and renewal to be useful documents.  
346 Pathologists, oncologists and other scientists are encouraged to submit suggestions  
347 and supporting data to enable thoughtful revision. Tumor types and behavior may differ  
348 in various geographic sites and we encourage communication from all points of the  
349 globe to enhance our overall understanding of tumor behavior. Protocols are needed for  
350 additional tumor types and appendices should be developed for other parameters such  
351 as cytological assessments to recognize and grade specific tumor types, cellular and  
352 nuclear pleomorphism and proliferative indices. Research needs to clarify which  
353 technique and modifications enhance diagnostic and prognostic accuracy and if they  
354 can be practically applied to diagnostic cases, and subsequently validated with robust  
355 data. As in most research endeavors, new technology should be directed to answer  
356 specific problems and not end up as a new method in search of a question to answer.

357 Prospective studies that follow rigorous guidelines are the standard we should strive for  
358 and which will help guide the way forward.(Webster, Dennis et al. 2011) We also  
359 propose a platform from which new data can be gathered and integrated into an  
360 ongoing approach to evaluate the practicality and utility of current, as well as newer  
361 methods of tumor evaluation. Publishers can aid this project by providing permission for  
362 authors to copy sections of manuscripts they authored without forcing them to rewrite  
363 their own sentences to avoid plagiarism. How long will it take to accomplish all of this is  
364 unknown, but we need to continue and expand upon what our colleagues started in  
365 2011.

366

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409

410 **Mitotic count (MC) and Histologic Morphology of Mitotic Figures (MF) (See**  
411 **Appendix 1 and Appendix 2)**

412 MC will remain an important parameter in the evaluation of tumors as it is easy to  
413 accomplish, incurs no additional costs, is predictive of tumor proliferation and is part of  
414 multiple grading schemes that help predict tumor behavior. However, certain  
415 components essential for performing reproducible MC must be defined including the  
416 region of the tumor where MC should be performed (ie “hot spot” or areas of highest  
417 mitotic density within a tumor)(van Diest, Baak et al. 1992, Baak, , Meyer, Cosatto et al.  
418 2009, Al-Janabi, van Slooten et al. 2013, , Veta, Van Diest et al. 2015, Meuten, Moore  
419 et al. 2016, ,) and the amount of tumor area in which MF will be counted, expressed in  
420 standard units of measure (ie mm<sup>2</sup>).(Meuten, Moore et al. 2016) Although enumerating  
421 MF has long been a mainstay of tumor assessment, until recently there has been no  
422 standardization of any element of this parameter in veterinary pathology. Perhaps, the  
423 assumptions were that we were all counting the correct structures with the same  
424 method, that these methods matched published manuscripts and therefore there was no  
425 need to standardize the technique. Unfortunately, some of those assumptions are false.  
426 Performing the MC is considered laborious but subjective with inter-pathologist  
427 variation.(, Tsuda, Akiyama et al. 2000, Meyer, Alvarez et al. 2005, Veta, Van Diest et  
428 al. 2015, Bertram, Gurtner et al. 2018,) Possible causes include counting differently  
429 sized areas, poorly defined methods, not following methods, counting too rapidly,  
430 counting ambiguous structures, experience level, tumor mitotic heterogeneity, inability  
431 to find hot spots, quality of sections (fixation, artifacts) and quality of images.

432 To achieve accurate and consistent counts the MC must be performed carefully  
433 following standardized procedures; when this is done, consistent counts can be  
434 achieved by pathologists.(van Diest, Baak et al. 1992) After we follow standardized  
435 methods, these criticisms should be re-evaluated for manual and automated MC. MC  
436 can be determined by partially automated means, using artificial intelligence (AI, more  
437 specifically, deep learning-based algorithms). MC performed with computer systems  
438 can correct for interobserver variations associated with manual counts. They can better

439 identify hot spots (Aubreville, Bertram Deep learning algorithms outperform...2020), and  
440 they can count thousands of fields but may introduce different hurdles. High quality  
441 training datasets that adequately reflect the variability of histopathology sections and  
442 scanned images, along with validation of AI methods are paramount for CPATH to  
443 produce accurate and verifiable counts. With high quality data sets that define MF,  
444 atypical mitotic figures (AMF) possibly along with hard negatives such as mitotic like  
445 figures (MLF such as inflammatory cells or cells undergoing necrosis or apoptosis),  
446 automated means to perform MC should eventually be able to address potential  
447 confounders. Regardless of which mode, manual or automated, we propose that each  
448 of these elements needs to be standardized: 1. definition of MF, AMF and MLF; 2. the  
449 size of the area in which MF and AMF are counted; 3. the area of the tumor to be  
450 evaluated and 4. how to handle confounders. Each of these is described in Appendix 1  
451 and 2, CPATH is in Appendix 5. At the end of all appendices are considerations for  
452 future studies which should help improve the method and clarify issues associated with  
453 assessing the parameter.

#### 454 **Histologic Morphology of Mitotic Figures (MF) (See Appendix 2)**

455 *What morphological features define a MF to be included in a MC?*

456 The morphologic characteristics of MF and AMF and features which distinguish these  
457 from MLF are detailed in a recent publication. Mitotic figures and AMF are most easily  
458 identified by the short “rods” of chromosomes protruding from the surface of aggregates  
459 of nuclear material (Figures 1-4). Identification of the different phases of mitosis or the  
460 type of AMF are not necessary, but an understanding of the mitotic continuum and that  
461 AMF may have prognostic significance should be appreciated. Counting AMF may  
462 correlate with poorer prognosis and outcome as seen in some human tumors.(Jin,  
463 Stewenius et al. 2007, Matsuda, Yoshimura et al. 2016) Definitive MF (figures 1-4) and  
464 AMF (figures 5-8) should be included in the MC; however, structures with ambiguous  
465 morphology create a dilemma in classification. This is not problematic if the MC is  
466 markedly high (e.g.  $>20 \text{ MF}/2.37\text{mm}^2$ ). However, if the MC is close to an established  
467 threshold which has clinical significance, then the identity of these candidate structures  
468 could be critical (see MC Appendix 1.0). New thresholds should be established following

469 the guidelines in Appendices 1 and 2 and those thresholds should be tiered (avoid  
470 thresholds based on a< or> single number). Clinicians that request recounts because  
471 the MC of a tumor is at a threshold should seek different parameters to help establish  
472 the prognosis or direct therapy. We all likely have “non-standardized methods” that we  
473 use while counting MF but would not necessarily like others to know we do them:  
474 enumerating doubtful structures under a column labelled “?”; looking at extra fields  
475 when no MF were seen; looking at extra fields because there were spaces created by  
476 blood vessels, ducts or cysts; what to do when the tissue sample is <2.37mm<sup>2</sup>; and/or  
477 looking for MF when the diagnosis of inflammation vs neoplasia is not clear. Practical  
478 considerations while performing a MC are listed in Appendix 1. Pathologists and  
479 laboratories will develop their own procedures to address MC reporting in non-routine  
480 situations. When solutions are found, the appendix will be updated accordingly. Correct  
481 identification of histologic structures will improve MC consistency and accuracy obtained  
482 from manual (glass or WSI) or CPATH modes.

483 *Does the FN of an ocular matter?*

484 For light microscopy, absolutely. It is the limiting factor that determines the diameter and  
485 therefore the area in the field of view (FOV) when objectives of the same magnification  
486 are used. Engraved or printed on some ocular eyepieces is a field number (FN) ranging  
487 from 6-28 mm. Higher numbers have larger FOV diameters and small increases in the  
488 FN will produce large increases in the area of FOV (see Appendix 1). The diameter of  
489 the FOV can be measured with a stage micrometer or it can be calculated by dividing  
490 the FN (mm) by the objective magnification. The formula for the area of a circle is used  
491 to calculate the area in the FOV. Therefore, a microscope with an ocular FN 18mm, 40X  
492 objective has a diameter of 0.45mm in the FOV and an area of 0.16 mm<sup>2</sup> per “hpf”; FN  
493 26.5mm, 40X objective has a diameter of 0.66 mm and an area of 0.34 mm<sup>2</sup> per “hpf”  
494 which is a 100% larger area, a two fold increase (see Figure 3; Table 3 in Appendix  
495 1).(Meuten, Moore et al. 2016)

496 Some objectives will have FN and/or NA (numerical aperture) numbers engraved or  
497 printed on them. Both are defined in the Appendix 9 “definitions and abbreviations”. NA  
498 is critical for resolution and depth of field but it is not used to calculate FOV. The higher

499 the NA the greater the resolution, or sharpness of features. All objectives have an FN  
500 but it may not be engraved on the objective. The FN of an objective can influence the  
501 FOV: however, it is the ocular FN which limits the maximum size of FOV in a standard  
502 microscope, not the objective FN.

503 *What is the area in 10 high power fields (hpf)?*

504 The area in 10 hpf is not a standard size as it varies up to 200% or more with the  
505 objective and the FN of the ocular.(Meuten, Moore et al. 2016) We proposed  
506 replacement of the imprecise phrase, *10 hpf* with  $2.37 \text{ mm}^2$  to reflect the area equating  
507 to 10 hpf using a 40X objective and a 10X ocular FN 22mm, the most common  
508 configuration of pathologists' microscopes today. Furthermore, 10 hpf is nebulous for  
509 whole slide imaging which is likely the number one means for diagnostic tumor  
510 evaluation worldwide. A standard size area in  $\text{mm}^2$  is required so the characteristics of  
511 the monitor and the magnification at which the image is reviewed can be configured to a  
512 specific area (see Appendix 1). Temporarily, retaining the phrase "10 hpf " together with  
513 accurate terminology ( $2.37 \text{ mm}^2$ ) clarifies communication with clinicians and permits MC  
514 to be determined with microscopes or WSI.

515 Mitotic counts (MC) reported in terms of high-power fields (hpf) without specific units of  
516 measurement ( $\text{mm}^2$ ) cannot be compared to other MC as the area within one or  
517 especially 10 hpf is too variable.(Meuten, Moore et al. 2016) Older microscopes were  
518 equipped with ocular FN 18 (smaller FOV) compared to current microscopes which  
519 commonly have oculars of FN 22 or greater. Most prior animal studies did not define  
520 the area ( $\text{mm}^2$ ) in which the MC or other histological features were enumerated, or  
521 defined the area incorrectly limiting the utility of this data for formulating prognoses for  
522 current cases. These studies need to be repeated with standardized methods of  
523 determining the basic histological parameters used to evaluate tumors. New methods  
524 should be considered and all must be correlated with outcome assessments.

525 *Does the standard area need to remain  $2.37 \text{ mm}^2$ ?*

526 No, it can be changed with data driven results. The total area evaluated can be  
527 amended for different tumors or unique situations. e.g. total tissue submitted is

528 <2.37mm<sup>2</sup>; cystic tumors etc. Perhaps tumors with low proliferative rates require larger  
529 areas to be enumerated (5-10mm<sup>2</sup>) or perhaps it is the opposite. What might be more  
530 important than a MC in one spot is what proportion of an entire tumor (or section) has  
531 low vs high proliferative rates. Greater than 85% of canine cutaneous MCT are indolent  
532 (Kiupel, Webster et al. 2011); perhaps determining the percent of a MCT that is “cold”  
533 (few hot spots, or areas of high mitotic activity) will predict how aggressive the tumor is.  
534 For canine oral melanoma, it might be the proportion of the tumor that is “hot” which is  
535 predictive. We also do not know how many sections of a tumor should be enumerated  
536 for the MC to be most predictive? This is true for other histologic parameters as well.  
537 These changes require correlating the different methods with known outcomes in many  
538 cases to show which method is predictive. Once a method is validated for a tumor type,  
539 the same size area, same region of the tumor and means to identify MF and AMF need  
540 to be validated if we want to compare results between labs or use published cutoffs of  
541 histologic parameters.

542 When multiple sections or regions are enumerated, should an average MC be reported  
543 or the ranges?(Meyer, Cosatto et al. 2009) Various guidelines have been proposed for  
544 determining the optimum tumor area for performing the MC in human tumors. Different  
545 sized areas are recommended to perform MC for different tumors. Some authors  
546 recommend counting a series of 5 or more sets of MC and reporting the average.  
547 Others report the highest MC. There are a multitude of scenarios that need investigation  
548 to change how we determine MC, and CPATH will greatly aid these studies because  
549 MC can be performed faster, more consistently, and can be performed over differently  
550 sized areas in different regions of the tumors. CPATH can report the proportion of a  
551 tumor that is *hot* or *cold*. Manual counts for these types of studies will be laborious.  
552 Studies using CPATH should also include the standard means of determining the MC  
553 and compare the various methodologies to known outcomes. Hopefully, these studies  
554 will avoid creating MC cutoffs that are based on a single number (above or below) and  
555 develop scoring systems, confidence intervals, and ranges of predictability for MC for  
556 different tumors.

557 Until data driven results provide new methods, an area equivalent to 2.37mm<sup>2</sup> should  
558 be used for MC and should be reported as mm<sup>2</sup> rather than stating the FN of the ocular  
559 or how the scope is configured.

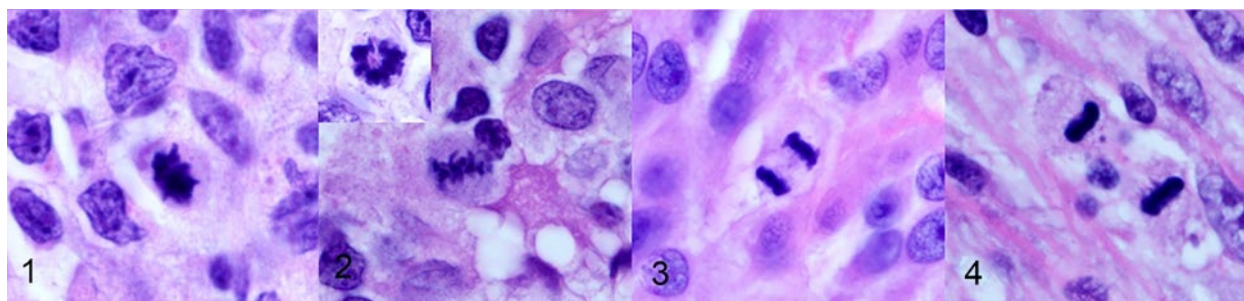
560 *Where in the tumor should the MC be performed?*

561 Presently MF and AMF should be counted in regions of *hot spots* or high mitotic activity  
562 in viable regions of tumor. It is logical to choose regions of high tumor cell proliferation  
563 because the cells in these areas may be more aggressive, they already may have the  
564 potential to metastasize or they have a greater opportunity to form a clone with  
565 metastatic potential. Until studies report that a different region is more predictive of  
566 outcomes, we should adhere to this method. There are no studies in animals that  
567 correlate MC determined in different regions with outcomes. Multiple studies in humans  
568 and one in dogs have demonstrated variability in the number of MF in different regions  
569 of tumors.(Bertram, Aubreville et al. 2020) We know there is heterogeneity of MF  
570 distribution in tumors, but we do not know if it matters, and we will not know until there  
571 are outcome assessments correlated to methods. Different regions and differently sized  
572 areas of different tumor types are used to perform MC in human tumors, and different  
573 cutoffs of MC are used to determine prognoses. Similar studies need to be done with  
574 animal tumors, and when these are performed, investigators should include newer  
575 technologies as well (molecular, CPATH etc., [https://www.cap.org/protocols-and-](https://www.cap.org/protocols-and-guidelines/cancer-reporting-tools/cancer-protocol-templates)  
576 [guidelines/cancer-reporting-tools/cancer-protocol-templates](https://www.cap.org/protocols-and-guidelines/cancer-reporting-tools/cancer-protocol-templates))

577 The periphery of some tumors is the preferred site because this is the invasive front,  
578 fixation is better, and there is a higher proliferative rate. A study of human breast  
579 carcinoma reported that the periphery contained more hot spots (using Ki67) than other  
580 regions and percentages of Ki67 positive nuclei obtained at the periphery changed the  
581 prognosis.(Gudlaugsson, Skaland et al. 2012) Other studies in humans reported that  
582 using Ki67 in hot spots, which were not just at the periphery of breast carcinoma,  
583 contributed the most prognostic information as compared to other  
584 methods.(Stålhammar, Robertson et al. 2018) Additionally, a study of canine cutaneous  
585 mast cell tumors did not find that the regions of highest mitotic activity were always at  
586 the periphery.(Bertram, Aubreville et al. 2020) Selecting the area of a tumor that is

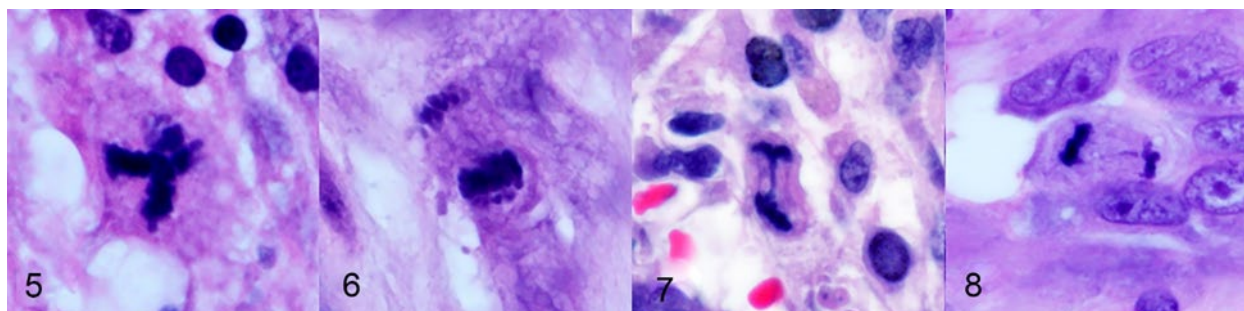
587 *predictive* of outcome(s) or treatments needs to be found for each tumor type. Until  
 588 those locations are identified, MC should be performed in regions of hot spots.  
 589 However, determination of hot spots by routine light microscopy is subjective and a  
 590 source of interobserver variation.(van Diest, Baak et al. 1992, Bertram, Aubreville et al.  
 591 2020) A study with canine MCT and one with canine melanoma showed that  
 592 pathologists were not as capable of finding the hotspots as compared with computer-  
 593 assisted localization of hot spots.)(Puri, Hoover et al. 2019, Aubreville, Bertram et al.  
 594 2020)

595 *Summary:* Appendix 1 and 2 detail the standard method of performing a MC  
 596 including: definitions of MF, AMF and MLF, contiguous 2.37 mm<sup>2</sup> area, hot spot,  
 597 practical considerations, and future considerations of how the MC can be improved.  
 598 The present standard means to perform the MC will be modified when data-driven  
 599 changes necessitate, and the appropriate appendices will subsequently be updated.



600  
 601 Figures 1-4: Mitotic Figures (MF) are characterized by dark aggregates of nuclear  
 602 material with short rods and projections. Figure 1: Prometaphase/metaphase (dense  
 603 nuclear cluster with short protruding rods). Figure 2: Metaphase with linear equatorial  
 604 plate of darkly staining nuclear material and short protruding rods and spikes. Inset:  
 605 Ring form of metaphase with end-on (non-perpendicular) view of the equatorial plate.  
 606 Figure 3: Anaphase MF with two separate nuclear aggregates with irregular contours  
 607 and short protruding spikes. Figure 4: Telophase MF with aggregates at opposite ends  
 608 of the cell and formation of a cleavage furrow.

609



610

611 Figures 5-8: Atypical MF (AMF). Figure 5: Tripolar AMF (more than two spindle poles  
 612 during any stage of mitosis). Figure 6: Asymmetric AMF (unequal sizes of the  
 613 metaphase axes or anaphase poles). Figure 7: AMF with anaphase bridging  
 614 (chromosomes stretching from one pole to the other). Figure 8: Lagging chromosomes  
 615 left behind during anaphase (small dark purple streak in center of cell).

616

617 **References** (A full list of references is available in Appendices 1 and 2)

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664

### 665 **Lymphovascular Invasion (See Appendix 3)**

666 Neoplastic cell invasion of blood vessels or lymphatics is widely recognized as evidence  
667 of tumor aggressiveness and potential malignancy in both humans (Falvo, Catania et al.  
668 2005, Mete and Asa 2011 ) and animals (Goldschmidt, Pena et al. 2011, Rasotto,  
669 Berlato et al. 2017) but, despite, the importance of this parameter, criteria to definitively  
670 identify lymphovascular invasion (LVI) and distinguish from pseudo-vascular invasion or  
671 retraction artifact are lacking in the veterinary literature. This lack of stringent,  
672 standardized criteria may have led to misdiagnosis of LVI in veterinary oncology  
673 studies. Assessing LVI with criteria of varying stringency has revealed key insights into  
674 the biological behavior of human cancers as has the distinction between blood vascular  
675 and lymphatic invasion.(Van den Eynden, Van der Auwera et al. 2006, Lin, Zhu et al.  
676 2010, Mete and Asa 2011) In veterinary medicine, LVI is recognized as a marker of  
677 potential tumor malignancy but this parameter has only been extensively evaluated in  
678 canine and feline mammary tumors(Goldschmidt, Pena et al. 2011, Rasotto, Berlato et  
679 al. 2017) without establishment of strict criteria for LVI diagnosis or comparison of blood  
680 vascular and lymphatic invasion.

681 Mimickers of LVI, such as pseudo-vascular invasion and retraction artifacts are not  
682 adequately addressed in the veterinary literature; images of each can be found in  
683 Appendix 3 and on the website. Pseudo-vascular invasion is the presence of neoplastic  
684 cells within vascular spaces, but the cells are not present because of tumor invasion of  
685 vessels. Displacement of neoplastic cells into vessels secondary to manipulation of the  
686 neoplasm at the time of biopsy, surgical excision, grossing procedure or tissue  
687 sectioning (ie, "floaters") can result in pseudo-vascular invasion.(Van den Eynden, Van  
688 der Auwera et al. 2006, Mete and Asa 2011) This is also reported for non-neoplastic  
689 lesions in the thyroid. (Mete and Asa, 2011) Neoplastic cells may protrude or impinge  
690 into adjacent vascular lumens without true invasion in which case endothelial cells cover  
691 the surface of the impinging tumor. However, endothelium may also line the surface of  
692 neoplastic cells which have invaded through the vascular endothelium but have  
693 undergone re-endothelialization, necessitating searching for other criteria of LVI to  
694 confirm which is the correct interpretation.

695 Distinguishing between these various manifestations of pseudo-vascular invasion and  
696 true LVI relies on identification of more robust LVI criteria. The two most definitive  
697 criteria used to define LVI in human tumors include: thrombus adherent to intravascular  
698 tumor and tumor cells invading through the vessel wall and endothelium. Additional  
699 criteria are listed in Appendix 3 along with a complete reference list.(Mete and Asa  
700 2011. These criteria should be used to assess LVI in tumors from animals.

701 Retraction artifact, another mimicker of LVI, forms an artifactual space surrounding  
702 tumor foci and can be distinguished from intravascular neoplasia by the absence of an  
703 endothelial cell lining. Retraction artifact is seen in epithelial tumors in which tumor cells  
704 retract from surrounding stroma (Figure 5 in Appendix 3).

705 Studies of human breast, thyroid and prostate cancer show widespread metastases are  
706 more commonly associated with blood vascular invasion in contrast to lymphatic  
707 invasion.(Mete and Asa 2011) Animal tumors may show similar distinctions between  
708 blood and lymphatic vascular invasion, warranting detailed descriptions of the type of  
709 vessels invaded (ie, if a muscular wall is discerned in the involved vessels) or use of  
710 immunohistochemical markers to distinguish blood from lymphatic vessels. A variety of  
711 immunohistochemical markers have been used to identify endothelial cells in blood and  
712 lymphatic vascular channels in humans and animals (Von Beust, Suter et al. 1988,  
713 Sleenckx, Van Brantegem et al. 2013, Wennogle, Priestnall et al. 2019, Fitzgibbons,  
714 Connolly et al. 2020) Some markers, such as CD31 and Factor VIII related antigen, do  
715 not discriminate between lymphatic and blood vascular endothelium, whereas others,  
716 such as Lymphatic vessel endothelial receptor 1 (LYVE-1), D2-40 and prospero –  
717 related homeobox gene-1 (PROX-1) are specific for lymphatic endothelium.(Von Beust,  
718 Suter et al. 1988, Pusztaszeri, Seelentag et al. 2006, Sleenckx, Van Brantegem et al.  
719 2013, Halsey, Worley et al. 2016, Wennogle, Priestnall et al. 2019, Fitzgibbons,  
720 Connolly et al. 2020) Use of IHC endothelial markers has been shown to facilitate  
721 identification of LVI in tumors in humans(O'Donnell, Feldman et al. 2008,) and in  
722 mammary and plasma cell tumors in dogs.(Sleenckx, Van Brantegem et al. 2013,  
723 Ehrensing and Craig 2018) Validation of IHC markers and antibodies used to  
724 differentiate lymphatic vs blood vessels for the different animal species is a necessity.

725 Although IHC confirms the identity of the vascular structure it does not confirm true LVI  
726 and, in fact, is not one of the more stringent criteria of LVI.

727 Studies of tumor lymphovascular density (LVD) in humans have been correlated with  
728 LVI in a number of human tumors.(complete list of references in Appendix 3) LVD is an  
729 enumeration of lymphatics within a defined area of a tumor and is used as an indicator  
730 of lymphangiogenesis and therefore probable lymph node metastasis. Both LVD and  
731 LVI are used as predictors of lymph node metastases in human breast cancer, and  
732 peritumoral lymphatic vessels may be the main route for dissemination of the tumor.  
733 Intratumoral microvascular density (IMD), the quantitation of blood vessels  
734 (number/mm<sup>2</sup>) in tumors, is used as an indicator of angiogenesis or vasculogenesis and  
735 by extension LVI and the ability of a tumor to metastasize. New blood vessels in a tumor  
736 are required for tumors to grow beyond several millimeters; they are believed to  
737 facilitate metastasis and are associated with more aggressive neoplasms in humans  
738 and animals. Although IMD has been assessed in a number of animal tumors and has  
739 been associated with higher grade or more malignant histological features (ie canine:  
740 soft tissue sarcomas, mammary gland tumors, seminomas, cutaneous squamous cell  
741 carcinoma,) and cutaneous mast cell tumors),(full reference listing in Appendix 3) there  
742 have been no comprehensive studies of intratumoral versus peritumoral vascular  
743 density nor associations between IMD and blood vascular or lymphatic vascular  
744 invasion in domestic animals. Future veterinary studies comparing intratumoral versus  
745 peritumoral microvascular density and correlation with nodal and systemic metastases  
746 are warranted.

747 A thorough reassessment of LVI is needed in veterinary oncology with attention to the  
748 specific details described in the *appendix LVI* and under future considerations. These  
749 studies should use the criteria outlined to determine if LVI is present, especially focusing  
750 on the more definitive features: invasion through vessel wall and endothelium and  
751 thrombus adherent to the tumor. Studies should include detailed descriptions of criteria  
752 used to establish presence of LVI and clarify the importance of lymphatic versus blood  
753 vascular invasion. Quantitation of blood and lymphatic vessels (IMD, LVD) may benefit  
754 from the use of CPATH, and both subjective and quantitative analyses should be

755 correlated with nodal and systemic metastases and, most importantly, known patient  
756 outcomes.

757

758 If individuals have images of true LVI and pseudo-vascular invasion please share them  
759 with the communication author of appendix 3.

760

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809

810 **Necrosis (See Appendix 4)**

811 The extent of tumor necrosis has been correlated with tumor biological behavior and is  
812 a parameter used in grading schemes in humans. Tumor necrosis has also been  
813 included as a grading scheme parameter in animals, primarily in dogs with STS/STT but  
814 is also used in other grading schemes (canine primary pulmonary carcinoma). Criteria  
815 for determining the percent of tumor necrosis in all species have not been adequately  
816 described (Kuntz, Dernell et al. 1997, Coindre 2006) Necrosis within a tumor is often  
817 subjectively and vaguely used to suggest a tumor is aggressive. In humans, the percent  
818 of tumor necrosis has been determined by estimating the amount seen grossly and  
819 histologically, whereas animal studies have not indicated if gross observations were  
820 used in combination with histological assessment, or if only histologic assessments  
821 were evaluated.(Trojani, Contesso et al. 1984, Kuntz, Dernell et al. 1997, Coindre 2006,  
822 McSporran 2009, Vayrynen, Vayrynen et al. 2016, Laurini, Blanke et al. 2017,  
823 Dobromylskyj, Richards et al. 2020) Many tumors are larger than a histological section,  
824 and measuring or estimating percentage of necrosis is more problematic.(Chiang and  
825 Oliva 2013)

826 In grading human soft tissue sarcomas, necrosis was found to be one of three  
827 parameters correlating with patient survival and tumor metastasis, along with tumor  
828 differentiation and mitotic count.(Trojani, Contesso et al. 1984) The thresholds for  
829 scoring necrosis histologically were no necrosis (score 0), less than 50% of tumor  
830 necrosis (score 1) and greater than 50% tumor necrosis (score 2) but how a pathologist  
831 was to estimate those percentages was not detailed. A grade of two could also be  
832 assessed for any neoplasm whose gross appearance was described as “mainly  
833 necrotic” by a surgeon or pathologist even if no necrosis was seen on the submitted  
834 sections.(Trojani, Contesso et al. 1984) We do not recommend this last criterion be  
835 adopted for animal tumors, and later authors and grading systems, as well as current  
836 College of American Pathologist protocol for assessment of soft tissue tumors in  
837 humans, require microscopic confirmation/validation of macroscopic evidence  
838 suggesting necrosis.(Coindre 2006, Laurini, Blanke et al. 2017) This brings us to the

839 problems associated with gross interpretation of necrosis (and to a lesser extent, its  
840 histologic interpretation). Even for an experienced pathologist, the gross diagnosis of  
841 necrosis may be problematic, and most pathologists in veterinary pathology will not see  
842 the gross specimen. Areas of edema or exudate may be interpreted as areas of  
843 necrosis grossly, and areas of hemorrhage, which are often associated with necrosis,  
844 may far exceed the boundaries of actual necrotic tissue. These problems are further  
845 compounded by certain histologic lesions such as myxomatous change, cystic space  
846 formation, edema, hemorrhage and exudate which can resemble or obscure necrotic  
847 areas. Gross/macrosopic assessment of necrosis requires histologic confirmation  
848 which, in large tumors, may not be practical for veterinary diagnosticians to submit an  
849 adequate number of sections (costs) but should be done in research studies. The  
850 number of sections examined at trimming and or submitted for histopathology for routine  
851 diagnostic cases is likely far fewer in veterinary than human pathology. If gross  
852 assessment is to be used as a parameter, numerous confounders must be clarified in  
853 future studies. This requires documentation of systematic sampling of both necrotic and  
854 viable tissue during the gross examination and confirmation of necrosis by histological  
855 evaluation. Alternatively, we can abandon the use of gross assessment and only use  
856 light microscopy. This would be straightforward, but if gross assessment of tumor  
857 necrosis improves the prognostic utility of grading systems then it would be lost as a  
858 parameter.

859 Although it seems obvious that the means to assess various histologic parameters need  
860 to be defined prior to implementation, this has not always happened, e.g. the area in  
861 which MF were counted was never standardized and the same seems true for percent  
862 necrosis. The percent of tumor necrosis in soft tissue mesenchymal tumors/soft tissue  
863 sarcomas (STT/STS) is included in grading schemes, yet the means to assess necrosis  
864 has not been clearly defined or standardized. Was the percent necrosis determined by  
865 examination of the tumor during gross sectioning, and were areas appearing necrotic  
866 confirmed microscopically? Was the percent necrosis used in the grading system based  
867 upon visual estimate of necrosis in random histologic tumor sections? Was a consistent  
868 portion of the tumor submitted for microscopic examination? A recent publication  
869 suggested preparation of 1 tissue block for each 2 cm diameter of soft tissue



870 tumors(Roccabianca, Schulman et al. 2020). Since no formulae for number of  
871 blocks/slides per tumor have been described in published grading systems for dogs this  
872 seems like a good starting point, but no studies using this guidance were referenced.

873 The necrosis appendix (Appendix 4) provides guidelines for recording and scoring  
874 extent of tumor necrosis on gross and histologic tumor evaluation which should enable  
875 evaluation of the utility of this parameter to assess tumor prognosis and patient  
876 outcomes. The scoring system proposed is based on prior reports and is indicated  
877 above but includes an unusual percentage of <10% for future studies and explains the  
878 logic for this. Additionally for necrosis to be objectively assessed as a parameter for  
879 future grading schemes, new studies must determine if gross assessment of necrosis  
880 can be documented in a standardized fashion and if this parameter correlates with  
881 outcome assessment independently or as part of a grading system. For this to be  
882 accomplished, grossing personnel must include sectioning of tumor sites which appear  
883 necrotic, hemorrhagic, or edematous, regions typically avoided in most grossing  
884 procedures. Most veterinary pathologists will only have microscopic sections to estimate  
885 necrosis and these sections are likely to be a small percentage of the entire tumor.  
886 Furthermore, in many cases, the gross description will be inadequate unless grossing  
887 personnel are instructed to search and report the percent of the entire tumor that  
888 appears necrotic. The usual practice of only sampling viable tissue for histological  
889 examination might bias the utility of tumor necrosis as an independent or a component  
890 parameter in grading systems. Importantly, the size of the tumor, method of sectioning,  
891 number of cut surfaces examined grossly and histologically must be documented and at  
892 some point, standardized. Based on size of tumor, a recommendation is needed for how  
893 many sections should be examined grossly and microscopically. It seems obvious that if  
894 pathologist A examines 5 histologic sections and pathologist B only 1 section of a tumor  
895 with 5cm<sup>3</sup> dimensions that the data gathered will not be comparable.

896 This brings us to the dilemma of how to currently approach reporting tumor necrosis.  
897 Given the lack of established guidance, the pathologist can estimate necrosis either  
898 visually with glass slides, WSI or measure necrosis with annotation software in WSI. If  
899 WSI has drawing software, simply outline the entire tumor circumference (X) as well as

900 the areas of necrosis (Y), followed by calculation of  $X/Y = \% \text{ necrosis}$  in one section (Fig  
901 1 Appendix 4). In the absence of software or if using a microscope then visually  
902 estimate with varying magnifications (to confirm areas are indeed necrotic) if the percent  
903 necrosis is <50%>. The range of <50%> seems like a wide target and perhaps that is  
904 sufficient for estimates. We “assume” prior studies that estimated necrosis in canine  
905 tumors only used histology. But how representative the slide(s) are of overall tumor  
906 necrosis is unknown and inconsistent sampling of the tumor, purposely avoiding areas  
907 of necrosis in tissue selection can skew any determination of percent necrosis in  
908 histologic sections. Given the wide target of greater than or less than 50% necrosis, it  
909 may be possible to assess this level of necrosis histologically, even with inconsistent  
910 sampling. However, determining a 10% threshold of necrosis may prove problematic,  
911 as reported in one study indicating that dogs with tumors with > 10% necrosis were 2.7  
912 times more likely to die of tumor related causes.(Kuntz, Dernell et al. 1997)

913 Future studies can clarify how to determine the percent of tumor necrosis, particularly in  
914 larger tumors, and establish a standardized means of gross tissue selection for  
915 histologic examination. Various means of assessing for necrosis in histologic sections  
916 can be compared and statistically evaluated. Results of standardized assessments for  
917 tumor necrosis can be compared to outcomes in univariate and multivariate analysis in  
918 concert with other histologic parameters and prognostic utility determined.

919

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945

## 946 **Computational pathology (CPATH) (See Appendix 5)**

947 Computational pathology (CPATH) is an umbrella term used to broadly encompass  
948 computerized/automated gathering of information on disease in patients.(Abels,  
949 Pantanowitz et al. 2019) Although CPATH may use a large variety of information  
950 sources (raw medical data: histology images, radiology images, gene sequences,  
951 clinical records ), Appendix 5 focuses only on *automated image analysis* (AIA) of  
952 microscopic tumor images, particularly whole slide images (WSI). When used  
953 appropriately, CPATH is an exciting tool which uses microscopic images (input data)  
954 and automatically produces output information (counts or scores of patterns,  
955 classification of images etc.). It allows the evaluation of large amounts of tumor data on  
956 an unprecedented scale, which is likely to reveal novel trends of prognostic importance.  
957 As AIA is a relatively new modality of analysis in veterinary pathology with a vast  
958 number of relevant methods, this field can be overwhelming with respect to terminology,  
959 technical aspects, requirements for developing algorithms, performance validation, and  
960 implementation strategies. Therefore, the associated appendix aims to give an overview  
961 of relevant terms, general considerations of CPATH methods and specific  
962 recommendations for individual prognostic parameters. Generally, two broad categories  
963 of AIA approaches are applicable for microscopic tumor prognostication: 1)  
964 thresholding-based and 2) advanced data-driven approaches. Thresholding-based  
965 algorithms use a set of simple, often programmer-designed image processing steps  
966 based on the color information of individual pixels, which are especially useful for  
967 scoring immunohistochemical labeling intensity. Data-driven approaches learn to  
968 retrieve meaningful patterns from images in order to derive the desired information  
969 using artificial intelligence (AI). AI can be used with traditional machine learning  
970 methods that require “hand-crafted” (by developer) information about relevant features  
971 of the pattern, or more sophisticated deep learning methods that autonomously extract  
972 relevant features (decision criteria are unknown to developers, “black box”). Deep  
973 learning is generally more powerful than traditional machine learning methods, but  
974 necessitates larger amounts of data. For histological images, supervised learning (as  
975 opposed to unsupervised learning) is a very useful method that learns by “feedback”

976 from ground truth labels assigned to the input images. Creating those labels is a very  
977 time-consuming task and is prone to several biases (see Appendix 5).

978 Possible applications of AIA for tumor prognostication are seemingly limitless and  
979 various benefits of these approaches have been determined in previous  
980 studies.(Stålhammar, Robertson et al. 2018, Steiner, MacDonald et al. 2018, Aubreville,  
981 Bertram et al. 2020) Compared to manual assessment by pathologists, algorithms have  
982 higher reproducibility, may have higher accuracy, may increase efficiency of repetitive  
983 tasks (such as counting of mitotic figures (MF)), and can carefully assess vast amounts  
984 of data per case (every image section of multiple WSIs at high magnification) without  
985 fatigue. AIA of immunohistochemical labeling intensity was reported to have higher  
986 reproducibility and improved prognostic value compared to the manual approach by  
987 pathologists for Ki-67 index in human breast cancer,(Stålhammar, Robertson et al.  
988 2018) and membrane-binding biomarkers in human esophageal  
989 adenocarcinomas.(Feuchtinger, Stiehler et al. 2015) An automated topometric  
990 segmentation mapping algorithm of immunolabeled MF (anti-phospho-histone H3) was  
991 used to identify mitotic 'hot spots' in canine melanomas and subsequently used image  
992 registration in order to assign the same region to H&E stained tumor sections(Puri,  
993 Hoover et al. 2019) Deep learning approaches for MF identification in H&E stained  
994 tumor sections have been developed for human(Veta, van Diest et al. 2016, Aubreville,  
995 Bertram et al. 2020) and canine(Aubreville, Bertram et al. 2020) breast cancer as well  
996 as canine mast cell tumors.(Bertram, Aubreville et al. 2019) Deep learning-based  
997 algorithms are comparable with pathologists for counting MF (in the same tumor  
998 regions)(Veta, van Diest et al. 2016) and outperform pathologists in identifying the 'hot  
999 spot' regions in WSI.(Aubreville, Bertram et al. 2020) However, correlation of algorithmic  
1000 MC to patient outcome has not yet been investigated in human and animal tumors. For  
1001 automated metastasis identification in H&E sections, deep learning-based algorithms  
1002 can be used for prescreening of images, and a computer-assisted approach has been  
1003 shown to have higher sensitivity and diagnostic speed compared to the unassisted  
1004 pathologist.(Steiner, MacDonald et al. 2018) Recent studies on tumors from humans  
1005 reported that the systems used could even predict if a tumor was benign, carcinoma in

1006 situ, or invasive carcinoma(Aresta, Araujo et al. 2019) as well as predict genetic  
1007 alterations and gene expression from H&E tumor sections.(Kather, Heij et al. 2020)

1008 Algorithms are not flawless, have multiple sources of error (depending on the  
1009 algorithmic approach and available dataset) and therefore require very careful validation  
1010 (see Appendix 5). While thresholding-based approaches have high explainability of  
1011 algorithmic predictions, data-driven approaches are often considered a “black box” as  
1012 decision criteria of the algorithms are typically unavailable. Although algorithms are  
1013 100% reproducible (same result for the same image using the same model), they may  
1014 not necessarily cope with variability introduced via biological and pre-analytic factors  
1015 (tumor type, tissue types present, section preparation and image acquisition ). For  
1016 example, a deep learning-based algorithm for MF may perform poorly on images  
1017 obtained from a WSI scanner that was not used for the training images.(Aubreville,  
1018 Bertram et al. 2020) If not part of the training data, algorithms can be compromised by  
1019 images with very poor tissue or image quality (artifacts, poor fixation etc.). In contrast to  
1020 thresholding-based approaches, data-driven algorithms are, however, capable of  
1021 learning a certain degree of image variability and training datasets should include  
1022 realistic variability that reflects the intended use. Performance evaluation should be  
1023 done with great care, and data-driven approaches can be assessed by mathematical  
1024 evaluation (see Appendix 5),(Abels, Pantanowitz et al. 2019) whereas thresholding-  
1025 based approaches are often only assessed visually by a pathologist.(Aeffner, Wilson et  
1026 al. 2016) As opposed to pathologists, current algorithms are not capable of modifying  
1027 their decision based on surrounding tissue (spatial awareness), which can lead to false  
1028 detections. For example, pathologists are more careful when classifying a MF in an area  
1029 of necrotic tissue as it may be a MLF but algorithms will not use surrounding tissue and  
1030 will use the decision criteria programmed to evaluate the candidate structure.

1031 Besides the numerous hurdles in development of AIA algorithms, there are practical  
1032 issues to consider for bringing AIA into diagnostic workflows. Basic requirements  
1033 include consistent tissue preparation steps, a digital image acquisition workflow,  
1034 appropriate IT infrastructure, and sufficient computational power. Increasing  
1035 implementation of digital microscopy in veterinary laboratories(Bertram and Klopffleisch

1036 2017) will augment access to WSI and facilitate AIA. Nevertheless, acceptance of AIA  
1037 may be hampered by unfamiliarity, limited research results and poor explainability of  
1038 machine learning-based algorithms (“black box”). However, there are approaches that  
1039 can convert the “black box” into a more transparent “glass box” that are likely to have  
1040 higher acceptance. For example, some algorithms can be implemented as computer-  
1041 assisted prognosis systems (as opposed to fully computerized decisions) that always  
1042 require review by a pathologist. These approaches will improve the reliability of the  
1043 computer assisted prognosis system and allow the reviewing pathologist to retain  
1044 responsibility in making final decisions with regards to these prognostic parameters. AIA  
1045 could greatly improve tumor prognostication by providing vast amounts of reproducible  
1046 and possibly accurate information on the tumor section, but interpretation of the result  
1047 remains the responsibility of the pathologist.

1048

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1096



1097

1098 **Margins (see Appendix 6)**

1099 Margin assessment is one of the most important histological parameters  
1100 evaluated in oncology.(Kamstock, Ehrhart et al. 2011, Stromberg and Meuten 2017,  
1101 Liptak 2020) Patient management decisions often hinge on the results of margin  
1102 assessment, and clinicians may value margin assessment as highly or more than a  
1103 diagnosis. Appendix 6 provides the types of data that are required to standardize the  
1104 reporting of margins for both clinical management and future studies.

1105 Histologic margin evaluation only needs to be reported on tumors where the aim  
1106 of surgery is to completely remove the neoplasm (achieve local control). Samples where  
1107 there was no intent to totally excise the tumor, including intralesional or incisional  
1108 biopsies are for diagnosis only. Certain tumors or the anatomic location of a tumor  
1109 dictate that excision for local control will be attempted but adjacent structures limit how  
1110 much margin can safely be taken, and therefore margin assessment by the pathologist  
1111 should not be requested, e.g. thyroid, anal sac tumors, adrenal glands. Consensus was  
1112 not reached whether margins should be reported for benign tumors. Appendix 6  
1113 provides contrasting philosophies (*Why not? vs Why bother?*) and the recommendation  
1114 that considerations such as this should be left at the discretion of the pathologist and or  
1115 their lab as there was no data to support either approach.

1116 For the overall evaluation of surgical margins, the members of the cancer  
1117 treatment team are the clinician, surgeon, laboratory technologist and pathologist. The  
1118 responsibilities of each are detailed in Appendix 6. Although terms such as complete,  
1119 clean, clean but close, narrow, and dirty are ingrained in the clinical and pathology  
1120 lexicon, practitioners, surgeons, and oncologists should discourage their use and not  
1121 expect these to be used in pathology reports. Ultimately it is the clinician and/or surgeon  
1122 that judges if the margin is deemed adequate after consideration of all factors.  
1123 Observations by the pathologist include 1) relationship of neoplastic cells to the  
1124 surrounding tissue including presence of a capsule, tissue compression, peripheral  
1125 invasion and lymphovascular invasion 2) the distance from neoplastic cells to the  
1126 narrowest or closest inked margin (histologic tumor-free distance (HTFD, Figure 1) and

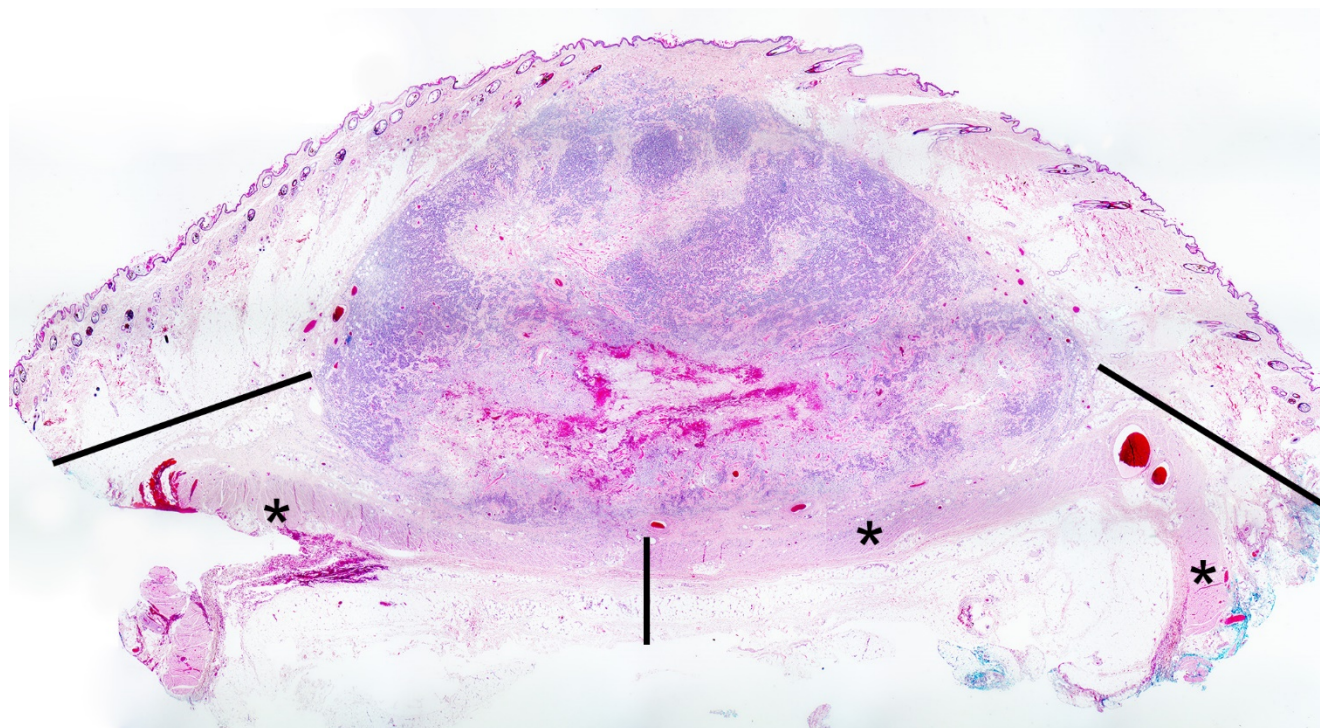
1127 3) the relationship of neoplastic cells to the boundaries of the *compartment* in which the  
1128 tumor is located. In many cases, measuring the HTFD alone is not enough to determine  
1129 the adequacy of surgical margins, yet it is the parameter that is often used to determine  
1130 ‘completeness’ of excision by clinician and pathologist. Inking the margin by the  
1131 clinician/surgeon immediately after tumor excision is required if a HTFD is expected.  
1132 Although surgical margin identification/inking is routinely performed by most surgeons,  
1133 this practice is not commonplace in general practice. Details of how to apply ink have  
1134 been reported (Kamstock, Ehrhart et al. 2011, Appendix 6) and this information should  
1135 be included in veterinary school curricula. If ink is not present when the sample arrives  
1136 at the lab this should be noted. Only a small portion of the circumferential surgical  
1137 margin is evaluated histologically (approximately 0.1- 0.01% of the total margin)(Rapini  
1138 1990, Becker 2007, Selmic and Ruple 2020). HTFD should be further studied by  
1139 comparing different methods of margin analysis (radial, tangential, parallel slicing) with  
1140 outcome assessments for different tumor types (Milovancev, Townsend et al. 2017,  
1141 Does, Milovancev et al. 2018). Until those studies provide comparative data, radial  
1142 sections are recommended. Regardless of the method used, any margin measured  
1143 histologically may not accurately represent the tumor and its relationship to the normal  
1144 surrounding tissue in the patient. It is important to note that HTFD is made on a  
1145 histopathology specimen that has undergone shrinkage, (ranges reported from 13-50%)  
1146 and can underestimate the surgically obtained margins by up to 40%.(Miller and Dark  
1147 2014, Upchurch, Klocke et al. 2018) Most of the shrinkage occurs immediately after  
1148 removal and prior to fixation.(Clarke, Banks et al. 2014, Miller and Dark 2014,  
1149 Upchurch, Klocke et al. 2018) The important margin is between neoplastic cells and  
1150 “normal tissues” (non-neoplastic) in the patient and this can only be estimated from  
1151 histopathology. It is recommended to use whole numbers and ranges when reporting  
1152 HTFD as reporting distances with decimals implies a level of precision and confidence  
1153 that could be misleading. Furthermore, data is accumulating that the biological behavior  
1154 of the tumor may be a more important predictor of recurrence than identification of  
1155 neoplastic cells at a margin. Certainly, this seems to be the case with low-grade canine  
1156 MCT and STS/STT. Most low grade MCT do not recur even with tumor cells at the  
1157 margin and approximately one-third of high-grade MCTs will recur when the histologic

1158 margins are free of tumor cells.(Donnelly, Mullin et al. 2015) Similarly, for canine soft  
1159 tissue tumors/sarcomas, greater than 95% of canine STT do not recur if margins greater  
1160 than 1mm are free of neoplastic cells and one study reported that when margins are  
1161 less than 1 mm, three of 41 grade 1 tumors (7%), 14 of 41 grade 2 tumors (34%), and 3  
1162 out of 4 grade 3 tumors recurred.(McSporran 2009) The biology of the tumor and the  
1163 host (immune system, genes) are important factors that influence tumor recurrence and  
1164 metastases.

1165         Of importance to surgeons is the concept of compartmental boundaries, which  
1166 are used to plan and perform surgical removal of tumors.(Enneking, Spanier et al. 1980,  
1167 Kawaguchi, Ahmed et al. 2004) The surgical margins for tumors can be planned  
1168 differently if the tumor is in a well-delineated anatomic compartment (such as bone,  
1169 joint, muscle) or is infiltrating poorly demarcated interfascial planes and  
1170 spaces.(Enneking, Spanier et al. 1980, Kawaguchi, Ahmed et al. 2004) For a well-  
1171 delineated compartment, it should be reported whether the tumor penetrated the  
1172 anatomic structure forming the boundary (e.g. periosteum, epimysium or cortical bone).  
1173 The deep fascia has been described as a barrier of the subcutaneous tissue  
1174 compartment(Enneking, Spanier et al. 1980) but this structure is not always included in  
1175 sections of cutaneous and subcutaneous tumor resections. Compartment boundaries  
1176 may be natural barriers to tumor extension.(Enneking, Spanier et al. 1980, Kawaguchi,  
1177 Ahmed et al. 2004) It is unclear if these structures function as a true barrier to tumor  
1178 growth; if they do then it is likely multifactorial and depends upon the aggressiveness of  
1179 the neoplasm as well as the components of the barrier (eg cortical bone vs adipose  
1180 tissue; cytokines). Furthermore, what a surgeon vs a pathologist sees as a *fascial plane*  
1181 may not be the same. If pathologists report the facts of what structures were seen  
1182 between the tumor and the inked margin, surgeons and oncologists can decide if they  
1183 are appropriate barriers, and if so, the clinical significance of their presence. Future  
1184 studies need to clarify if anatomic structures can prevent tumor infiltration, if so how and  
1185 what the pathologist should identify for skin and subcutaneous “tissue barriers” and  
1186 fascial planes.(Fulcher, Ludwig et al. 2006) Appendix 6 lists references that describe  
1187 using CT and MRI for visualizing tissue compartments and assessing the relationship of  
1188 tumor to adjacent structures, even differentiating aggressive from benign soft tissue

1189 tumors in humans. It is reported that the tunica serosa fascia in peritoneal cavities is a  
1190 barrier to migration of tumor cells using an in vitro system.(Gao, Ye et al. 2013)

1191 When a delineated anatomic compartment is not obvious, the HTFD is of critical  
1192 importance. HTFD for lateral and deep margins in samples from skin and subcutis  
1193 tumors should be reported separately. In a review of surgical biopsy reports of canine  
1194 cutaneous mast cell tumors, details about the margins and consistency of how  
1195 histologic margins were reported were generally lacking.(Reagan, Selmic et al. 2018)  
1196 For example, while some margins were reported in 92% of cases, lateral and deep  
1197 margins were described separately in 77% of cases, margin direction was only given in  
1198 16% of cases and descriptions of the deep margin were only available in 11% of  
1199 cases.(Reagan, Selmic et al. 2018) The deep margin is difficult for surgeons to visualize  
1200 intraoperatively. At the end of appendix 6 are considerations for future studies (M1-M4  
1201 or R0-RX)(Stromberg and Meuten 2017, Liptak 2020)



1202

1203

1204 Figure 9: Canine cutaneous mast cell tumor involving the dermis and subcutaneous  
1205 tissues. The histologic tumor free distance (HTFD) is depicted with horizontal and  
1206 vertical black lines and can be measured with manual or digital means. Note that ink

1207 can be observed at the lateral (or peripheral) margins, but is not visible at the deep  
 1208 margin. Therefore, the deep margin measurement represents an approximation given  
 1209 the lack of ink. Additional sections into the formalin fixed, paraffin embedded block may  
 1210 resolve this issue. A potential tissue barrier within the subcutaneous tissue is the  
 1211 striated muscle (also called panniculus carnosus or cutaneous trunci in the truncal  
 1212 region, denoted by the asterisks). This muscle is not always visible in histologic sections  
 1213 of cutaneous and subcutaneous tumors; it has variable distribution and continuity in  
 1214 different body regions.(Ahmed, Kulikowska et al. 2019) The subcutaneous fat and loose  
 1215 connective tissue are considered a weak barrier as compared to epimysium,  
 1216 epineurium, or periosteum. The effectiveness of tissue barriers is likely multifactorial  
 1217 and depends upon the aggressiveness of the neoplasm as well as the components of  
 1218 the barrier.

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1277

1278 **Outcome Assessment (See Appendix 7)**

1279

1280 Standardized methods of histologic and outcome assessment parameters for animal  
1281 tumors are essential if we wish to compare studies and apply the data to clinical cases.  
1282 The protocols and the appendices herein are an attempt to address this goal and  
1283 complement existing literature. Outcome assessment of clinical patients is required for  
1284 determining the predictability of histologically determined prognostic measures (e.g.,  
1285 tumor classification, grade, etc.) Outcome assessment data need to be collected as  
1286 carefully and accurately as the techniques used to assess tumors.(Webster, Dennis et  
1287 al. 2011) Some criteria are subjective, clinical, and out of the realm of pathology.  
1288 Clinicians must carefully select and standardize clinical outcome measures to avoid  
1289 potential confounders. For example, reporting either disease- or progression-free  
1290 interval is preferable to median survival time, in order to avoid the confounding effect of  
1291 timing of euthanasia, which reflects individual biases present within owners and  
1292 clinicians. Pathologists play a critical role in accurately determining both progression-  
1293 and disease-free intervals by allowing definitive determination of whether the same  
1294 tumor recurred and/or metastasized given the appropriate tissue. Obtaining samples for  
1295 histopathology presents more of a challenge than non-invasive imaging modalities.  
1296 Although many advances have been made in this realm, microscopic examination of  
1297 tissues remains the gold-standard. Histopathologic assessment has advantages over  
1298 cytologic evaluation as more definitive information regarding tumor type can be gained  
1299 from histopathology. Spindle cell tumors pose a particular problem for cytologic  
1300 evaluation as it is difficult (impossible) to distinguish reactive fibroplasia (granulation  
1301 tissue) from sarcomas and difficult to specifically identify tumor type. If we want to know  
1302 if there is reactive fibroplasia or recurrent perivascular wall tumor at the original excision  
1303 site, histologic assessment is ideal. However, even with histopathology it is difficult to  
1304 differentiate these two processes and can be difficult to find tumor cells in re-excision  
1305 specimens. There is no standard means to evaluate these cases (clinically and  
1306 histologically) and in at least one study of STTs, presence or absence of tumor in re-

1307 excision specimens did not accurately predict recurrence.(Bacon, Dernell et al. 2007)  
1308 Future studies could include imaging modalities, and correlate outcome with the  
1309 presence of normal tissue at the margins of resected samples (eg. no spindle cells of  
1310 any type). The type of tumor being evaluated will influence the feasibility of visualizing  
1311 residual tumor cells in margin excisions as well as the concern for local recurrence.

1312 Genetic studies have shown human and animal breed susceptibilities to develop  
1313 specific tumor types and multiple tumors in the same host. We know that multiple  
1314 aggressive tumors can be present in the same dog, (Golden Retriever, Rottweiler,  
1315 Bernese Mountain dogs and others)(Cullen and Breen 2016) . Given this tendency, it is  
1316 essential to make a definitive diagnosis of tumors in metastatic sites. Combining  
1317 methodologies is ideal but practical considerations of costs and emotional factors  
1318 impact study results. Imaging can provide an alternative means to assess for suspected  
1319 metastatic lesions and can provide useful clinical information for patient management  
1320 but leaves a gap in outcome assessment studies provided no other confirmatory data is  
1321 available. Imaging cannot determine whether the tumor suspected of being in the lungs  
1322 is the same tumor as was excised previously. These methods to identify suspected  
1323 neoplasia are the methods of choice for clinical settings but not research models. It is  
1324 important to differentiate the information from a test being used to help treat one patient  
1325 or predict how a population of animals with the same tumor will react to that tumor type.  
1326 The latter will be applied to the former when we gather and analyze data carefully.  
1327 Histopathology remains the gold standard to develop ground truths if the tumor type is  
1328 the same. We can substitute other methods for histopathology, but the data should be  
1329 labelled suspected neoplasia/metastases (e.g., as determined by imaging or physical  
1330 exam) but not confirmed unless histopathology is used. In the future, molecular testing  
1331 of suspected tumor tissue may be superior to histopathology.

1332 Other appendices have detailed how to assess parameters used to evaluate a tumor,  
1333 recurrence, margins and metastases. In order to use morphologic diagnoses, margins,  
1334 LVI, MC, lymph node status, or CPATH to predict tumor behavior and/or to select  
1335 treatment options, oncologists must acquire sufficient outcome assessment information  
1336 to allow interpretation of tumor parameters. Knowing actual survival times of geriatric



1337 pets or including pets in which no treatments were performed provides control groups to  
1338 which treatments and outcomes can be compared. Determining the least invasive  
1339 means to characterize tumor behavior is ideal but cannot be accomplished without  
1340 adequate outcome assessment studies. Appendix 7 utilizes and expands upon  
1341 published guidelines for conduct and evaluation of prognostic studies(Webster, Dennis  
1342 et al. 2011) and for response assessment in canine solid tumors,(Nguyen, Thamm et al.  
1343 2015) citing specific information gained from studies of canine soft tissue sarcoma and  
1344 canine mast cell tumor.

1345 Standardized criteria, such as RECIST and RECIST 1.1(Therasse, Arbuck et al. 2000,  
1346 Schwartz, Seymour et al. 2016) should be used to document the patient's response to  
1347 treatment and progressive disease. The RECIST 1.1 criteria have been robustly  
1348 evaluated for use in human clinical trials and can be easily adapted to the evaluation of  
1349 veterinary patients. Pathologists, oncologists, surgeons, clinicians and students should  
1350 be familiar with the terms explained in these manuscripts which indicate response to  
1351 treatment and include Complete remission (CR), Partial response (PR), Progressive  
1352 disease (PD), Stable disease (SD) and Not evaluable (NE).(Nguyen, Thamm et al.  
1353 2015) Documented progression is needed in the cases of questionable lesions, or a  
1354 minimum size is required to determine whether neoplastic disease is present within a  
1355 lymph node. Additionally, there may be specific anatomical locations evaluated  
1356 depending on the tumor type. For example, prostate cancer may favor bone  
1357 metastases, pulmonary carcinoma in cats requires assessment of all digits, and  
1358 hemangiosarcoma is the most common metastatic tumor to the brain of dogs. Ideally,  
1359 imaging will be used in concert with biopsy or autopsy in order to confirm recurrence  
1360 and metastasis with the utmost accuracy.

1361 Metastasis should be subdivided into confirmed and suspected. Metastases determined  
1362 by imaging only should be labelled suspected. Histopathology is required to confirm  
1363 metastases are present and are of the same tumor type. The preferred methodology of  
1364 evaluation in humans, the CT scan, should be used if possible as it avoids some of the  
1365 technical problems associated with the use of radiographs, whereas ultrasound is not  
1366 an acceptable method of assessing disease state(Nguyen, Thamm et al. 2015) The use

1367 of functional imaging (PET scans) is increasingly common to better determine sites of  
1368 disease; however, it cannot be used for measuring purposes. Following these  
1369 standardized criteria will ensure that studies can be reproduced and compared between  
1370 institutions, resulting in more useful correlates of clinical data to prognostic information,  
1371 and ensuring progress in veterinary oncologic pathology.

1372 Euthanasia is a reality of veterinary medicine, and oncology studies that use pets must  
1373 carefully evaluate how decisions to euthanize influenced survival times. Reported  
1374 patient survival times are impacted by euthanasia which may be elected due to  
1375 perceived pet value, owner income, primary vs referral centers or other factors which do  
1376 not reflect tumor behavior. When patients are euthanized, clinicians should determine  
1377 and/or record the cause of death with as much accuracy as possible. If euthanasia is  
1378 due to an unrelated disease process, this must be noted. If euthanasia is caused by the  
1379 neoplasm being studied, and cachexia is present, then histologic confirmation of the  
1380 extent of the neoplastic disease helps verify clinical observations and reliability of study  
1381 conclusions. Oncology studies no longer include results of autopsy, the perceived value  
1382 of which seems to have hit a nadir. Permission to perform autopsies should be pursued  
1383 as autopsy greatly increases the confidence in results from the case. Studies should set  
1384 a goal of autopsies on at least 20% of the cases.

1385

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1410

1411 **Synoptic Reporting in Veterinary Medicine (See Appendix 8)**

1412

1413 Synoptic reporting (as opposed to the traditional narrative reporting) is a method for  
1414 reporting specific pieces of prognostically-relevant data in a discrete format in pathology  
1415 reports (Renshaw, Mena-Allauca et al. 2018). In human medicine, these have  
1416 progressed from individual efforts (Markel and Hirsch 1991) to being mandated by the  
1417 College of American Pathologists (CAP) for accreditation (College of American  
1418 Pathologists 2020). In general, a synoptic pathology report consists of data elements  
1419 and responses (see Figures S1, S2 Appendix 8, supplemental), which may be either  
1420 required or optional. For CAP purposes, the report must have all required core  
1421 components reported, all conditional core components reported when applicable, must  
1422 be listed with the element next to its associated response, and all elements and  
1423 responses must be on separate lines and in one place in a report. Synoptic reporting  
1424 has been shown to make pathology reports more readable to clinicians and patients  
1425 (Renshaw, Mena-Allauca et al. 2018), as well as making reports more likely to include  
1426 all data elements needed (Karim, van den Berg et al. 2008, Kang, Devine et al. 2009,  
1427 Srigley, McGowan et al. 2009, Messenger, McLeod et al. 2011) To develop an effective  
1428 synoptic report typically requires the efforts of pathologists and clinicians, who develop  
1429 the checklist of required and recommended items after reviewing the relevant literature  
1430 (Chamberlain, Wenckebach et al. 2000). Currently, there are two main groups  
1431 producing templates in human medicine, CAP and the International Collaboration on  
1432 Cancer Reporting (ICCR). Both require a committee of pathologists, oncologists, and  
1433 other interested representatives (e.g., World Health Organization working groups, etc.)  
1434 to develop a new protocol.

1435 A number of studies have found that synoptic reporting produces reports that are more  
1436 likely to contain all significant pieces of information than narrative reports. For  
1437 pancreatic tumors, 100% of synoptic reports had information about small vessel and  
1438 perineural invasion, compared to 66% and 84% of narrative reports, respectively (Gill,  
1439 Johns et al. 2009). In addition, the stage could be determined in 100% of synoptic

1440 reports compared to 56% of narrative reports. In a comparison of melanoma reports,  
1441 mitotic count, histologic subtype, predominant cell type, vascular and lymphatic  
1442 invasion, neurotropism, desmoplasia, and distance to the nearest margin were all  
1443 reported significantly more frequently in synoptic reports than narrative reports, both at  
1444 the teaching institution responsible for the study and the outside reports sent in to the  
1445 teaching institution for a second opinion(Karim, van den Berg et al. 2008).

1446 While full implementation of standardized reporting would allow for easy automated data  
1447 collection(Ellis and Srigley 2016), even simple implementations of synoptic reporting  
1448 can allow for significant automated information extraction. For example, if all deep  
1449 margins are listed as “DEEP MARGIN: <xx>mm” on a line by itself, it is comparatively  
1450 easy to extract all margins from reports using standard text search and manipulation  
1451 tools (e.g., grep, cut, etc.). Not only can this improve retrospective studies, but can also  
1452 provide valuable clinical information, as extracted information can be compared  
1453 between services, clinicians, and other variables to determine if these influence patient  
1454 outcomes.

1455 From the beginning of synoptic reporting, clinicians have reported increased satisfaction  
1456 with synoptic vs. narrative reports (Markel and Hirsch 1991). A study of treating  
1457 physicians and pathologists in Canada found that both groups found synoptic reports  
1458 easier to find information in, facilitate a consistent approach to interpretation of  
1459 diagnostic and prognostic factors, and provide higher overall satisfaction (Lankshear,  
1460 Srigley et al. 2013). While pathologists felt that reports took approximately 25-50%  
1461 longer to complete, treating physicians did not notice a difference in the length of time it  
1462 took pathology reports to be completed.

1463 The major problem in veterinary medicine is a lack of knowledge about factors involved  
1464 in prognosis. As discussed in the other appendices in this document, there is little  
1465 standardization of methods used in determining prognostic factors. There are also no  
1466 standards for terminology, such as immunohistochemical findings (e.g., “positive” vs.  
1467 “immunoreactive” vs. “present”), which hinders design of standardized reports. Another  
1468 issue for many pathologists, particularly in academia, is the effect switching to synoptic  
1469 reports would have on resident training. Given the necessity of writing descriptions for

1470 boards and the lack of universal adoption of synoptic reporting, residents still require  
1471 significant experience in writing narrative reports. This can be mitigated by requiring  
1472 narrative reports in other resident educational settings (such as rounds) to provide  
1473 practice in writing narrative reports for neoplasms.

1474 Many pathologists are concerned about increased time to finish reports with synoptic  
1475 reporting, including physicians (Lankshear, Srigley et al. 2013); however, when synoptic  
1476 reports have been implemented many of these concerns have been deemed  
1477 technology related rather than issues with the reporting format. As with many new  
1478 processes, we assume that once the pathologists become familiarized with the new  
1479 format, there will be a decrease in time to write these types of reports. A standardized  
1480 formatted template will be created and added to the website we propose. In veterinary  
1481 medicine, no current laboratory information management system (LIMS) can use  
1482 synoptic reporting, which may seem like an obstacle to implementation of synoptic  
1483 reporting. However, any word processor can be used to implement synoptic reporting  
1484 without specialized software (Ellis and Srigley 2016); all that is required is to type the  
1485 data element, a separator (such as TAB), and the response. Templates can be saved  
1486 containing required and optional data elements, making it easier for pathologists to fill  
1487 out reports quickly. These can then be copied and pasted into any LIMS or word  
1488 processor for subsequent reporting.

1489 Finally, another major obstacle to implementation of synoptic reporting is a lack of  
1490 awareness of synoptic reporting and its benefits in veterinary medicine. Establishing  
1491 working groups with pathologists and oncologists to develop guidelines for specific  
1492 neoplasms would help promote awareness and develop reporting checklists that would  
1493 benefit both pathologists and treating clinicians.

1494 The next step beyond synoptic reporting is standardized reporting, that is, having a  
1495 standardized, specific set of responses for each required question (Srigley, McGowan et  
1496 al. 2009). Ultimately, this can lead to automated staging and grading, as well as  
1497 improving data harvesting for future research and clinical applications. The addition of  
1498 free text fields associated with standardized options would allow for customization of  
1499 reports while retaining standardization for further applications.

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- 1530

**Synoptic Report**

MASS SIZE:	3 cm x 2 cm x 2 cm Gross measurement
BIOPSY TYPE:	Excisional
LOCATION:	Forelimb proximal to elbow
ASSESSMENT METHOD:	Manual light microscopy with glass slides
HISTOLOGIC TYPE:	Nerve sheath tumor
DEEPEST LAYER INFILTRATED:	Dermis Via histology
DIFFERENTIATION SCORE:	2
MITOTIC COUNT:	12 per 2.37mm <sup>2</sup>
NECROSIS:	11-50%
TOTAL SCORE:	5
HISTOLOGIC GRADE:	2 Kuntz system
LYMPHOVASCULAR INVASION:	None
METASTASIS:	None
MARGINS INKED:	By laboratory
MARGIN TYPE:	Radial
DEEP MARGIN:	Complete
DEEP MARGIN HTFD:	3 mm
LATERAL MARGIN:	Complete
LATERAL MARGIN HTFD:	6 mm

**Narrative Report**

In one transverse and two longitudinal sections (from a 3 x 2 x 2cm mass from the left forelimb, per submitter), the dermis is disrupted by a highly cellular, infiltrative, unencapsulated mass. The mass is composed of cells forming bundles and whorls surrounding empty capillaries. The cells have indistinct borders and eosinophilic cytoplasm. The nuclei are medium to large and fusiform, with finely stippled chromatin. Mitoses average 12 per ten 400x fields (2.37mm<sup>2</sup>). The central 30% of the mass is necrotic. The mass is separated from the deep and lateral sample margins by 3mm and 6mm, respectively.

**DIAGNOSIS:**

Nerve sheath tumor, grade II, left forelimb

1531

1532 Figure 10: Comparison of synoptic and narrative reports. The same information in each

1533 report is in the same color.

1534



**1535 Skin and Subcutaneous Soft Tissue Tumors (STT/STS) (See Protocol 1)**

1536 This protocol is intended for use with soft tissue tumors arising in the skin and  
1537 subcutaneous tissues which are predominantly of mesenchymal tissue origin and which  
1538 are commonly referred to as soft tissue sarcomas (STS).(Bostock and Dye 1980, Kuntz,  
1539 Dernell et al. 1997, McSporran 2009, Roccabianca, Schulman et al. 2020) Modifying a  
1540 name generally meets with resistance and lack of unanimity. The term sarcoma  
1541 suggests the group of neoplasms are aggressive (malignant), however present outcome  
1542 assessment data does not indicate that is the case.(Bostock and Dye 1980, Kuntz,  
1543 Dernell et al. 1997, McSporran 2009, Roccabianca, Schulman et al. 2020) Thus, it is  
1544 proposed to remove sarcoma from the acronym. These neoplasms are predominantly  
1545 mesenchymal, however, a subset (namely nerve sheath tumors) are not solely derived  
1546 from the mesoderm, therefore, soft tissue mesenchymal tumor is not entirely accurate.  
1547 These neoplasms can be accurately encompassed by the term soft tissue tumors (STT)  
1548 (which is admittedly vague), however, ensures that more users of this term will be  
1549 satisfied. The purpose of this protocol is to provide standards for accruing data so that,  
1550 over time, large data sets with comparable information can be evaluated to enable  
1551 meaningful conclusions and accurate prognostic information.

1552 The term STT/STS encompasses a wide range of benign and malignant tumor types in  
1553 humans (Byerly S, Chopra S, Nassif NA et al, 2016) The different types are much more  
1554 limited in animals and, although the veterinary terminology and various grading  
1555 schemes have, in many instances, been borrowed from the human literature, the types  
1556 of neoplasms which commonly comprise soft tissue tumors in humans are very different  
1557 from the tumor types typically encountered in animals. This is exemplified by  
1558 liposarcomas, which are common in humans and rare in dogs, and perivascular wall  
1559 tumors (PWT), very common in dogs, are rare in humans. Furthermore, STS in humans  
1560 have extensive molecular profiles to help subtype them, which is not established for  
1561 canine tumors. The common denominators between species appears to be an origin in  
1562 non-epithelial, extraskelatal soft tissues exclusive of hematopoietic system. (Bostock  
1563 and Dye 1980, Trojani, Contesso et al. 1984, Kuntz, Dernell et al. 1997, Coindre 2006,  
1564 McSporran 2009, Dennis, McSporran et al. 2011, Roccabianca, Schulman et al. 2020)

1565 This protocol is intended for use with the following types of tumors: Perivascular wall  
1566 tumors (PWT), nerve sheath tumors (NST), fibrosarcoma, myxosarcoma,  
1567 leiomyosarcoma, liposarcoma, rhabdomyosarcoma or unclassified spindle cell  
1568 tumor/sarcoma arising in the dermis or subcutis. PWT and NST are the most common  
1569 types of STT/STS and their biological behavior is primarily indolent.(Roccabianca,  
1570 Schulman et al. 2020 )The effect of grouping of disparate tumors within the same  
1571 grading scheme needs to be compared to grading tumors segmented into specific  
1572 histological diagnoses so that important predictive parameters may be determined.

1573 The current scheme used for grading dog STT/STS is patterned after Trojani's grading  
1574 of human STS.(Trojani, Contesso et al. 1984) Unlike the human grading scheme,  
1575 however, the studies of dog STT/STS only evaluated three histological features. Some  
1576 criteria, such as determination of the percentage of necrosis via gross and/or  
1577 histological criteria, are poorly defined in the human literature and were not clarified in  
1578 the veterinary manuscripts.(Bostock and Dye 1980, Kuntz, Dernell et al. 1997,  
1579 McSporran 2009) Percent necrosis for human tumors was determined by estimating the  
1580 amount seen grossly and histologically (see Appendix 4).(Trojani, Contesso et al. 1984,  
1581 Coindre 2006, Rubin, Cooper et al. 2010, Nguyen, Thamm et al. 2015) There are a  
1582 number of distinctions between the grading systems used for human tumors and how  
1583 the they are applied to dogs, which have not been addressed in the canine  
1584 papers;(Bostock and Dye 1980, Trojani, Contesso et al. 1984, Kuntz, Dernell et al.  
1585 1997, Coindre 2006, McSporran 2009) in particular, the need to determine histological  
1586 tumor type and confirmation of the diagnosis of sarcoma *prior* to applying the human  
1587 grading systems. Four additional histological features evaluated by Trojani but not  
1588 found useful for human tumors were not assessed in the dog STT/STS grading studies.  
1589 Our existing scheme needs to be broadened to determine if parameters originally  
1590 rejected for human STS may, in fact, be predictive in dogs. The methods described to  
1591 assign scores for necrosis, MC and differentiation for canine tumors are not detailed  
1592 enough that others can replicate them, and the number of dogs reported with high-  
1593 grade STT/STS that have outcome assessments is small. These studies need to be  
1594 repeated with additional parameters evaluated, more detailed description of methods  
1595 and greater case numbers paired with standardized outcome assessments. The

1596 protocol in this appendix provides details of the histological findings that should be  
1597 noted in STT/STS which will enable more thorough assessment of these tumors and  
1598 should provide a database for performance of studies and validation of grading  
1599 schemes.

1600 For any proposed veterinary tumor grading system, the tumor type should be  
1601 designated as precisely as possible and the criteria used to designate that diagnosis be  
1602 provided (H&E, IHC etc). Each graded element must be clearly defined. For instance,  
1603 the means to assess percent necrosis (gross, histology, both; Appendix 4) must be  
1604 clarified if this is an element of a grading system and others are expected to duplicate  
1605 the method.(Kuntz, Dernell et al. 1997) Histologic classification of some types of  
1606 STT/STS is difficult. A particular conundrum is differentiating PWT from NST.  
1607 Histological features characteristic of PWT and NST have been described, but there is  
1608 overlap of histological patterns found in these two tumor types(Avallone, Helmbold et al.  
1609 2007, Suzuki, Uchida et al. 2014, Loures, Conceição et al. 2019, Vučićević, Marinković  
1610 et al. 2019, Avallone, Stefanello et al. 2020, Roccabianca, Schulman et al. 2020) which  
1611 can complicate definitive diagnosis in routinely stained sections. How specific can, or  
1612 should our diagnoses be from HE slides and how does this influence differentiation  
1613 scores used to grade these tumors? Examples: Should PWT be subtyped, and similarly  
1614 as NST is not just one tumor, should neurofibroma, Schwannoma and malignant NST  
1615 be identified? Classification of some tumors, including some cases of PWT, may  
1616 require IHC or other ancillary tests. In veterinary medicine, the costs for these tests are  
1617 incurred by owners and, if the tests are declined, it is unreasonable to expect a precise  
1618 classification of some of these tumors with H&E. These practical factors influence our  
1619 diagnoses and grading systems.

1620 Present canine studies have not determined if identifying tumor type is predictive of  
1621 tumor behavior. Until we use a grading system for specific tumor types as well as for the  
1622 entire group of STT/STS, we will not know which approach is more predictive. A  
1623 grading scheme that can be applied to any tumor within the STT/STS group is easier to  
1624 apply then requiring identification of the specific tumor type before grading, particularly  
1625 in instances in which a definitive diagnosis cannot be made with evaluation of routinely

1626 stained tissue sections. However, future studies should validate if this is “best practice”.  
1627 On the one hand, identifying the precise tumor type may have prognostic information  
1628 unrelated to a specific grade and, on the other hand, perhaps all tumors within either the  
1629 entire STT/STS group or within specified subsets of the group (for instance PWT/NST)  
1630 may behave according to assigned grades. For instance, group PWT and NST  
1631 together, based on H&E histologic morphology and determine outcome and determine if  
1632 there is a difference in outcome assessment if these two tumor types are evaluated  
1633 separately. Identification of these two tumor types may involve IHC or electron  
1634 microscopy. If the biological behavior of these two tumors was such that distinguishing  
1635 them at the H&E level was not needed that would have practical use for a diagnostic  
1636 pathologist and oncologist. The only means to determine the prognostic utility of  
1637 grouping or separating tumor types within the STT/STS category is to perform studies  
1638 which evaluate outcomes related to the STT/STS group as a whole and ALSO evaluate  
1639 outcomes in relation to specific histologic type of tumors. Studies must have sufficient  
1640 numbers of animals within each tumor grade to generate statistically significant findings.  
1641 This latter issue will be a problem for uncommon tumors, such as liposarcoma, for  
1642 which it may be problematic to find enough high-grade tumors with accurate outcome  
1643 assessments, but using criteria in which two tumor types (ie, PWT and NST) comprise  
1644 more than 80% of the cases to predict how uncommon tumors behave needs to be  
1645 validated.

1646 Future considerations should address existing and new grading systems for STT/STS  
1647 (see protocol 1). The present grading system should be followed with methods  
1648 described in sufficient detail to permit other investigators to duplicate the methods and  
1649 the scoring systems. Consideration should be given to assessment of weighted scores  
1650 for parameters, such as differentiation or mitotic count, in determining grade and  
1651 correlation with outcome assessment. Additional histological features should be  
1652 evaluated for their prognostic utility, for instance, tumor cellularity, presence of atypical  
1653 nuclei or multinucleated giant cells and presence of lymphovascular invasion (see  
1654 Appendix 3). The benefit of applying a new, better-detailed scoring system for  
1655 histological differentiation should be assessed as this is the most subjective parameter

1656 in human tumors and likely canine tumors. The use of a defined area in mm<sup>2</sup> should be  
 1657 applied to all parameters enumerated in a grading system. New grading systems  
 1658 should be compared to older systems, and there must be sufficient numbers of animals  
 1659 in each tumor grade to enable interpretation of results. Studies should be initiated to  
 1660 assess the criteria for diagnosis of NST and PWT and the reproducibility of the criteria.  
 1661 Finally, the use of computational pathology and molecular profiling should be explored  
 1662 in determining grades and outcomes of STT/STS.

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