Cutaneous Canine Mast Cell Tumors: Is Cytology Up to Grade?
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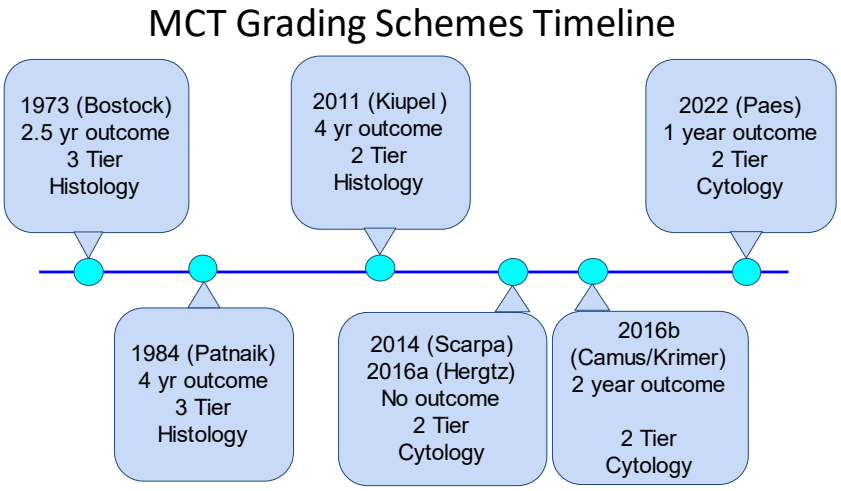
The goals of this talk are to:

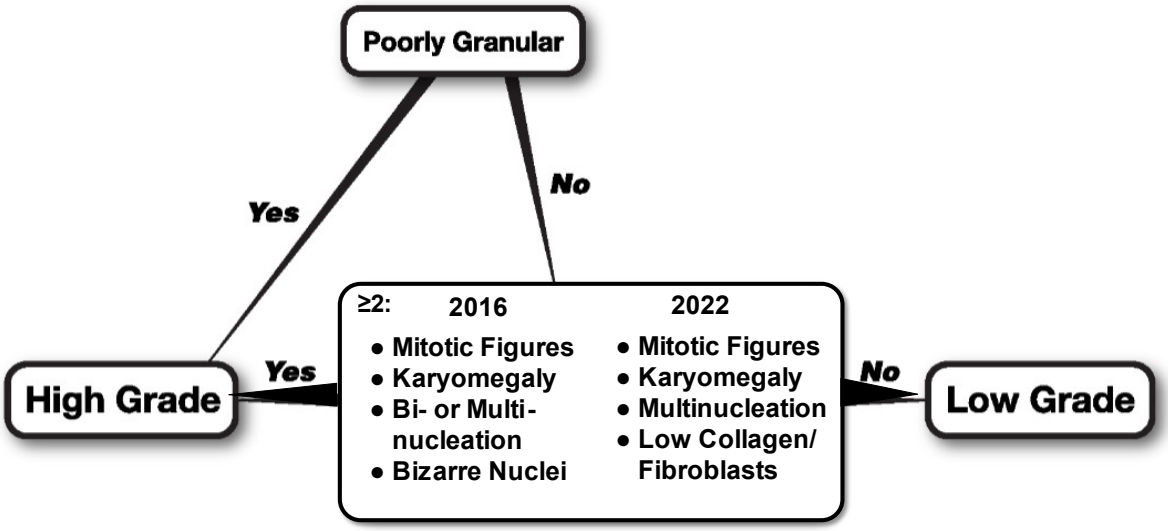
- Understand the different cytologic and histologic grading systems
- Be able to apply the criteria for cytologic grading
- Understand the limitations of cytologic and histologic grading
- Inspire cytologists to consider grading canine cutaneous mast cell tumors
- Inspire clinical and anatomic pathologists to work together for prognostication of mast cell tumors

Histology has a long tradition of grading neoplasms and inflammatory disorders; however, this concept does not exist with cytology specimens (with the possible exception of estrus staging on vaginal swabs). Due to its reliance solely on cytologic criteria of individual mast cells, the 2011 proposal and current adoption of a two-tier histology grading system for canine cutaneous mast cell tumors (Kiupel et al, 2011) has opened the door to this possibility rather than the previously standard 3-tier system (Patnaik et al, 1984). Recent studies on the direct application of the 2011 two-tier histologic system to cytology specimens yielded mixed results (Scarpa et al 2014, Hergtz et al, 2016) but two novel cytology grading systems were proposed in 2016 and 2022 (Camus et al 2016, and Paes et al 2022). Both the latter have high sensitivity with few false negatives (histologic high-grade tumors that are low grade on cytology) and a 2021 consensus statement by the Oncology-Pathology Working Group indicates cytology grading is promising (Berlato et al, 2021). A timeline of canine cutaneous mast cell tumor grading schemes is outlined below.

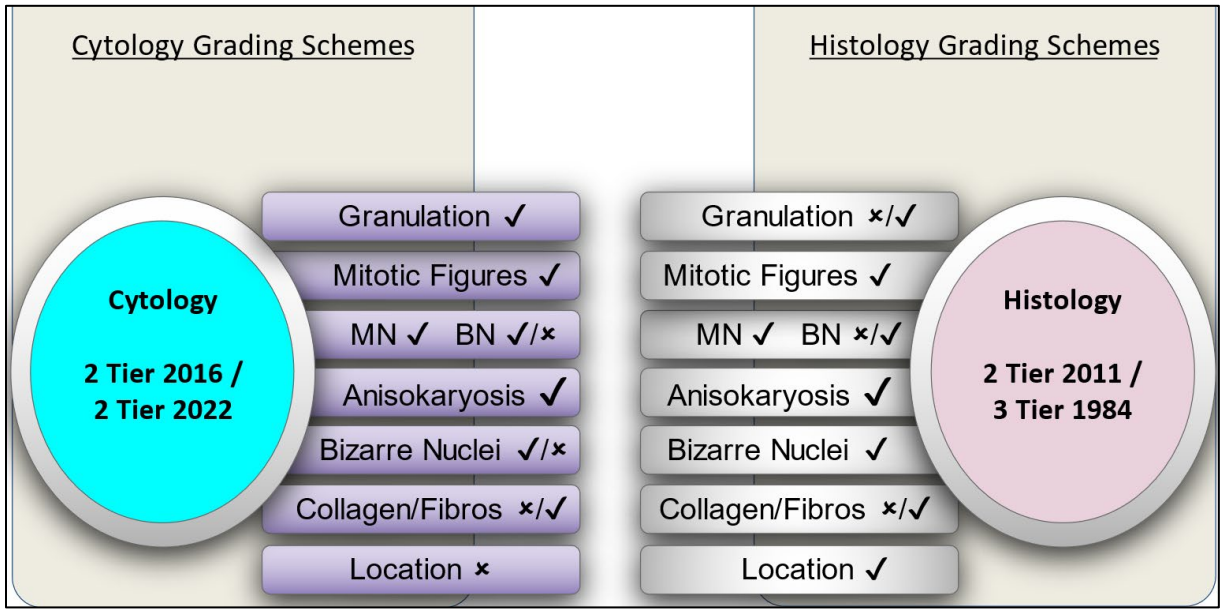


It is important to emphasize that histology grading systems are not perfect, nor should they be considered a gold standard. Cellular morphology is a manifestation of genetic abnormalities, and it is these mutations combined with host response that predict recurrence and survival, the ultimate gold standard. So why should cytologists attempt to create a cutaneous mast cell tumor cytology grading system? There are several reasons. Non-surgical treatment is available and though not ideal, it is an option for difficult surgical sites (perianal, perinasal, etc..) and patients with a poor anesthetic risk. Preliminary cytology grading can encourage more aggressive clinical staging such as aspirating nodes, marrow, or internal organs while patient is already under anesthesia for mass removal. Finally, the best overall prognostication for survival may in fact be a combination of cytology & histology factors. Cytology would likely be used as a screening test for these tumors, where a **high sensitivity is most desirable**, (i.e. there are fewer false negatives so high grade cases are less likely to be missed), and this should be the deciding factor rather than overall agreement with histologic grade (i.e. kappa).

The proposed cytologic grading systems are summarized below. In both, poorly granular tumors are considered high grade. In both, the presence of two or more other factors also classifies a tumor as high grade. Both use mitotic figures, karyomegaly/anisokaryosis, and multinucleation as high grade factors. The 2016 system adds bizarre nuclei or binucleated cells as a high grade factor, while the 2022 system uses the absence of collagen/fibroblasts as a high grade factor. Compared to the histology grading on the same tumor, the 2016 system had a sensitivity of 88% with a false negatives of 1.6%, and false positive rate of 31.8%, while the 2022 system had a sensitivity of 89.8%, false negative rate of 10.2%, and false positive rate of 45.5%.



A comparison of prognostic criteria used in the cytology and histology grading schemes is summarized below.



Granulation is the most significant cytologic feature correlated with grade and outcome in both the 2016 and 2022 cytology grading systems, is part of the 1984 3-tier histologic system, but is not considered in the 2011 histologic system. Poor cytologic granulation alone classifies the tumor as high grade. However, grading should only be performed on Romanowsky-type methanolic stains as aqueous rapid cytologic stains have variable

cytoplasmic granule staining. It is possible that abundant granules may mask nuclear criteria in both cytology and histology, leading to potential false negatives. Images of highly granular, mixed granularity, and poorly granular mast cell tumors are provided below.

Mitotic figures can be detected on cytology, but a mitotic count (MC) cannot be performed. With histologic evaluation, the MC is a strong predictor of tumor grade. Standardization of the counting area in histology is important (2.37 mm^2). The area with the highest concentration of mitotic figures (hot-spot) should be counted but there is variation within the sample and between pathologists. Computer assistance may be a helpful aid for pathologists by improving accuracy and reproducibility of the mitotic count (Bertram, 2022). With cytologic evaluation, the presence of any mitotic figure counts towards the grade and is an independent factor for survival.

Binucleation and Multinucleation are treated differently in the different cytology and histology grading systems. Binucleation was found to be significant in the 2016 cytology grading system, but not the 2022 cytology grading system, and is not used in histology. Multinucleation is a factor for high grade tumors in all systems. The 2 tier 2011 histology scheme uses multinucleation (cells with 3 or more nuclei) as a criterion for high grade cutaneous mast cell tumors (Kiupel, 2011). Binucleation is not a criterion in the 2 tier 2011 scheme. The 3 tier 1984 scheme mentions binucleated cells in Grade II tumors, common binucleated cells in Grade III tumors, and scattered multinucleated cells in Grade III tumors. Preliminary studies using computer assistance to identify binucleated and multinucleated mast cells in whole slide images (WSI) found a positive correlation with mitotic density, but a high inter-rater variability in identifying binucleated and multinucleated mast cells between pathologists (Bertram 2021).

Anisokaryosis (aka karyomegaly): The evaluation of mast cell nuclei was defined in both cytologic papers as a variation of nuclear size greater than 50%, but whether it is equivalent to a two-fold change in size used in the 2011 histology system is uncertain given the different preparation techniques (fixation vs smears) and potential angle of cuts on ovoid nuclei in three dimensions. The 2 tier 2011 scheme uses objective criteria for anisokaryosis (10% of the cells with at least 2-fold anisokaryosis), however, scoring of nuclear pleomorphism has poor agreement between pathologists for other tumors (Casanova, 2021). Automated image analysis and morphometry of digitized slides increases the reproducibility and accuracy when evaluating prognostic parameters, and may be useful when incorporated into grading schemes (Casanova 2021, Strefezzi 2009), but should also be compared with pathologist estimates.

Nuclear Pleomorphism is a variably defined parameter in histology grading, included in the 2016 cytology grading system, but not evaluated in the 2022 cytology grading systems. In the cytology system, pleomorphic nuclei are defined as non-round and non-oval nuclei. In the histology systems, this parameter is defined as "highly atypical nuclei with indentations, segmentation and irregular shape or spindle shaped." We propose the more accurate term **Anisokaryoschema** instead of "nuclear pleomorphism" or "bizarre nuclei". The Greek etymology of this term are *aniso*- not same, *karyo* – nucleus, *schema* – shape. Once again, potential angle of cuts on ovoid nuclei in three dimensions or artifact caused by fixation versus flattening of nuclei on cytology preparations may influence the utility of this parameter on cytology. In the 2016 cytology grading study, it correlated most poorly with histologic grade but still had a small but significant association with patient outcome and survival.

Collagen & Fibroblasts: The 2022 cytologic grading system uses the absence of collagen and fibroblasts to indicate a potential high-grade tumor. In the original data presented in the 2016 cytology system, the presence of collagen/fibrocytes was significant but because it was a negative result, it was not included in the grading system. A re-evaluation of the 2016 data set by applying the 2022 cytologic grading system yielded 82%

sensitivity, 97% specificity, 3.0% false positives, and 18% false negatives (Krimer, in press). While dermal collagen is described in the 1984 3 tier scheme, it is not a criterion used often for grading amongst anatomic pathologists. Grade I tumors have mature collagen fibers with minimal edema, Grade II tumors have some areas of thick collagenous stroma with hyalinization, and Grade III tumors have fibrovascular or thick collagenous stroma with areas of hyalinization. Interestingly, this means the histologic scheme uses collagen and fibrosis in the opposite way as cytology (history lower grades have less collagen, cytology lower grades have more collagen).

Cytology Limitations: Cytologic grading does have limitations inherent to the methodology. There are some potentially important factors for patient survival that cannot be evaluated on cytology, specifically the location of the tumor, invasion, and surgical margins. There is controversy regarding the prognosis for subcutaneous versus cutaneous mast cell tumors, therefore the impact to cytologic grading is uncertain.

Location: The 1984 3 tier system defines low grade (Grade I) dermal mast cell tumors as being confined to the dermis and interfollicular spaces. Grade II tumors infiltrate into the deep dermis and subcutaneous tissues, while Grade III tumors are present in the subcutaneous and deep tissues. Since subcutaneous tumors would automatically be graded as III using this scheme, subcutaneous mast cell tumors are currently excluded from 3-tier or 2-tier grading systems. Subcutaneous location is defined as: “a location within the subcutaneous tissue and no invasion of the dermis.” (Thompson et al. 2011). Although subcutaneous MCTs are considered to have a less aggressive clinical course, the biological behavior of cutaneous and subcutaneous MCT are often not directly compared (deNardi, 2022) with no correlation to outcome in one study (Horta 2018). Tumor depth (degree of infiltration) cannot be appreciated with cytology. In some studies, tumor depth was not of prognostic significance for dogs with cutaneous MCTs (Kiupel 2005, 2011 papers). In other papers, asymmetric invasion was associated with incomplete excision (Russell et al. 2017). Some anatomic locations are reported to confer a worse prognosis (mucosa, scrotum, prepuce, perineum and vulva).

Margins: Margins have been shown as an independent prognostic factor for survival. Peripheral margins of 1-2 cm and a deep safety margin of at least 4mm including a fascial plane are recommended for grade 1 and 2 tumors, and up to 4 cm in diameter to provide effective local control with low recurrence rates (deNardi 2022, Donnelly 2015, Schultheiss 2011). However, multiple studies have shown that grade is also an important predictor of recurrence; low grade tumors may not recur even when margins are not clear of neoplastic cells or are classified as narrow, while high grade mast cell tumors can recur even when margins are free of neoplastic cells (deNardi 2022, Scarpa 2012, Sledge 2016, Donnelly 2015). These findings should be further validated and investigated in future studies, as the outcomes in dogs with grade II MCT vary between studies and accurate prediction of the risks of local recurrence have proven challenging to achieve (deNardi 2022).

Not Useful: Factors that have been investigated but were found not useful in cytology grading studies include proportion of eosinophils or neutrophils and presence of necrosis. In the 2022 cytology study, cell clustering was also evaluated but not found valuable to predict outcome. In one study reviewing multiple histologic parameters and correlating with clinical outcome, no correlation was found with tumor extent (subcutaneous, dermal, ulcerated, in muscle), presence of neoplastic cells at the surgical margin, presence/intensity of edema, necrosis, hemorrhage, collagenolysis, cystic apocrine glands, desmoplasia, eosinophilic infiltrate or numbers of binucleate cells (Horta, 2018).

Inter-Operator Variability (IOV): Grading systems are only useful if repeatable, reliable, and realistic for a pathologist to perform in reasonable time frame. An important factor in the implementation of any grading system is inter-operator variability, which is similar to the concept of Coefficient of Variation (CV). This was evaluated in the 2016 cytology paper, but not the 2022 cytology grading study. Cytology grading is new and did

not have established standards; the IOV may improve over time but was higher than the 1984 3-tier histology system and approached the 2011 histology system.

Method	Grading System	IOV
Histology	**1984**	**62.1-72.9%**
Histology	**2011**	**77.0-96.8%**
Cytology	**2016**	**73.6% - 81.8%**
Cytology	**2022**	**not evaluated**

Combining Histology and Cytology: In the 2016 cytology grading study, all histologic and cytologic factors were combined to determine those most important to patient survival. These were a combination of patient demographics, two histologic factors (margins and mitotic count), and a cytologic factor (multinucleation). Cytology and histology are complementary diagnostic methods, and future studies should evaluate them concurrently to develop a database for further studies.

Risk Factor	Cox Hazard Ratio (95% CI)	p-value**
Age	**1.4 (1.1-1.8)**	**0.0117**
Surgical margins (narrow vs wide)	**13.4 (2.1-86.6)**	**0.0065**
Histology: Mitotic figures	**42.8 (8.4-216.7)**	**<0.0001**
Cytology: Multinucleation	**48.5 (7.2-327.1)**	**<0.0001**

Oncology-Pathology Working Group Statement (Berlato et al 2021)

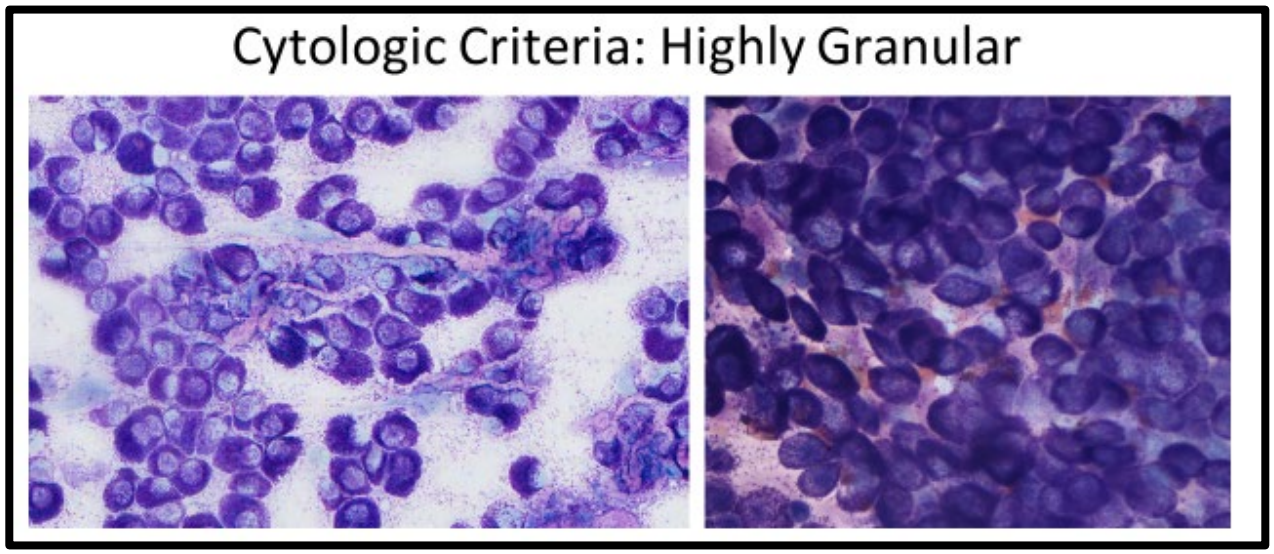
"Cytological grading is promising. This grading system should be further validated but may provide valuable preoperative information. A cytological diagnosis of low grade MCT correlates well with histologic grading and clinical outcome. However, a diagnosis of high-grade MCT should be received with caution if only based on 2 morphological criteria, mainly if anisokaryosis and nuclear pleomorphism is one of them, because of the risk of false positives when compared to histological grading."

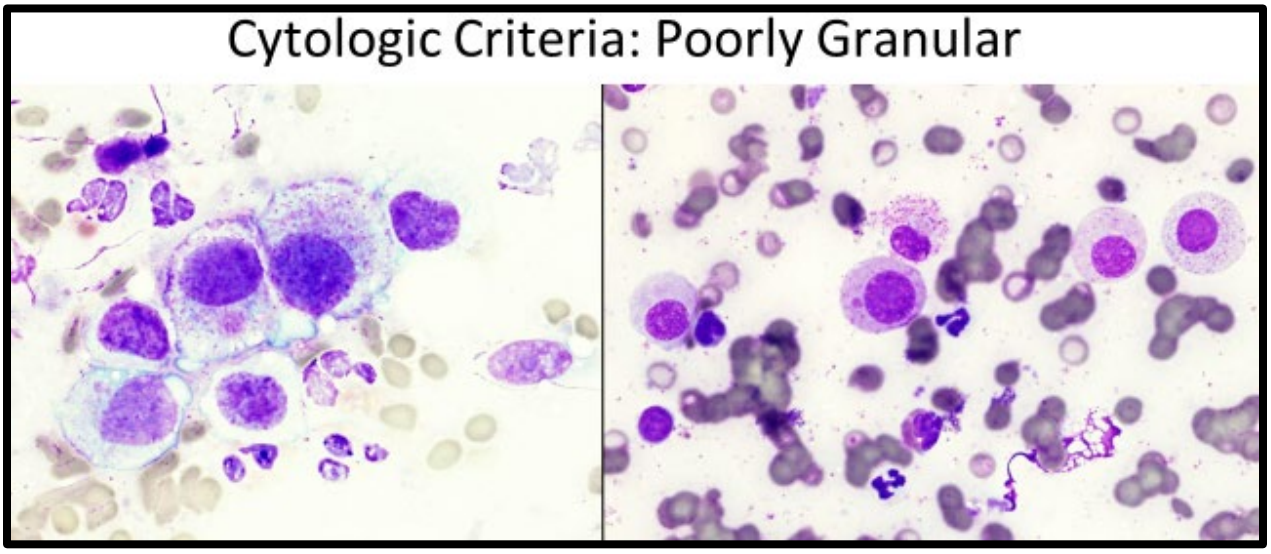
Final Thoughts

- Cytology grading systems have a very high sensitivity to detect high grade tumors; important for a screening test
- Cytology cannot determine location, invasion, or margins
- Cytology and Histology may complement each other for providing prognostic information
- Grade is only part or the picture: Staging critical for prognosis
- True gold standard is survival/outcome, not histology grade

Our recommendation is to **report both 2016 and 2022 cytologic grades on methanolic stains**, providing detailed information on specific grading criteria, to allow for future studies on survival and ongoing refinement of canine cutaneous mast cell tumor prognostication using both cytologic and histologic criteria.

IMAGES


Cytologic Criteria: Highly Granular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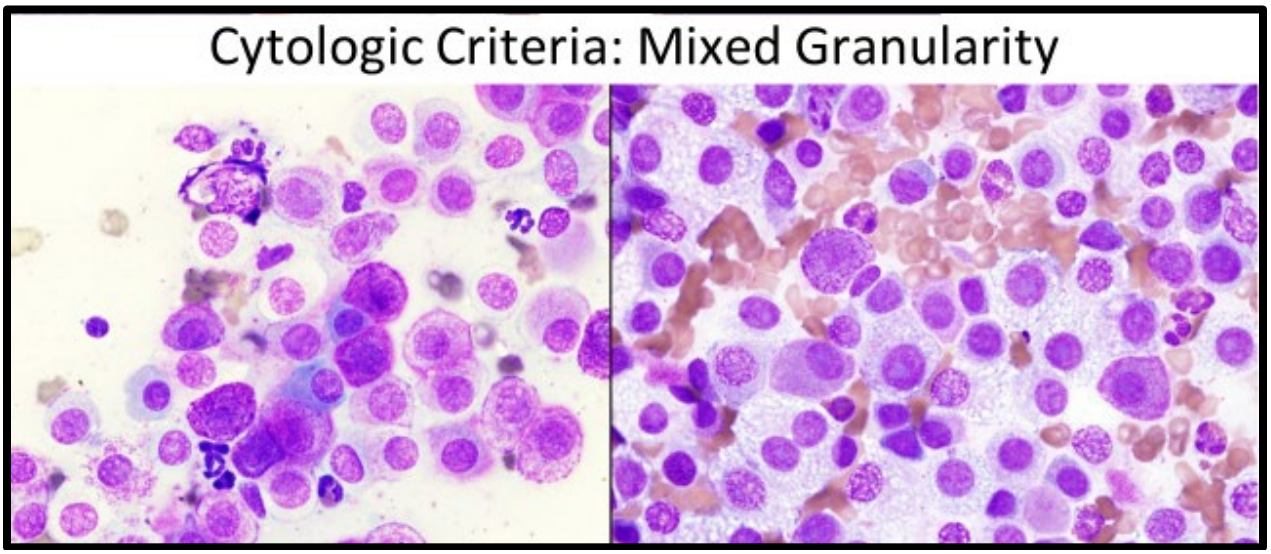
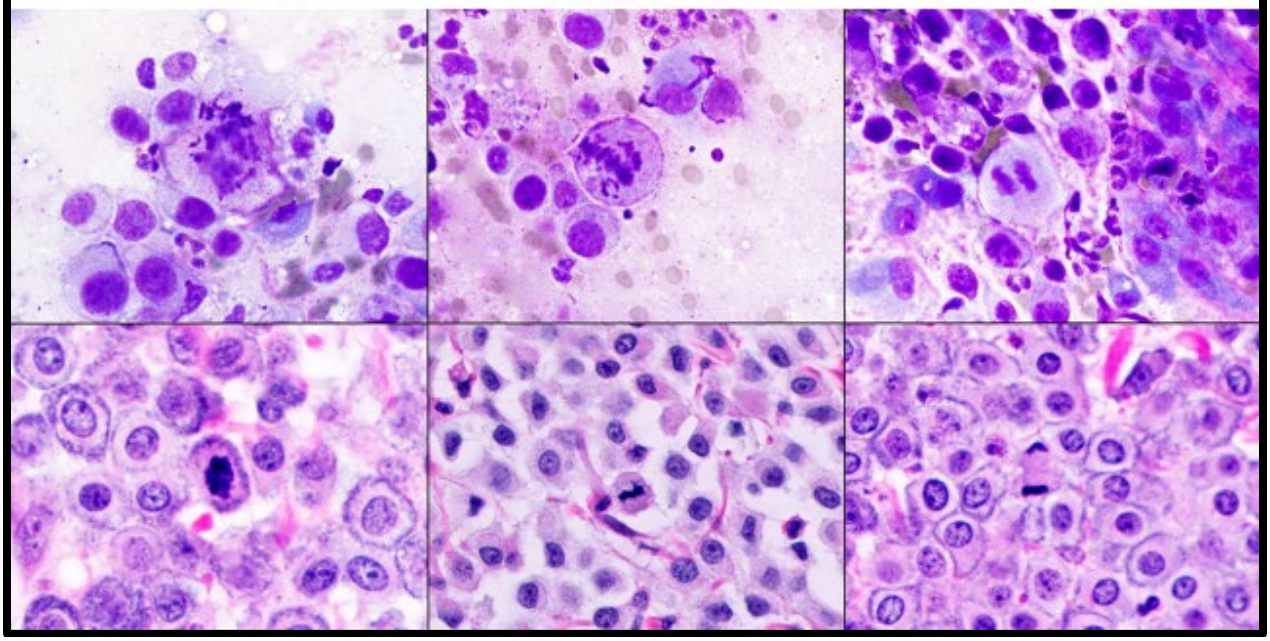
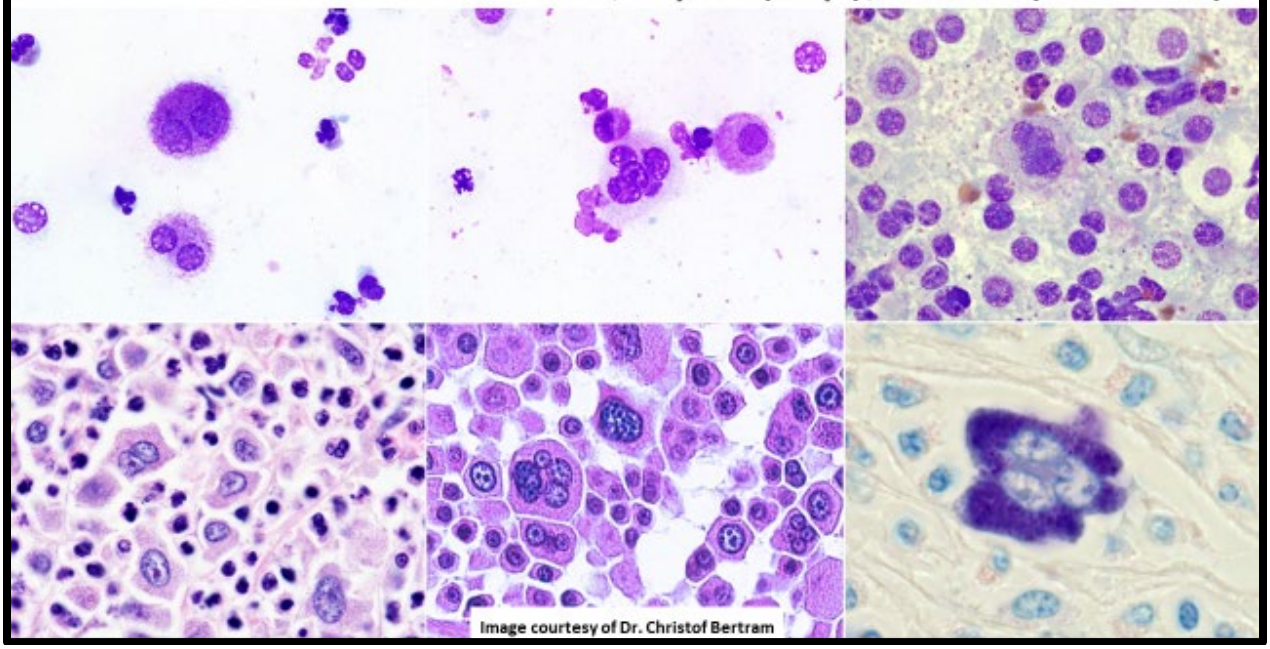
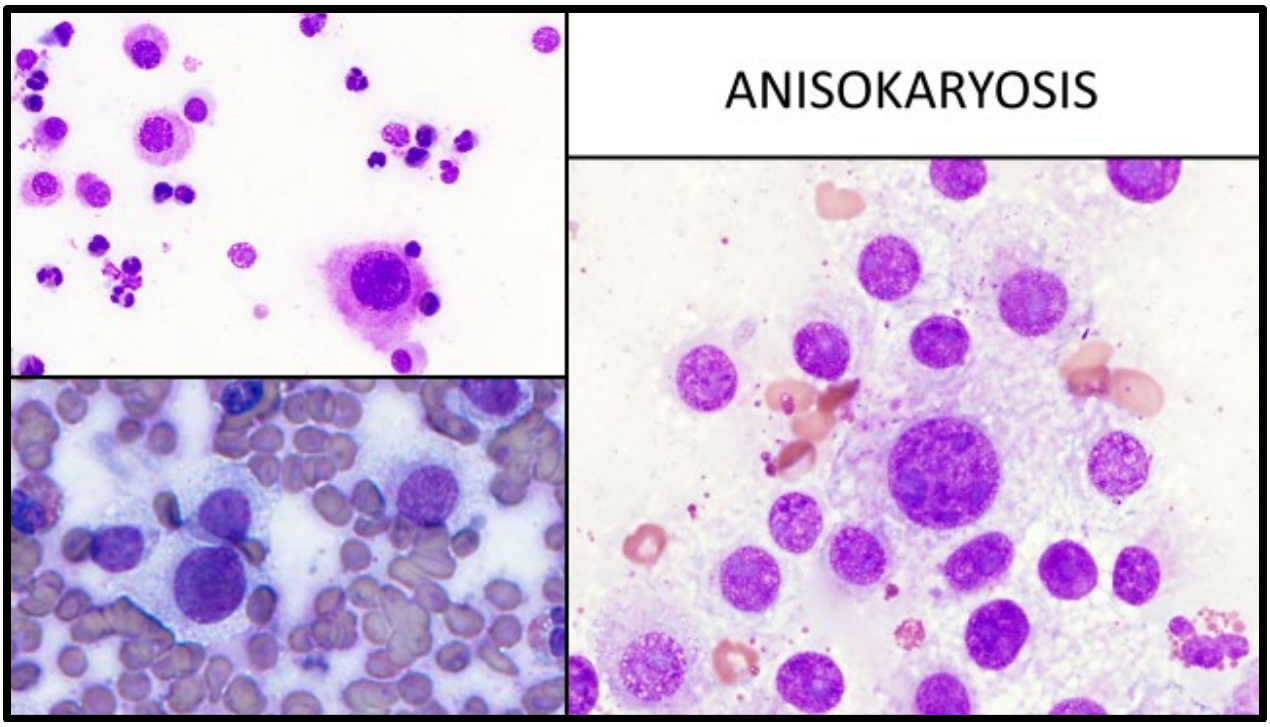

Cytologic Criteria: Mixed Granularity

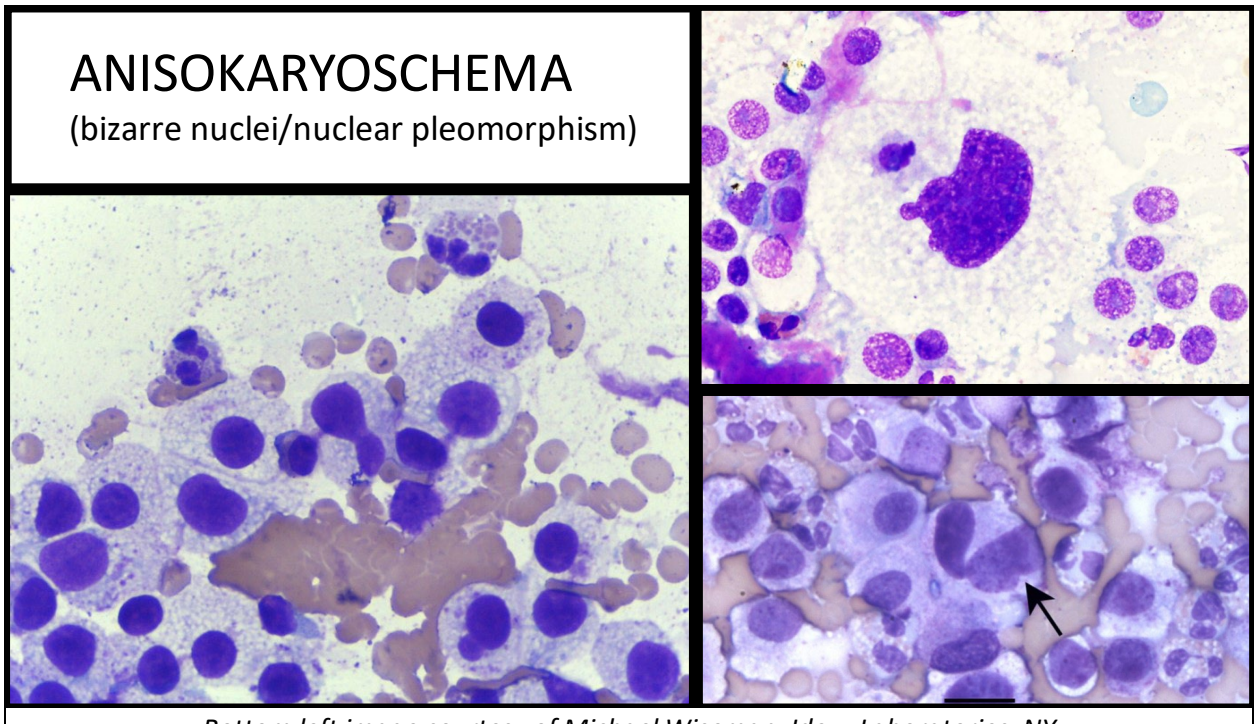
Mitotic Figures: Cytology (top) Histology (bottom)



Bi- & Multi-nucleate cells; Cyto (top), Histo (bottom)

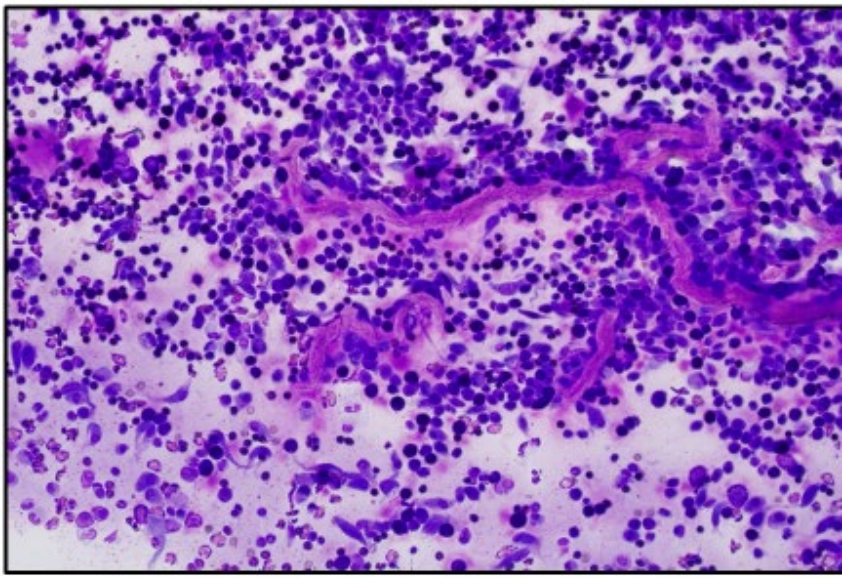






Bottom left image courtesy of Michael Wiseman, Idexx Laboratories, NY

Collagen
&
Fibrocytes



References

- Berlato D, Bulman-Fleming J, Clifford CA, et al. Value, Limitations, and Recommendations for Grading of Canine Cutaneous Mast Cell Tumors: A Consensus of the Oncology-Pathology Working Group. Vet Pathol. 2021 Sep;58(5):858-863
- Bertram, C.A. *et al.* (2021). Dataset on Bi- and Multi-nucleated Tumor Cells in Canine Cutaneous Mast Cell Tumors. In: Palm, C., Deserno, T.M., Handels, H., Maier, A., Maier-Hein, K., Tolxdorff, T. (eds) Bildverarbeitung für die Medizin 2021. Informatik aktuell. Springer Vieweg, Wiesbaden.
- Bertram CA, Aubreville M, Donovan TA, et al. Computer-assisted mitotic count using a deep learning-based algorithm improves interobserver reproducibility and accuracy. Vet Pathol. 2022 Mar;59(2):211-226.
- Bertram CA, Aubreville M, Gurtner C, et al. Computerized Calculation of Mitotic Count Distribution in Canine Cutaneous Mast Cell Tumor Sections: Mitotic Count Is Area Dependent. Vet Pathol. 2020 Mar;57(2):214-226.
- Camus MS, Priest HL, Koehler JW, et al. Cytologic Criteria for Mast Cell Tumor Grading in Dogs With Evaluation of Clinical Outcome. Vet Pathol. 2016 Nov;53(6):1117-1123.
- Casanova M, Branco S, Veiga IB, et al. Stereology in Grading and Prognosis of Canine Cutaneous Mast Cell Tumors. Vet Pathol. 2021 May;58(3):483-490.
- de Nardi AB, Dos Santos Horta R, Fonseca-Alves CE, et al. Diagnosis, Prognosis and Treatment of Canine Cutaneous and Subcutaneous Mast Cell Tumors. Cells. 2022 Feb 10;11(4):618.
- Donnelly L, Mullin C, Balko J, et al. Evaluation of histological grade and histologically tumour-free margins as predictors of local recurrence in completely excised canine mast cell tumours. Vet Comp Oncol. 2015 Mar;13(1):70-6.
- Hergt F, von Bomhard W, Kent MS, et al. Use of a histologic grading system for canine cutaneous mast cell tumors on cytology specimens. Vet Clin Pathol. 2016 Sep;45(3):477-83. doi: 10.1111/vcp.12387. Epub 2016 Aug 2. Erratum in: Vet Clin Pathol. 2017 Mar;46(1):202. PMID: 27483044.
- Horta RS, Lavalle GE, Monteiro LN, et al. Assessment of Canine Mast Cell Tumor Mortality Risk Based on Clinical, Histologic, Immunohistochemical, and Molecular Features. Vet Pathol. 2018 Mar;55(2):212-223.
- Kiupel M, Webster JD, Bailey KL, et al. Proposal of a histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. *Vet Pathol*. 2011 Jan;48(1):147-55.

- Krimer, PM. Response to Paes et al, Inclusion of fibroblasts and collagen fibrils in the cytologic grading of canine cutaneous mast cell tumors, Vet Clin Pathol. 2022 Apr 13. *in press*.
- Paes PRO, Horta RS, Luza LC, et al. Inclusion of fibroblasts and collagen fibrils in the cytologic grading of canine cutaneous mast cell tumors. Vet Clin Pathol. 2022 Sep;51(3):339-348. doi: 10.1111/vcp.13098. Epub 2022 Apr 13.
- Patnaik AK, Ehler WJ, MacEwen EG. Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. Vet Pathol. 1984 Sep;21(5):469-74.
- Russell DS, Townsend KL, Gorman E, et al. Characterizing Microscopical Invasion Patterns in Canine Mast Cell Tumours and Soft Tissue Sarcomas. J Comp Pathol. 2017 Nov;157(4):231-240.
- Schultheiss PC, Gardiner DW, Rao S, et al. Association of histologic tumor characteristics and size of surgical margins with clinical outcome after surgical removal of cutaneous mast cell tumors in dogs. J Am Vet Med Assoc. 2011 Jun 1;238(11):1464-9.
- Scarpa F, Sabattini S, Bettini G. Cytological grading of canine cutaneous mast cell tumours. Vet Comp Oncol. 2016 Sep;14(3):245-51.
- Scarpa F, Sabattini S, Marconato L, et al. Use of histologic margin evaluation to predict recurrence of cutaneous malignant tumors in dogs and cats after surgical excision. J Am Vet Med Assoc. 2012 May 15;240(10):1181-7.
- Sledge DG, Webster J, Kiupel M. Canine cutaneous mast cell tumors: A combined clinical and pathologic approach to diagnosis, prognosis, and treatment selection. Vet J. 2016 Sep;215:43-54.
- Thompson JJ, Pearl DL, Yager JA, et al. Canine subcutaneous mast cell tumor: characterization and prognostic indices. Vet Pathol. 2011 Jan;48(1):156-68.
- Willmann M, Yuzbasiyan-Gurkan V, Marconato L, et al. Proposed Diagnostic Criteria and Classification of Canine Mast Cell Neoplasms: A Consensus Proposal. Front Vet Sci. 2021 Dec 10;8:755258.

Cytology of Bone, is it really that hard?
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INTRODUCTION

Fine needle aspiration of lytic lesions of bone is becoming more common. Advantages of cytology over biopsy include rapid turnaround time (in some locations time from aspiration to evaluation can be less than 24 hours) lower risk of pathologic fracture, minimal patient discomfort, decreased procedure and recovery times, and decreased risk of wound infection. Cytology, however, also has its limitations. These include difficulties in differentiating reactive from neoplastic tissues, poor cell exfoliation resulting in nondiagnostic specimens, inability to characterize architecture of a lesion and finally, difficulty in distinguishing the different tumors of bone. In particular, osteosarcoma can appear cytologically similar to chondrosarcoma, synovial cell sarcoma and fibrosarcoma. Cytology should not be used as a stand-alone diagnostic test but should be used in conjunction with radiographs, clinical presentation and confirmation with histopathology.

Fine needle aspiration of bone is indicated if an osteolytic or osteoproliferative lesion is present. Radiographic changes may include cortical lysis or periosteal bone proliferation with or without soft tissue swelling. Methods of cytology include fine needle aspiration with a needle as large as 18 gauge however, in highly lytic lesions smaller gauge needles can be used. Also, roll preparations or imprints can be made from Jamshidi or Michele trephine biopsies. Radiographic evaluation of the lytic lesion is recommended to identify the best location for aspiration or biopsy. Ideally, the center of the lytic lesion rather than the transitional area between abnormal and normal bone should be aspirated. This will increase the chance of aspirating tumor and not just reactive bone. A study by Britt et al demonstrated that 89% of samples were diagnostic when ultrasound-guided aspiration was used to obtain a cytologic specimen of lytic or proliferative lesions.

Normal and Reactive bone

One of the most important steps to interpreting cytology is recognizing what is normal. Normal bone consists of osteocytes housed in lacunae, low numbers of osteoblasts and osteoclasts in remodeling bone. Osteoblasts produce osteoid which mineralizes through deposition of hydroxyapatite crystals to form bone. The osteoblasts get trapped in the mineralized osteoid, mature to osteocytes, and are then responsible for maintenance of the bone matrix. The outer surface of bone consists of condensed fibrous connective tissue (periosteum). The periosteum also contains osteoprogenitor cells which are difficult to distinguish from fibroblasts. Osteoclasts are multinucleated and contain several evenly spaced, uniform nuclei. Mineralized bone is very difficult to aspirate or biopsy. Therefore, in the absence of a lytic lesion, cytology of bone is generally low to acellular. Few mesenchymal cells from the periosteum may be observed, but these cells often appear small and mature and if more than one cell is observed, these cells are uniform in cell and nuclear size.

Trauma with or without fracture and bone surrounding lytic lesions either from neoplasia or inflammation will result in areas of reactive bone. Cells from this tissue can exfoliate well and may result in a fairly cellular sample which can be deceiving as normal, healthy bone does not exfoliate well at all. Therefore, close attention must be paid to the cellular morphology. The cells of reactive bone consist of osteoblasts. These cells have abundant basophilic cytoplasm, an eccentrically placed nuclei with prominent nucleoli. Osteoclasts can also be observed. Minimal criteria of malignancy are observed in reactive bone. With the exception of prominent nucleoli, the cells look uniform and have generally more cytoplasm than neoplastic osteoblasts. Presence of osteoblasts in the absence of inflammation and with minimal criteria of malignancy should be interpreted as reactive bone.

When bone is lytic, it exfoliates much more readily and often results in a cellular cytologic preparation. Broad categories of processes which can result in lysis or proliferation of bone include, inflammation, neoplasia (either primary or metastatic), hypertrophic osteopathy, aneurismal bone cyst or periosteal response to trauma.

Histopathology correlate:

Normal bone: Bone is present as cortical bone, making up the outer supportive and containing portions of a bone, or as trabecular bone, which in long bones is found in the medulla and is most concentrated in the epiphysis and metaphysis. Normal bone has a fairly bland microscopic appearance with a major matrix component and a minor cellular component. The majority of normal bone is mineralized matrix that has smooth margins and is organized with collagen fibers of the matrix in alignment with one another. There are periodic lacunae that contain a small osteocyte that has little to no cellular atypia. Normal bone may have individual or small groups of osteoblasts on its margins periodically, but not at the numbers seen in reactive bone. Infrequent osteoclasts may also be observed with normal bone.

Reactive vs. Neoplastic bone: Differentiation of reactive and neoplastic bone is easier with histopathology as there is better spatial awareness and architectural separation in histology specimens. Reactive bone lesions are typically found within the periosteal or endosteal space adjacent to the bone lesion, be it a traumatic, neoplastic or inflammatory/infectious process. Reactive bone maintains an organized overall architecture and may compress but does not infiltrate adjacent structures or tissues. Reactive osteoblasts form a single and often uninterrupted layer along immature osteoid which will progress to immature woven bone, which has disorganized collagen fibers going in different directions in the matrix; these collagen fibers will later align and the new bone becomes mature lamellar bone. In osteosarcoma, growth is disorganized, infiltrative, and regions between osteoid deposition are filled with neoplastic osteoblasts (rather than the single layer formed in reactive bone).

Cytology of specific diseases of bone

Osteomyelitis

Osteomyelitis can be caused by fungal and bacterial agents. Depending upon the cause, the cytologic appearance can be quite different. Fungal osteomyelitis consists of more of a pyogranulomatous inflammatory population with varying numbers of neutrophils, macrophages and multinucleated giant cells. Additionally, in lesions with proliferative bone, osteoblasts and occasional osteoclasts may be observed. Osteoblasts identified with inflammatory lesions are often reactive and will have deeply basophilic cytoplasm and prominent nucleoli with an eccentrically placed nucleus. Osteoclasts can be very difficult to distinguish from multinucleated giant cells associated with inflammation but can have eosinophilic, granular material within their cytoplasm.

Agents known to cause osteomyelitis include *Coccidioides*, *Blastomyces*, *Histoplasma*, *Cryptococcus*, *Aspergillus* and *Candida*. These organisms are commonly observed within aspirates. *Blastomyces* is a round yeast organism with a double contoured wall and broad-based bud. *Coccidioides* organisms are large (10-100μ) blue or clear spheres with finely granular protoplasm. *Histoplasma* organisms by comparison are quite small (2-4μ) and are easily phagocitised by macrophages and can be observed within the cytoplasm of macrophages. The organisms are round with a thin capsul and crescent shaped, eccentrically placed, eosinophilic nuclei. Cryptococcal organisms are round with a narrow-based bud and thick, nonstaining (with Wright's stain), mucoid capsule. Often the organisms outnumber the inflammatory cells in preparations. *Candida* is a yeast organism, oval with a deeply basophilic nucleus, with narrow based budding organisms and occasional pseudohyphae. *Aspergillus* sp. form hyphal structures and are difficult to distinguish from other fungal organisms cytologically. Culture is recommended with these organisms.

There are many causes of bacterial osteomyelitis, however organisms commonly associated with osteomyelitis include *Actinomyces* sp. and *Nocardia* sp. The inflammatory process associated with bacterial osteomyelitis generally have a stronger suppurative component to the inflammatory

response. It is important to remember when aspirating bone that there is often peripheral blood contamination and some white blood cells will be observed secondary to the hemodilution. It may be necessary to evaluate a CBC or peripheral blood smear on the patient to determine if there are truly increased numbers of neutrophils within the sample. Observation of intracellular bacteria is diagnostic for bacterial osteomyelitis however culture is recommended for all inflammatory bone aspirates.

> Histopathology correlate: Many of the changes that can be identified with cytology of osteomyelitis lesions are also present in histologic preparations of the same lesions. Inflammatory cells, infectious agents, and reactive and/or proliferative changes can routinely be identified in both types of examination. In addition to changes seen cytologically, histopathology allows for visualization of architectural changes to the bone, bone marrow, and adjacent soft tissues. As well, the inflammatory process present can be described in more detail. Some examples include determination of the extent of inflammation and tissues affected (e.g., bone and associated soft tissues affected rather than just bone), specific inflammatory structures being formed (e.g., classic granulomas), bone loss and osteonecrosis, and vascular presence of infectious organisms (e.g., ascomycete and zygomycete fungal infections). The appearance and morphology of infectious organisms, both fungal and bacterial, are similar in cytologic and histologic specimens.

Neoplasia

Neoplastic processes of bone can result from metastasis, local invasion or as a primary bone tumor. Primary bone tumors consist primarily of osteosarcomas, chondrosarcomas, synovial cell sarcomas, fibrosarcomas and less common tumors, such as multilobulated tumor of bone, hemangiosarcoma and liposarcoma. Additionally, multiple myeloma and lymphoma are classified as tumors of bone marrow which can result in boney lysis. Benign tumors of bone have been reported and include osteoma, chondroma, osteochondroma, and ossifying fibroma.

Primary bone tumors

Cytologically, many of these tumors often have similar characteristics and can be difficult to distinguish with cytology alone. The cells of primary bone tumors consist of a population of round to spindle shaped cells with varying amounts of pale basophilic cytoplasm. Cells of osteosarcoma and chondrosarcoma often have eccentrically placed nuclei giving them a plasmacytoid appearance. Prominent and often multiple nucleoli are commonly observed. These samples are generally highly cellular compared to what would be expected if normal bone were aspirated. Often within the background is a pale, eosinophilic, proteinaceous material consistent with matrix. This is most seen with osteosarcoma and chondrosarcoma but can also be observed with fibrosarcoma and synovial cell sarcoma. Chondrosarcomas often will have an abundant, deeply eosinophilic matrix, often surrounding the cells, making it difficult to evaluate the individual cell morphology. Fibrosarcomas and hemangiosarcomas, in general, are spindle shaped rather than round but otherwise have many of the same cellular characteristics. One of the most difficult challenges is differentiating neoplastic from reactive bone. A pure population of mesenchymal cells may be consistent with a neoplastic process or with reactive bone so close examination of individual cell features is essential. Primary bone tumors such as osteosarcoma show criteria of malignancy such as anisocytosis, anisokaryosis, variation in the nuclear to cytoplasmic ratio and binucleation. Prominent nucleoli can be observed reactive as well as neoplastic bone. A recent study compared specific cytologic differences between osteosarcoma and reactive bone and found that presence of mitoses, more than one nucleus/cell and increased N:C ratio were observed more commonly with osteosarcoma than reactive bone. Histopathologically, the matrix produced by the tumor, particularly with osteosarcoma, chondrosarcoma and fibrosarcoma are utilized to distinguish the tumor types.

> Histopathology correlates: Histopathology is currently the gold standard for differentiating primary bone tumors. Much like with infectious disease, architecture of the

cells and their supporting matrix are what allows for differentiation of primary bone tumors. Each primary bone tumor produces a matrix or pattern that allows for differentiation.

Osteosarcoma: **Production of osteoid** by neoplastic cells allows for definitive diagnosis of this tumor. Osteosarcomas can be characterized as non-/mildly, moderately, or highly productive neoplasms based on the amount of osteoid produced; these levels are subjectively applied by anatomic pathologists. There are six different subtypes of osteosarcoma which are based on architectural make up:

Osteoblastic osteosarcoma: the most common type. This neoplasm is made up of anaplastic osteoblasts and produce variable amounts of osteoid.

Chondroblastic osteosarcoma: neoplastic cells produce both osteoid and chondroid matrices. The production of osteoid in this tumor is given precedence over cartilage production for diagnostic purposes, hence "chondroblastic osteosarcoma" rather than "osteoblastic chondrosarcoma." Careful examination of this tumor is required to detect both types of matrices.

Fibroblastic osteosarcoma: neoplastic cells are highly spindloid and produce bundles and streams of neoplastic cells, resembling a fibrosarcoma. These tumors produce mostly small amounts of osteoid and variable amounts of collagen. Care must be taken to differentiate collagenous matrix from osteoid production.

Telangiectatic osteosarcoma: An osteosarcoma that has intermixed caverns/spaces filled with hemorrhage. Osteoid production helps to differentiate this neoplasm from its most common differential, hemangiosarcoma. CD31 or factor VII-related antigen immunohistochemistry (IHC) can assist in ruling out hemangiosarcoma for tumors that have sparse osteoid production. This subtype of osteosarcoma carries the poorest prognosis.

Giant cell-rich osteosarcoma: resembles osteoblastic osteosarcoma but possesses regions of neoplastic giant cells that usually have multiple nuclei.

Poorly differentiated osteosarcoma: a tumor with variable cell morphology and small amounts of osteoid production.

Chondrosarcoma: **Production of cartilage without osteoid production** by neoplastic cells defines a chondrosarcoma. The most common appearance of this tumor is multiple nodules of disorganized cartilage proliferation with entrapped neoplastic chondrocytes within lacunae and plasmacytoid to spindloid neoplastic chondrocytes on the margins of the nodules. These tumors can be difficult to differentiate from their benign counterpart, chondroma, so cellular features of malignancy are often relied upon to differentiate the two. These include the presence of many tumor cells surrounding cartilage lobules, multiple neoplastic cells or multinucleate neoplastic cells within lacunae of the cartilage, significant anisocytosis/anisokaryosis between neoplastic cells, and mitotic figures. Mitoses are uncommon in cartilage tumors; identification of a single mitotic figure strongly supports diagnosis of a malignant chondrosarcoma.

Fibrosarcoma: Fibrosarcomas arising from bone resemble those arising from other tissues. Absence of osteoid production by tumor cells is required for definitive diagnosis and differentiation from fibroblastic osteosarcoma. Fibrosarcomas can produce collagen, form long interlacing streams and bundles, and often have regions where neoplastic cells form a herringbone pattern. Fibrosarcomas arising from the mandible and maxilla carry special consideration as part of the oral fibrosarcoma group of tumors. These tumors frequently have a deceptively benign appearance, but are highly aggressive and spread rapidly, hence the designation "histologically low-grade/biologically high-grade" fibrosarcoma.

Hemangiosarcoma: Skeletal hemangiosarcoma resembles hemangiosarcoma from other tissues, forming vascular channels lined by malignant endothelial cells. This is an aggressive tumor that usually causes bone destruction through infiltration and lysis.

This neoplasm can be difficult to differentiate from telangiectatic osteosarcoma; IHC (most commonly CD31) can be used to differentiate the two.

Primary tumors of bone marrow

Multiple myeloma consists of a neoplastic proliferation of plasma cells. These cells also have an eccentrically placed nucleus often with a prominent golgi apparatus. Binucleation and anisocytosis are common. Generally, the cells are smaller than osteoblasts however an anaplastic plasma cell tumor can have giant, poorly differentiated cells in addition to the more typical plasma cells. Usually, this tumor is fairly easy to differentiate from osteosarcoma. Lymphoma of the bone is not common and is a round cell tumor that consists of a monomorphic population of lymphocytes which may be large, intermediate or small in size. Whether lymphoma occurs in a lymph node or the bone, it will appear cytologically similar.

Histopathology correlates:

Multiple myeloma: Typically produces multicentric lytic lesions in bone. The histologic appearance of plasma cell neoplasia is similar to the cytologic appearance described above; histology allows for better determination of the extent of infiltration by neoplastic cells, including vascular invasion, extension to adjacent tissues, and excision status (in resected masses). These tumors can have associated deposition of amyloid (AL amyloid produced by misfolding of the light chain of immunoglobulin). MUM1 IHC can be used to differentiate poorly differentiated or anaplastic plasma cell tumors that have large cells that resemble neoplastic osteoblasts; absence of osteoid production also helps to differentiate these two.

Lymphoma: Primary marrow lymphoma is less common than spread from a multicentric lymphoma. As with plasma cell neoplasia, lymphoma has a similar histologic/cytologic appearance in bone. CD3 IHC is specific for T cell lymphoma and CD20(mature neoplasms), CD79a, and PAX5 IHC are specific for B cell lymphoma.

Tumors which invade bone

Oral squamous cell carcinomas are often associated with lytic bone. These have been shown in people to be locally invasive rather than arising from or metastasizing to bone. Cytology of these samples look typical of a squamous cell carcinoma in any other location. A population of neoplastic epithelial cells is observed. These cells will occasionally have keratinized cytoplasm and a progression towards mature squamous epithelial cells may be observed. These more mature cells will be seen in concert with a more immature population with large nuclei and prominent nucleoli. The predominance of mature looking cells versus more anaplastic appearing cells will vary with the level of differentiation of the tumor.

Histopathology correlate: With a few uncommon exceptions, oral squamous cell carcinoma has a similar microscopic appearance to other locations. Neoplastic cells form trabeculae and nests of squamous cells. Neoplastic cells can have prominent intercellular bridging and often surround clusters of compact lamellar keratin (termed keratin pearls). Neoplastic cells themselves are variably sized with moderate to abundant eosinophilic cytoplasm and round to oval nuclei containing vesicular chromatin and a central nucleolus. Neoplastic squamous cells can have uneven distribution of brightly eosinophilic keratin within their cytoplasm, which is called dyskeratosis, and dyskeratotic cells can be present in pre-neoplastic (dysplastic) squamous cell populations as well. Histopathology can be used to determine the extent of neoplastic infiltration and as well examine excision status in resections.

Multiple other neoplastic processes are capable of infiltrating bone, particularly in the mouth, including odontogenic tumors (e.g., ameloblastoma), melanoma, oral fibrosarcoma, and others.

Metastatic neoplasia

Many tumors can metastasize to bone but the common tumors we think of are prostatic, lung and mammary carcinomas. Identification of metastatic neoplasms can be difficult because cytology is often accompanied by reactive osteoblasts and osteoclasts. However, a second population of cells can be differentiated from the reactive population. Epithelial neoplasms are usually clustered but when they metastasize, they may appear more poorly differentiated. Additional stains are very helpful for diagnosis.

Histopathology correlate: Histologic examination of metastatic tumors within bone usually allows for definitive diagnosis, although additional histochemical and immunohistochemical stains can be used for poorly differentiated or anaplastic tumors. As mentioned above, various carcinomas are well known for their propensity to spread to bone. The presence of a primary tumor elsewhere in the body and identification of specific features unique to a given carcinoma type can be used to definitively subtype a metastatic carcinoma. Some examples of specific features include the presence of ciliated cells in certain types of lung carcinoma, formation of tubules indicative of adenocarcinoma, and dyskeratotic cells and/or keratin pearl formation in squamous cell carcinoma. Pancytokeratin IHC staining can be used to confirm an epithelial origin, while specific cytokeratin markers (e.g., cytokeratin 7, cytokeratin 14) can sometimes be used to differentiate between different carcinomas (e.g., differentiating an apocrine-derived neoplasm from a holocrine-derived neoplasm). In some cases, neoplastic epithelial cells will be very poorly differentiated or anaplastic, meaning they lack unique or determining features, in which case a diagnosis of “anaplastic carcinoma” may be as far as a pathologist can go in differentiation.

CYTOCHEMISTRY AND IMMUNOHISTOCHEMICAL STAINING

Cytologic differentiation of osteosarcoma from other mesenchymal tumors of bone is challenging however recent evaluation of a cytochemical stain shows promise. Staining of cells for alkaline phosphatase activity with a phosphate substrate allows osteosarcoma to be identified with 100% sensitivity and 89% specificity. These results were supported by another study with a sensitivity of 100% and a specificity of 67%. The ALP stain essentially consists of a phosphate salt (Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt) which acts as a substrate for ALP and undergoes an oxidation/reduction reaction after dephosphorylation by the enzyme. This results in a color change of the insoluble product at the site of the reaction (the cell membrane). The primary limitation of this stain is that reactive osteoblasts will stain the same as neoplastic osteoblasts. Therefore, the ALP stain should only be used on samples where a cytologic diagnosis of sarcoma has been made. With such a high sensitivity, the stain is an excellent screening test, however other tumors and reactive bone will also stain positively with ALP, decreasing its specificity. In one study, 1 out of 4 chondrosarcomas, 1 of 2 amelanotic melanomas and one multilobulated tumor of bone stained positive for ALP activity. Generally, the cytologic and clinical features of the tumors can assist in diagnosis. Often chondrosarcomas have excessive eosinophilic matrix, melanomas often will have pigment, (even small amounts in amelanotic melanoma), and multilobular tumors of bone have a distinct clinical appearance. The combination of cytology, clinical appearance, location of the tumor and radiographic changes are all important in the diagnosis of OSA. The additional information of ALP activity only improves the ability of the clinician and pathologist to appropriately diagnose primary tumors of bone.

Antibodies directed against ALP and runx2 have recently been evaluated as potential diagnostic markers in histologic sections of bone to distinguish OSA from other primary bone tumors6. The ALP antibody was not nearly as sensitive or specific as the cytochemical stain for ALP activity but the combination with runx2 may have some benefits.

REFERENCES

1. Britt T, Clifford C, Barger A, et al. J Small Anim Pract. 2007;48(3):145.
2. Fielder SE. In: Diagnostic Cytology and Hematology of the Dog and Cat. 4th ed. Valenciano AC and Cowell RL, eds. Elsevier, St. Louis, MO, 2014.
3. Akerman M, Domanski HA, Jonsson K. Monogr Clin Cytol, 2014;19:13.
4. Reinhardt S, Stockhaus C, Teske E, et al. J Small An Pract 2005;46:65.
5. Tada T, Jimi E, Okamoto M. Int J Cancer 2005;116:253.
6. Moore PF, Vet Pathol, 2014;51:167.
7. Barger A, Graca R, Bailey K, et al. Vet Pathol 2005;42:161.
8. Barger A, Baker K, Driskell E, et al. Vet Pathol 2022;59:427.
9. Craig, LE, Dittmer, KE, Thompson, KG. In: Pathology of Domestic Animals. 6th ed. Maxie MG, ed. Elsevier, St. Louis, MO, 2016.