# FELINE ORAL NEOPLASMS: A TWENTY-YEAR RETROSPECTIVE SURVEY AND EXPRESSION OF AMELOGENIN AND AMELOBLASTIN IN FELINE CONVENTIONAL (KERATINIZING) AMELOBLASTOMA AND ORAL SQUAMOUS CELL CARCINOMA.

A thesis submitted to the College of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Veterinary Pathology University of Saskatchewan Saskatoon

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### ABSTRACT

Feline oral neoplasms are underrepresented in scientific studies and reviews when compared with similar canine neoplasms. Oral neoplasms include those of the oral cavity, pharynx, gingiva, dental structures (odontogenic neoplasms), tongue, tonsils, and salivary glands. Oral neoplasms are common in cats representing 10-60% of all neoplasms in previous publications.

In Chapter 2 of the thesis, 569 surgical biopsies obtained from feline oral cavities submitted for routine diagnostic purposes between January 1998 and December 2019 were reviewed. Twenty-two different neoplasms were found. A majority of neoplasms were malignant (85%). The most frequently diagnosed were: squamous cell carcinoma (68.8%), peripheral odontogenic fibroma (5.3%), fibrosarcoma (4.4%), peripheral giant cell granuloma (3.5%), conventional (keratinizing) ameloblastoma (3.5%), and adenocarcinoma of the salivary gland (2.46%). The current study is the first one of its type conducted in Canada and the second one in North America. Compared to a previous North American study, fewer cases of fibrosarcoma (4.4% vs 12.9%), and significantly more cases of conventional (keratinizing) ameloblastoma (3.5% vs 0.3%) were reported. Several neoplasms were identified in this study that were not seen in the previous study, these included: plasma cell neoplasm, hemangiosarcoma, and osteoma.

Oral squamous cell carcinoma (OSCC) and conventional ameloblastoma (CA) represent two epithelium-derived neoplasms that affect the oral cavity of cats and histologically may look similar. In Chapter 3, two immunohistochemical (IHC) markers, amelogenin and ameloblastin, were compared to determine usefulness in differentiation of the two neoplasms. The expression of amelogenin and ameloblastin has been previously established in the feline tooth bud and canine and human odontogenic tumors. The aim of this study was to characterize the amelogenin and ameloblastin expression profile of OSCC in comparison to CA. Samples from 15 OSCC and 15 CA cases were examined. Amelogenin expression was intranuclear in 15 OSCC cases, with all cases demonstrating high staining intensity. 14 of 15 CA cases demonstrated mild-moderate intranuclear staining intensity. Neither CA nor SCC expressed ameloblastin. Ki67 stained SCC samples had proliferation index 29.80% and CA had proliferation index 16.51%.

The difference in staining pattern and intensity of amelogenin and ameloblastin along with proliferation index of Ki76 in OSCC and CA did not help distinguish between the two neoplasia types.

The combined conclusions of the investigations are feline oral neoplasms are still an under researched area, ameloblastoma might be more common than previously thought, amelogenin and ameloblastin are not specifically expressed in odontogenic neoplasia, and Ki67 labeling index is not significantly different between OSCC and CA.

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# LIST OF ABBREVIATIONS.

α-SMA	α-smooth muscle actin
APOT	Amyloid producing odontogenic tumor
CA	Conventional ameloblastoma
CAA	Canine acanthomatous ameloblastoma
СК	Cytokeratin
COX	Cyclooxygenase
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EMP	Extramedullary plasmacytoma
FcaPV	Felis catus papillomavirus
FEPLO	Fibromatous epulis of peripheral odontogenic ligament
FIOT	Feline inductive odontogenic tumor
FNA	Fine needle aspirate
FNI	Fine needle insertion
FOSCC	Feline oral squamous cell carcinoma
FSA	Fibrosarcoma
GFAP	Glial fibrillary acidic protein
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IG	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IS	Impression smear
ISH	In situ hybridisation
LI	Labelling index
MUM1/ IRF4	Multiple myeloma 1 / interferon regulatory factor 4
NQO1	NAD(P)H Quinone Dehydrogenase 1
PA	Peripheral ameloblastoma
PAS	Periodic acid–Schiff

PCR	Polymerase chain reaction
PGCG	Peripheral giant cell granuloma
PI	Proliferation index
PNST	Peripheral nerve sheath tumor
POF	Peripheral odontogenic fibroma
OBCC	Oral basal cell carcinoma
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
STS	Soft tissue sarcoma
TERT	Telomerase reverse transcriptase
VEGF	Vascular endothelial growth factor

### CHAPTER 1 LITERATURE REVIEW AND INTRODUCTION.

### 2.1 Introduction

### 2.1.1 Context

What is a neoplasm? Merriam Webster dictionary defines neoplasm as an abnormal benign or malignant new growth of tissue that possesses no physiological function and arises from uncontrolled usually rapid cellular proliferation. A benign neoplasm is usually localized and does not migrate to another part of the body, and in most cases responds well to treatment. Malignant neoplasms also called cancer, can spread to other body parts and are very often resistant to treatment.

Feline oral neoplasms are underrepresented in scientific studies and reviews when compared with similar canine neoplasms. For instance, searching PubMed data base (PubMed.gov) using key words, "canine oral neoplasia" provides 1,713 results, "human neoplasia" 150,223 results and "feline oral neoplasia" gives only 424 results

Oral neoplasms include those of the oral cavity, and its structures: pharynx, gingiva, dental structures (odontogenic neoplasms), tongue, tonsils, salivary glands (buccal, lingual, sublingual, minor), mandibular and maxillary bones. Oral neoplasms are common in cats representing 10-60% of all neoplasms and their diagnosis requires histopathological examination.<sup>1</sup> A retrospective study conducted in Poland examined 146 feline neoplasms and neoplasm-like oral lesions and obtained the following results: 4.78% benign neoplasms, 15.07% hyperplastic lesions, 57.53% inflammatory lesions and 21.91% malignant neoplasms. Oral neoplasms or neoplasm-like lesions are encountered commonly during routine oral exam or/and dental procedures, often accidentally. They might cause discomfort, reduced appetite, and hypersalivation to the affected animal and give distress to the owner. <sup>2</sup> A correct diagnosis can be very important to the well-being of the patient and client alike.

Benign oral masses in cats are often from proliferative inflammatory tissue; but numerous benign neoplasms have been identified including occasionally giant cell epulis, osteoma, plasmacytoma; and rarely peripheral odontogenic fibroma, acanthomatous ameloblastoma, inductive ameloblastoma (feline inductive odontogenic neoplasm), amyloid-producing odontogenic neoplasm (APOT), and odontomas.

Malignant oral neoplasms in cats include squamous cell carcinoma (SCC), fibrosarcoma, osteosarcoma, hemangiosarcoma, and malignant melanoma (rare). Feline oral squamous cell carcinoma (OSCC) is by far the prevailing oral malignancy in cats. It is also the most represented and researched one. PubMed gives 171 results researching "oral feline squamous cell carcinoma and 30 results using "feline oral fibrosarcoma".

Because many oral masses, especially mesenchymal ones, do not exfoliate well on fineneedle aspiration,<sup>3</sup> or increased vascularity may result in hemodilution, diagnosis of oral masses usually requires histopathologic analysis of a biopsy which remains a gold standard.<sup>4</sup>

In addition to this, feline patients with naturally occurring malignant neoplasms might be useful as animal models for developing treatments of human neoplasia. It was proposed feline OSCC be used as a model of head and neck SCC (HNSCC) in humans.<sup>5</sup> In particular, they are useful potential models for the more aggressive human papilloma virus negative human HNSCCs.<sup>6,7</sup> This common feline oral neoplasm can be also be used to develop therapeutics that could target NAD(P)H:quinone oxidoreductase 1 (NQO1)<sup>8</sup> and to evaluate other anticancer strategies.

### 2.2 Types of oral neoplasms

Oral neoplasms are very diverse and are currently grouped<sup>9</sup> in recent literature based on their histomophological features in the following categories: odontogenic, neoplasms arising from soft tissue, neoplasms of the jaws, and neoplasm-like proliferative lesions of mucosa and jaws. Most common oral neoplasms are summarized in Table 1.1 and Table 1.2. Neoplasms of odontogenic origin are summarized in Table 1.3.

Table 1.1 Neoplasms of the oral cavity reported in oral cavity of cats. (ad	lapted from Histological
classification of the neoplasms of alimentary system of domestic animals	) <sup>63</sup>

Epithelial	neoplasms	Neuroendoc	Melanocytic	Mesenchym	al neoplasms
		rine	neoplasms		
		neoplasms			
Benign	Malignant			Benign	Malignant
Viral filiform	Squamous cell	Carcinoid	Malignant	Fibroma	Fibrosarcoma
papilloma	carcinoma		melanoma		
Squamous	Adenocarcinoma				Rhabdomyosar
papilloma	of salivary gland				coma
Viral	Undifferentiated			Hemangioma	Hemangiosarc
fibropapilloma	carcinoma				oma
Adenoma of				Schwannoma	Malignant
salivary gland					schwannoma
					Undifferentiate
					d sarcoma

Table 1.2 Neoplasms of the upper alimentary tract reported in oral cavity of cats(continued).

Granular	Neoplasms of bone		Neoplasms of	Neoplasm-like	
cell	-		hematopoietic and	lesions	
neoplasms				related tissues	
Benign	Benign	Malignant	Tumor like		
			lesion		
Granular cell	Osteoma	Osteosarcoma	Fibrous	Lymphoma	Lymphoid
tumor			dysplasia		hyperplasia
	Ossifying	Chondrosarcoma		Plasmacytoma	Nasopharyngeal
	fibroma				polyp
		Multilobular		Mast cell tumor	Eosinophilic
		tumor of bone			granuloma
					Calcinosis
					circumscripta

 Table 1.3 Neoplasms of odontogenic origin.

Odontogenic epithelium without odontogenic mesenchyme	Odontogenic epithelium with odontogenic mesenchyme	Derived from periodontal ligament	Cyst of the jaw	Neoplasm-like lesions
Ameloblastoma	Ameloblastic	POF	Dentigerous cyst	Inflammation of
(including CA)	fibroma			odontogenic structures
APOT	Feline inductive		Radicular cyst	Peripheral giant cell
	odontogenic tumor			granuloma
Acanthomatous	Complex odontoma			Gingival hyperplasia
ameloblastoma				_

The naming and classification of some oral neoplasms can be confusing. For example, in human oral pathology peripheral nerve sheath tumors (PNSFs) are classified and named based on clinicopathological features as malignant PNSTs, neurofibromas and schwannomas, but with cats there is often overlap in those features, therefore the general term PNSTs is preferred.<sup>10</sup> Peripheral nerve sheath tumors in the oral cavity are less common in cats than on skin. In a study of 53 cats with PNSTs, the tongue was affected in two animals and another two had labial lesions; no metastases were documented.<sup>10</sup>

Another group of neoplastic lesions with frequently changing naming terminology are those arising from the odontogenic epithelium. The neoplasm previously called calcifying epithelial odontogenic neoplasms, was re-named as amyloid producing odontogenic tumor (APOT) and amyloid producing ameloblastoma in a recent veterinary oral pathology texboook.<sup>9,11–13</sup> Feline inductive odontogenic tumor (FIOT) is a rare neoplasm that was first reported in 1979 under the name inductive fibroamelobastoma<sup>14</sup> and also published under the name adamantinoma.<sup>15</sup> In 1995 the neoplasm was reviewed and compared with human ameloblastic fibroma. It was concluded the neoplasm is unique to cats and the new name of FIOT was proposed that is currently in use.<sup>16</sup> Several publications since, use the name feline inductive odontogenic tumors including the recent text book of oral pathology.<sup>9,16–18</sup>

Ameloblastoma was previously called adamantinoma (this name was also used in reference for FIOT mentioned above) and enameloblastoma.<sup>9</sup> Ameloblastomas in cats are a rare neoplasm and only a few cases have been reported in the literature to date,<sup>16,19,20</sup> including wild felids. Authors of the recent veterinary oral pathology textbook preferred the name conventional ameloblastoma (CA).<sup>9</sup>

### 2.3 Prevalence of feline oral neoplasia.

Oral neoplasia is common in cats, with neoplasms of the oral cavity and tongue accounting for 3% to 12% of all neoplasms and 88% of these being malignant. <sup>21–23</sup> The incidence of oral neoplasms in feline species was calculated as 4.9 per 1,000 cats in another study.<sup>24</sup> Neutered males were almost as frequently affected as spayed females, but intact males were twice as often affected compared to intact females. The most common locations were lingual, followed by gingival locations. Domestic short hair, along with American short hair and mixed breeds were the most

represented.<sup>24</sup> A more recent epidemiological 6-year retrospective study from Europe included 297 oral cavity lesions from cats. While inflammatory lesions were the most common; OSCC accounted for 16.5% of all lesions.<sup>25</sup>

OSCC remains the most common neoplasia across all publications ranging from 60 to 80% of reported neoplasms. <sup>1,2,21,26</sup> Unfortunately wild felids are not spared from this invasive tumor with two reports in lynx (*Lynx lynx* and *Lynx canadensis*) and one in a bobcat (*Lynx rufus*)were published.<sup>27–29</sup> A California study reported an incidence rate of 9 cases of oral SCC per 100,000 cats.<sup>30</sup> In a Swiss large scale retrospective study of 18,375 diagnosed neoplasms, 5.3% were in the oral cavity/pharynx, and of these 88% were malignant with SCC and fibrosarcoma being the most common.

Fibrosarcomas are the second most frequently reported at 10-12 % of oral neoplasms. <sup>1,2,31</sup>

Odontogenic neoplasms accounted for up to 8 % of total oral neoplastic lesions in cats including peripheral odontogenic fibroma (POF) and ameloblastoma. <sup>1,25</sup> A large scale review of odontogenic neoplasms from Germany reported 3.2% as being of odontogenic origin .<sup>32</sup> Epithelial odontogenic neoplasms on the other hand are quite rare in domestic animals accounting for less than 0.7% of all oral neoplasms and in cats were represented by calcifying epithelial odontogenic neoplasms, ameloblastic fibroma, and complex odontoma in 1987 review.<sup>33</sup>

Tumor like lesions peripheral odontogenic fibroma (POF) and peripheral giant cell granulomas (PGCG) are relatively uncommon finding in cats. There are only a handful of PGCG reports in cats.<sup>34–36</sup>

Oral lymphoma composed 3 to 6% of reported neoplasms in several studies. <sup>1,2,25,37</sup>

Osteosarcoma are represented 2.4% to 4.5% of oral neoplasms  $^{1,2,37}$  Plasma cell neoplasms were 5% of those reported in one small scale survey from Europe. <sup>2</sup>

Melanocytic neoplasms are uncommon in cats, and are reported to represent less than 1% of all oral neoplasms.<sup>1,38</sup>In contrast to what is found in dogs 35.8%<sup>2</sup>, melanomas are rare in cats 3.1% of all malignant oral neoplasms<sup>2</sup>. Seventeen percent (56/324) were arising from the oral cavity in a recent large scale report on non-ocular melanocytic neoplasm in cats.<sup>39</sup>

In a recent study, the incidence of salivary neoplasia was 26.3 per 100,000 cats and no breed predilection was found.<sup>40</sup> In other studies, simple adenocarcinoma was the most common, no benign neoplasms were found, and unlike the more recent study, Siamese/Siamese cross cats were overrepresented (30%).<sup>41,42</sup>

Several other feline oral neoplasms have been reported such as mast cell neoplasms, peripheral nerve sheath neoplasms, granular cell neoplasms, osteomas, fibromas, hemangiomas, and hemangiosarcoma. <sup>1,2,25,31,37</sup>

### 2.4 Risk factors for development of feline oral neoplasms.

Breed, age, mutations, and environmental factors may have a certain role in initiation and development of feline oral neoplasia.

Siamese cats in the past were thought at increased risk for Salivary gland neoplasms. <sup>41,42</sup> However, a more recent, 2020 retrospective study found no breed predisposion.<sup>40</sup> There was no breed predilection found in the development of other oral neoplasms in cats.<sup>1,22</sup>

In general, males (54%) were slightly more affected compared to females (46%) according to Polish researchers<sup>43</sup> and in neutered males vs intact males, according to a Swiss publication.<sup>22</sup> Also, according to the same paper chances of developing oral fibrosarcoma were higher in female cats.<sup>22</sup>

Chance of developing a malignant growth in the oral cavity of cats also increases with age (mean age 12.2 years).<sup>22</sup>

Squamous cell carcinoma is by far the most common feline oral neoplasia and the most researched one.

Promoter mutation and associated increased expression of telomerase reverse transcriptase (TERT) is noted in many neoplasms including OSCC, which contributes to neoplastic cell immortality.<sup>44</sup> Expression of cMyc (TERT transcriptional activator) and several matrix metalloproteinases (important in cell migration and contribute to invasiveness) are also observed in a few SCC cell lines.<sup>44</sup> It is common knowledge that p53 prevents the replication of cells with damaged genetic material by counteracting the oncogenic transformation and neoplasm growth. Increase of p53 dysregulation and mutations in exons 5–8 of TP53 were detected in 69%<sup>45</sup> of specimens and 79% (7/9) of feline OSCC in a recent publication.<sup>46</sup> Abnormal accumulation of p53 was reported in feline OSCC by other authors.<sup>47</sup>

There were several other risk factors associated with feline OSCC occurrence, including environmental (rural environment, outdoor access, environmental tabaco smoke) and dietary components (wet diet, petfood containing chemical additives).<sup>48</sup> Among environmental, use of a flea collar was associated with a fivefold increase in development, and exposure to tobacco smoke

a two fold increase.<sup>47</sup> SCC from cats exposed to environmental tobacco smoke were 4.5 times more likely to have increased expression of p53 compared to unexposed cats.<sup>49</sup> But, more recent work found no association between tobacco smoke exposure and increased p53 or TP53 mutations.<sup>45</sup> Certain diets such as consumption of canned tuna have been reported to increase the chances of neoplasia by three times.<sup>47</sup>

Papilloma viral DNA was amplified from 6% of oral and auricular feline SCC that were not associated with UV radiation. In the same study human papilloma virus (HPV) was detected in 2 feline OSCC.<sup>50</sup> A more recent study demonstrated FcaPV-2 DNA and FcaPV-2 mRNA in 31% and 70% of feline SCC samples respectively, but viral DNA was also detected in nonneoplastic ulcerative lesions of feline oral mucosa in 36%.<sup>51</sup> It appears that viral load was different as detected by qPCR between ulcerated mucosa and SCC, although not statistically significant. Contrary to feline OSCC, *Felis catus* Papillomavirus Type 1, DNA was amplified from feline oral papillomas.<sup>52</sup> Contrary to human SCC the overexpression of TERT in feline OSCC is not associated with infection by papilloma virus.<sup>44</sup>

### 2.5 Molecular pathogenesis of feline OSCC.

Chronic inflammation can also promote epigenetic and genetic aberrations through different mechanisms.<sup>53</sup> Infection and inflammation potentially account for around 25% of the factors associated with the development of neoplasia.<sup>53</sup> Reactive oxygen and nitrogen species released during inflammation can cause DNA damage.<sup>53,54</sup> The role of both cyclooxygenase-1 and 2 (COX-1,2) mediators of inflammation in pathogenesis of feline OSCC was established in 2006. Normal tissues express no COX-2 and little COX-1, but COX-2 expression was identified in feline OSCC.<sup>55</sup> COX-1 IHC staining was associated with approximately a four fold increase in risk and being a pedigree cat more than eight fold.<sup>55,56</sup> COX-2 and CD147 in feline OSCC was expressed in approximately half of cases compared with normal adjacent mucosa and stroma.<sup>57,58</sup>

One of the membrane-bound receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR) had a membrane associated pattern in 69% of feline OSCC in one study of 13 neoplasms,<sup>59</sup> and in 14 out of 19 cases (73.7%) in another study and had poor prognosis among EGFR-positive malignancies compared to EGFR-negative ones.<sup>60</sup> EGFR signaling pathway may also be involved in pathogenesis and progression of SCC in cats according to the same paper.<sup>60</sup> Somewhat confusing, a study from the UK supported the notion that feline OSCC often express

EGFR, but contrary to the above data, hypothesized that cats with high EGFR expression may have a more favorable prognosis.<sup>61</sup>

### 2.6 Diagnostic challenges of oral neoplasms and neoplasia like lesions.

There are many differential diagnoses for feline oral neoplasms which can make arriving at the correct diagnosis particularly challenging, time consuming and often frustrating. For instance, there are many neoplasms that may be confused with OSCC. These include melanomas, odontogenic neoplastic lesions (including CA), and amyloid producing odontogenic neoplasms and although comparatively rare, can sometimes present a significant diagnostic challenge to a pathologist.<sup>33</sup> Odontogenic neoplasms are composed of odontogenic epithelium and fibrous stroma and two types are recognized, conventional ameloblastoma (CA), and amyloid producing ameloblastoma.<sup>9</sup> Keratinization is sometimes present in such neoplasms and previously they have been referred to as keratinizing ameloblastoma. They are only rarely reported in cats and can be confused with feline OSCC. <sup>20</sup>

Feline OSCC itself can be confused with other neoplasms. For example OSCC may occasionally present as primarily a proliferative to lytic bony process mimicking an osteosarcoma with little to no surface mucosal alterations.<sup>62</sup>

Adenocarcinoma is the most common salivary gland malignancy in felids.<sup>63</sup> Carcinomas from other sites could also metastasize to the oral cavity. The location and clinical history are important in helping determine if primary salivary gland carcinoma or a metastasis.<sup>9</sup>

Multiple feline epulides morphologically/histologically are of periodontal ligament origin, as is peripheral odontogenic fibroma (also known as Fibromatous Epulides of Periodontal Ligament Origin) and needs to be differentiated from the morphologically similar feline fibrosarcoma (FSA).<sup>9</sup>

Occasionally, dentigerous cysts can be clinically or grossly mistaken for neoplasms, abscesses or granulomatous lesions.<sup>64</sup>

Finally, with some neoplasms there is still no agreement between pathologists on specific diagnosis and naming. Odontogenic myxoma is one example where, odontogenic epithelium is not required for diagnosis in humans. Veterinary pathologists might have different opinions whether odontogenic epithelium is required to make this diagnosis opposed to myxoma or fibromyxoma.<sup>65</sup>

### 1.6.1 Immunohistochemical aid in diagnosis and prognosis.

IHC can play a role in prognostication and diagnosis of feline oral neoplasms. For example, EGFR expression can be a useful prognostic factor for survival time and when deciding on the treatment plan and its outcome in feline OSCC. <sup>61</sup> Three Wnt β-catenin transcription targets, namely CD1, FRAT1 and c-Myc were several times increased in feline OSCC compared to normal control tissues and can serve as a marker for this neoplasm.<sup>66</sup> Cancer associated fibroblasts were found in 75% of SCC in cats using an IHC for α-smooth muscle actin and their presence was associated with a shorter (2 weeks) survival time.<sup>67</sup> COX-1 positive IHC staining in FOSCC was correlated with a negative prognosis (fourfold increased hazard).<sup>68,69</sup> A 2012 paper reveals a significant correlation between the mitotic index and Ki67 intensity in FOSCC.<sup>70</sup> Ki67, a marker of proliferation was correlated with a worse outcome in feline OSCC.<sup>61</sup>

Extramedullary plasmacytomas (EMP) were reported in the oral cavity of three cats. Neoplastic cells were positive for CD79 $\alpha$ , but negative to CD 18 (attributed to a lack of cross-reactivity of anti-canine antibody with the feline antigens) <sup>43</sup>. Another publication reported feline oral EMP with monoclonal expression of an Ig  $\lambda$  light-chain type, which is considered to be a decisive diagnostic criterion in plasma cell origin neoplasms.<sup>71</sup>

Malignant melanomas in cats are reported at a much lower rate compared to dogs<sup>2</sup> and surprisingly have several publications available. Metastases are relatively common, and were reported in one third of affected cats.<sup>72</sup> If the typical histological pattern of packeting and pigmentation are present the diagnosis of melanoma is not difficult.<sup>73</sup> However, feline amelanotic melanoma can represent a diagnostic challenge and might necessitate the use of IHC to avoid misdiagnosis.<sup>74</sup> Amelanotic neoplasms in cats generally have a poorer prognosis therefore correct diagnosis is important, unfortunately there was no correlation of mitotic rate, nuclear atypia, and junctional activity with survival time. <sup>72</sup> Melan A is likely to be more specific but less sensitive compared to S100 in all species including cats. <sup>38,75,72,74,76</sup> Melan-A and PNL-2 IHC markers are reported to be reliable help in diagnosing melanocytic origin neoplasia in cats, especially for amelanotic ones, in a recent study and recommended to use as part of IHC panel.<sup>39</sup> Inclusion of CD34 (expressed by perivascular wall tumors) and laminin (expressed by peripheral nerve sheath tumors) is also recommended to rule in/out feline oral soft tissue sarcomas, a common differential diagnosis for amelanotic melanoma.<sup>74,77–79</sup>

Gingival inflammatory and proliferative lesion secondary to trauma can sometimes present a diagnostic predicament as well. For example in one report lesions initially diagnosed as poorly differentiated spindle cell sarcomas or carcinomas were interpreted as inflammatory lesions following IHC testing for cytokeratin and vimentin.<sup>80</sup>

### 1.6.2 Ancillary diagnostic tools.

Cytology can be relatively inexpensive, minimally invasive, and an accurate mean of diagnosis of oral neoplastic and non-neoplastic lesions in cats. A 2015 paper explored the accuracy of three cytological diagnostic techniques compared to histology in 85 dogs and 29 cats with oral cavity lesions. For cytology they used fine needle aspiration (FNA), fine needle insertion (FNI) and impression smear (IS).<sup>81</sup> Among the cats, 18 (62.1%) had malignant oral neoplasms, three cats (10.3%) had a benign oral neoplasm, and eight cats (27.6%) had non-neoplastic lesions. While in some cases diagnosis was impossible due to inadequate cellularity or necrosis, in the remainder had high sensitivity and specificity for the diagnosis of both neoplastic and non-neoplastic lesions (94.1–100%) using any of three techniques when compared to histopathological diagnosis.<sup>81</sup> Cytology can also allow for mandibular node screening for metastasis of SCC, as in one study five out of 14 cats had mandibular lymph node metastasis.<sup>82</sup>

Histochemistry can also often provide valuable diagnostic information at low cost to the client. The distinctive features of a benign amyloid producing odontogenic neoplasm (aka calcifying epithelial odontogenic neoplasms) in cats and dogs are the presence of odontogenic epithelium and the spherical extracellular amyloid-like deposits, which stains positive with histochemical stain Congo Red, Thioflavin S, <sup>83</sup>Dylon,<sup>84</sup>and Sirius red<sup>85</sup>.

### 2.7 Conclusions.

Although much research has been conducted on feline oral neoplasms, the field is still lagging compared to the studies done on canine and human oral neoplasia. For instance, the most resent large scale feline neoplasia prevalence study in North America was done over thirty years ago. No comparable study has been published in Canada. It also appears that despite progress achieved in diagnosis, there are still several grey areas such as differentiation of feline OSCC from other neoplasms, CA, diagnosing amelanotic melanoma and determining non-neoplastic from neoplastic proliferation of fibroblasts.

Further research into these and other areas, including the causative factors of feline oral neoplasia, would help determine new diagnostic, therapeutic and prevention strategies would benefit our feline friends and their owners

# CHAPTER 2 FELINE ORAL NEOPLASMS: A TWENTY-YEAR RETROSPECTIVE SURVEY.

### 3.1 Abstract.

**Objectives.** The aim of this survey was to determine the frequency and types of feline oral neoplasms submitted to a large diagnostic laboratory in Canada.

*Methods.* 569 surgical biopsies obtained from feline oral cavities submitted for routine diagnostic purposes between January 1998 and December 2019 were reviewed.

**Results.** Twenty-two different neoplasms were found. The majority of neoplasms were malignant (85%). The most frequently diagnosed were squamous cell carcinoma (68.8%), peripheral odontogenic fibroma (5.3%), fibrosarcoma (4.4%), peripheral giant cell granuloma (3.5%), conventional (keratinizing) ameloblastoma (3.5%), and adenocarcinoma of salivary gland (2.5%).

*Conclusions and relevance.* The current study is the first one of its type conducted in Canada and the largest one in North America. Compared to a previous North American study fewer cases of fibrosarcoma (4.4% vs 12.9 %), and significantly more cases of conventional (keratinizing) ameloblastoma (3.5% vs 0.3%) were reported. Several neoplasms were identified in this study that were not seen in the previous study, including: plasma cell neoplasm, hemangiosarcoma, and osteoma.

**Keywords:** cats, feline, soft tissue, oral neoplasms, odontogenic neoplasms, squamous cell carcinoma, ameloblastoma.

### 3.2 Introduction.

Oral neoplasms are very diverse and are grouped<sup>9</sup> in the recent literature based on their histomophological features into the following categories: odontogenic, neoplasms arising from soft tissue, neoplasms of the jaws, and neoplasm-like proliferative lesions of the mucosa and jaws. Oral neoplasms are common in the cat, with neoplasms of the oral cavity accounting for 3% to 12% of all neoplasms.<sup>21</sup> Despite being common, feline oral neoplasms are a relatively under researched field compared to other companion animal species. The last major retrospective study of feline oral neoplasms was published in 1989 from the University of Pennsylvania School of

Veterinary Medicine.<sup>1</sup> It is the sole source reference in most current veterinary literature. No comparable retrospective study has been published from Canada.

### 3.3 Material and Methods.

A search of the Prairie Diagnostic Services Inc. (PDS) and WCVM computerized databases beginning from January 1998 to December 2019 was conducted. Only surgical biopsies from spayed and intact females, neutered and intact males of domestic cats of all ages were included in the search. The databases were searched using several key words (oropharynx tumors, acanthomatous epulis, epulis (nonacanthomatous), odontogenic tumors, oncocytoma, squamous cell carcinoma, oropharynx) and their combination, and then manually reviewed. Five hundred and sixty nine cases with a final diagnosis of neoplasm were found. The diagnoses by the pathologist were recorded and if available the cat's age, gender, and breed, and clinical features, such as location, and extension.

### **Results.**

The most frequently reported breed of cats was the American domestic shorthair. The male to female ratio was 1:1, and the age varied from 7 months to 25 years. Twenty-two different types of neoplasms were identified (Table 2.1). Malignant neoplasms accounted for 84% of all diagnosed oral neoplasms in this 20-year review. There were no significant breed predilections found for any of the neoplasms reviewed.

Neoplasm type	Number of animals*	Number of Males	Number of Females	Age range (years)	Mean age (years)
Squamous cell carcinoma	391 (68.7%)	202 (52.5%)	183 (47.5%)	4-25	12.9
Peripheral odontogenic fibroma	30 (5.3%)	19 (65.5%)	10 (34.5%)	8m-17	8.9
Fibrosarcoma	25 (4.4%)	14 (56.0%)	11 (44.0%)	5-18	11.1
Conventional ameloblastoma	20 (3.5%)	9 (45.0%)	11 (55.0%)	2-19	14.3
Peripheral giant cell granuloma	20 (3.5%)	11 (55.0%)	9 (45.0%)	6-17	9.7
Adenocarcinoma salivary origin	14 (2.5%)	8 (57.1%)	6 (42.9%)	11-18	13.6
Undifferentiated sarcoma	9 (1.6%)	5 (55.6%)	4 (44.4%)	5-15	10.1
Undifferentiated round cell sarcoma	9 (1.6%)	5 (5.56%)	4 (44.4%)	6-15	10.8
Malignant melanoma	8 (1.4%)	4 (50.0%)	4 (50.0%)	9-17	11.9
Lymphosarcoma	7 (1.2%)	3 (42.9%)	4 (57.1%)	7-16	12.0
Adenocarcinoma unknown origin	6 (1.1%)	4 (66.7%)	2 (33.3%)	9-12	10.4
Amyloid producing odontogenic tumor	5 (0.88%)	4 (80.0%)	1 (20.0%)	7-13	10.8
Undifferentiated carcinoma	5 (0.9%)	1 (20.0%)	4 (80.0%)	7-20	14.8
Mast cell neoplasm	5 (0.9%)	1 (20.0%)	4 (80.0%)	7-18	11.6
Osteoma	4 (0.7%)	4 (100.0%)	0	7m-13	7.8
Plasmacytoma (Fig11-12)	3 (0.5%)	2 (100.0%)	0	8-12	10.3
Osteosarcoma (Fig13)	2 (0.35%)	0	2 (100.0%)	11-15	13
Feline inductive odontogenic tumor	2 (0.5%)	1 (50.0%)	1 (50.0%)	9-11 m	10 months
Fibroma	1 (0.2%)	0	1 (100.0%)	-	3
Hemangiosarcoma	1 (0.2%)	1 (100.0%)	0	-	16
Perivascular spindle cell tumor	1 (0.2%)	1 (100.0%)	0	-	13
Multiple feline epulides	1 (0.2%)	0	1 (100.0%)	-	6

**Table 2.1** Feline oral masses submitted for biopsies to a veterinary diagnostic laboratory from 1998 to 2019 by diagnosis, sex, and age.

\*number of animals and a sum of males and females may be different as the sex of animals were not always recorded.

**Squamous cell carcinoma** (SCC, Fig. 2.1) was by far the most commonly diagnosed neoplasm (391/569) (Table 1). The reported locations were as follows: maxillary gingiva (95/391) 24.30%, mandibular gingiva (99/391)25.32%, tongue (70/391) 17.90%, sublingual (46/391) 11.76% of SCC cases. There was no gender predilection. The breed of cats was predominantly American domestic shorthair (272/391), but domestic longhair (56/391), domestic medium hair

(17/391), Siamese (11/391), Himalayan (10/391), Maine Coon (five), Rag doll (three), Manx (two), Persian (two), Burmese (one), Turkish Van (one), and Bengal (one) were also represented. The breed was unknown in some instances (50/391). SCC was diagnosed as early as 4-years old and as late as 25 years with a mean age of 13 years. Invasion of maxilla or mandible with bony lytic changes was often reported (128/128, 32.74%). Emperipolesis of neutrophils, and nests of neoplastic cells along the scalloped edges of the alveolar bone were also noted. Frequently there was secondary suppurative or mixed inflammation.



Figure 2.1 Squamous cell carcinoma with evidence of bony spicules to support bone invasion

**Peripheral odontogenic fibroma** (POF, Fig.2.2) was the second most reported condition and accounted for 5.3% (30/569) of total cases. Affected cats age ranged from 8 months to 17 years with an average age of 8.9 years. Predominantly affected breeds were American domestic shorthair (14/30), Domestic long hair (five), Domestic medium hair (two), British shorthair (one), Himalayan (one), Siamese (one), Angora (one), Main Coon (one) were also represented. The breed was unknown in four instances. There was slight edge 65% vs 35% toward male cats being affected, which was not statistically significant (p = 0.2).



Figure 2.2 Peripheral odontogenic fibroma that has three concurrent histological features: immature fibrous mesenchyme (periodontal ligament like stroma), odontogenic epithelium and cemento-osseus matrix.

**Fibrosarcoma** (Fig.2.3 and 2.4) was diagnosed in 4.4% (25/569) of total cases. Age range varied from 5 years to 18 years and average age of occurrence was 11 years. The affected breeds were American domestic shorthair (16/25), Domestic long hair (three), Himalayan (two), Siamese

(one), Persian (one), and Domestic medium hair (one). Bone or muscle invasion and lysis were noted in half of cases.



Figure 2.3 Fibrosarcoma. Interlacing fascicle of spindle cells.



Figure 2.4 Fibrosarcoma higher magnification, mitotic figures.

**Conventional (keratinizing) ameloblastoma** (CA, Fig.2.5) represented 3.5% of the total submissions and were almost equally split between males (nine) and females (eleven). The affected breeds were American domestic shorthair (ten), Domestic longhair (six) and Himalayan (one). The breed was not reported in three instances. The average age of affected animals was 14.26 years and the age of occurrence ranged from 2 to 19 years.



Figure 2.5 Conventional (keratinizing) ameloblastoma with bone spicules supporting invasion of bone.

**Peripheral giant cell granuloma** (Fig.2.6) was also diagnosed in 3.5% of the cases (20/569); the same as for convential ameloblastoma. The breed of cats was predominantly American domestic shorthair (15/20), but American domestic medium hair (one), American domestic long hair (one) and Rex (one) were also represented. The breed was not reported in one case. Males and female were almost equally split (11 and 9 accordingly). The age of occurrence ranged from 6 to 17 years with mean age of 10.22 years.



Figure 2.6 Peripheral giant cell granuloma. Numerous MNGC and streams of spindloid cells.

**Salivary adenocarcinoma** (Fig.2.7) from buccal, lingual, sublingual, and minor salivary glands was diagnosed in 2.46% (14/569) of cases. Males were reported in eight cases and females in six. The mean age was 13.61 years and ranged from 11 to 18 years. The breeds reported were Domestic short hair (8/14) and, Siamese (3/14) Domestic long hair (1/14) and the breed was unknown(1/14). Several other relatively uncommon oral neoplasms were found.



Figure 2.7 Oral adenocarcinoma (lip).

**Malignant melanoma** (Fig. 2.8 and 2.9) represented 1.41% of the feline oral neoplasms. The age of occurrence ranged from 9 to 17 years, with an average age of 11.87 years. In three of eight cases, these neoplasms were amelanotic. They were reported to arise from the palatine area and lip in two cases each, gingiva and maxillae in one each and without a specific location given in the remaining three instances.



Figure 2.8 Malignant melanoma.



Figure 2.9 Higher magnification of Figure 2-8.

**Lymphoma** was diagnosed in seven cats, three males and four females. The age range was from 7 to 16 years, with an average age of 12 years. The predominate breed was Domestic short hair (four), followed by Siamese (two) and Domestic long hair (one). Immunohistochemistry was performed in two instances and in both a neoplasm of B cell origin was confirmed.

**Amyloid producing odontogenic tumors** (APOT Fig. 2.10 and 2.11) were diagnosed in five animals (0.88% of total submissions). The affected breeds were American domestic shorthair (two), domestic longhair (one) and Siamese (one). The breed was unknown in one. The age range of affected animals was from 7 years to 13 years, with average age of 10.8 years. No predilection site was noted. Amyloid was confirmed by Congo Red staining in all cases.



Figure 2.10 Amyloid producing odontogenic tumor. Congo Red.



Figure 2.11 Same case as Figure 2-10 under polarized light.

**Mast cell neoplasms** were found in five animals, arising from gums or ventral tongue in two cases each, and from inside the lip in one case.

### 3.4 Discussion.

Oral neoplasms are common in companion animals and are usually detected during routine clinical examination. Benign odontogenic neoplasia in cats, similar to the other species, can develop spontaneously while malignant neoplasms are thought to develop either as a result of spontaneous mutation or chronic irritation and persistent antigenic stimulation may contribute to malignant transformation.<sup>53</sup> Feline oral neoplasms account for 6% to 10% of all neoplasms in the specie.<sup>1,2,86–88</sup> Most oral neoplasms in this study were malignant which is similar to previous large<sup>1</sup>

and small<sup>2,31,37</sup> scale surveys. Neoplastic lesions of the oral mucosa were identified relatively frequently and should be differential diagnoses when dealing with oral masses in feline patients. Some inflammatory lesions such as pyogenic, giant cell or eosinophilic granulomas, and stomatitis can mimic neoplasia and make clinical diagnosis more challenging.

As with most retrospective studies, cases diagnosed by multiple different pathologists over the course of many years could potentially lead to variations in the diagnoses made. In the current study, all diagnoses were recorded as those made by the original pathologist. This approach is the same used in the small study done at Arizona Veterinary Dental Specialists.<sup>31</sup> In the Polish small scale retrospective study diagnoses were made by two of the authors.<sup>2</sup> It is unclear how diagnoses were made in the large scale study from University of Pennsylvania it is only mentioned that case records were reviewed<sup>1</sup> and another small study from University of California Davis the summary of biopsies from its Dentistry and Oral Surgery Service was used.<sup>37</sup> Therefore, it seems fair to assume that a similar approach was used in most of the published studies.

The results of the current feline retrospective study are in some ways similar to other studies and in some ways are different. There is only one large scale study<sup>1</sup> published, which analyzed 317 feline neoplasms and did not include inflammatory lesions similar to our survey. Smaller scale studies included inflammatory and hyperplastic lesion in addition to neoplasms; University of California- Davis scrutinized 107 biopsy specimens.<sup>37</sup> Arizona Veterinary Dental Specialists reviewed and categorized 73 biopsies of cats submitted for histopathology.<sup>31</sup> A more recent retrospective study from Europe analyzed 146 feline oral cavity tumors and tumor-like lesions.<sup>2</sup> See Table 2.2 for more details.

**Table 2.2** The frequency of feline neoplastic oral biopsy specimens submitted to a Canadian veterinary diagnostic laboratory between 1998 and 2019, as compared with the frequencies of oral neplasia in cats in previous publications. Number of cases (%).

Histological diagnoses	Current study	Stebbins et al	Regezi et al <sup>37</sup>	Wingo <sup>31</sup>	Mikiewicz et al
Squamous cell carcinoma	391 (68.7%)	227 (61.2%)	20 (45.5%)	27 (87%)	24 (60%)
Peripheral odontogenic fibroma	30 (5.3%)	29 (7.8%)	6 (13.6%) *	1 (3.2%)	2 (5%)
Fibrosarcoma	25 (4.4%)	48 (12.9%)	1 (2.3%)	1 (3.2%)	4 (10%)
Conventional (keratinizing) ameloblastoma	20 (3.5%)	1 (0.3%)	-	-	-
Peripheral giant cell granuloma	20 (3.5%)	2 (0.5%)	-	-	1 (2.5%)
Salivary Adenocarcinoma	14 (2.5%)	8 (2.2%)	1 (2.3%)	-	-
	20 (3.5%) **	17 (4.6%) **	3 (6.8%) ***		
Malignant melanoma	8 (1.4%)	3 (0.8%)		1 (3.2%)	1 (2.5%)
Amyloid producing odontogenic	5 (0.88%)	1 (0.3%)	2 (4.5%)	-	-
tumor					
Mast cell neoplasm	5 (0.88%)	3 (0.8%)	3 (6.8%)	-	-
Lymphosarcoma	4 (0.7%)	11 (2.9%)	3 (6.8%)	-	2 (5%)
Osteoma	4 (0.7%)	-	1 (2.3%)	-	-
Plasma cell neoplasm	3 (0.5%)	-	-	-	2 (5%)
Osteosarcoma	2 (0.35%)	9 (2.4%)	2 (4.5%)	-	1 (2.5%)
Feline inductive odontogenic	2 (0.5%)	3 (0.8%)	1 (2.3%)	-	-
tumor					

\* peripheral fibroma/fibrous hyperplasia. \*\* including adenocarcinoma of unknown origin,

\*\*\* including metastatic adenocarcinoma/carcinoma

Oral squamous cell carcinoma (OSCC) is a common neoplasia among domestic cats and reported in wild felids<sup>27</sup>. This study found OSCC in 68.8% (n=391) of total submissions, which is in line with some of the previous surveys (Table 2). This malignancy in cats has a reported multifactorial etiology with cigarette smoke<sup>49</sup>, consumption of canned tuna, chemical residues<sup>49</sup>, or viral etiology<sup>6,51,89</sup> being among suggested potential risk factors. The prognosis is generally unfavorable with a reported median survival time of 44 days and only 9.5% cats lived at least 1 year.<sup>69</sup> It is often a locally invasive and destructive neoplasia with reported metastasis in 37.5% of cases typically to the mandibular lymph nodes.<sup>82</sup>

Peripheral odontogenic fibroma (POF) is also known as a fibromatous/ossifying epulis of periodontal ligament origin (FEPLO) depending on the degree of mineralization. Of the submitted samples 5.3% were diagnosed as POFs. A 2007 survey of 52 feline "epulides" revealed that POF was the most common type (57.7%), followed by "giant cell epulis"(peripheral giant cell granuloma) (28.8%), "acanthomatous epulis" and ossifying epulis<sup>90</sup>. According to the recent

literature "fibromatous" and "ossifying epulis" are now considered synonyms for POFs. Acanthomatous epulis is now recognized as (canine) acanthomatous ameloblastoma and is relatively unique to dogs.<sup>9</sup> In contrast to this, feline acantomatous ameloblastomas (epulis) are a very rare<sup>90</sup> entity. A unique feature common to most feline epulids is the presence of three components (fibromatous, ossifying and acanthomatous) within the same lesion, which makes classification a difficult task.<sup>91</sup>

The naming terminology of "epulides" has been a thorny topic for some time, with two names (older name FEPLO and newer POF) currently in use, neither of which has gained a complete acceptance among veterinary oral pathologists.<sup>9,91</sup>

Although POFs have a variable presence of odontogenic epithelium and mineralized cemento-osseous matrix, a distinctive type of more cellular fibrous mesenchyme is the primary histological diagnostic criterion that must be in the masses to make the diagnosis.<sup>9</sup>

Fibrosarcoma (FSA) was diagnosed in 4.4% of cases and this neoplasm was the third most common (after SCC, POF). The previous 10-year survey reported 12.9% making it the second most of all oral neoplasms .<sup>1</sup> Small scale surveys from University of California-Davis and Arizona Veterinary Specialist each reported a single case of FSA 2.27% (1/44) and 3.22% (1/31). At the same time researchers from Poland and Ukraine found 4 cases of FSA or 10% (4/40).<sup>2</sup> Such variability among publications can possibly be a result of the diagnostic challenge especially when additional features of the case such as signalment, gross appearance, location, imaging data were not completely provided by a submitter or lost. Differentiating FSA against other feline epulids (lesions that share both bone and fibrous tissue features), fibromatous gingival hyperplasia, or inflammatory/fibromatous polyps can be difficult as well.

20 cases were identified in the current study that either had a principal diagnosis or one of the differentials as conventional keratinizing ameloblastoma. Of these, 8 had a maxillary location and 10 had a mandibular location, and in 2 cases no specific location was indicated. Conventional (keratinizing) ameloblastoma (formerly known as "adamantinomas"<sup>15</sup>) were thought to be a rare neoplasm with only a single case reported in the previous 10-year survey<sup>1</sup>. Since then several other cases have been published that will fall under the category of either cystic or keratinizing ameloblastoma, including one reported from Italy in 2010.<sup>19,20</sup> In the past, this tumor has been potentially mistakenly reported as amyloid producing odontogenic neoplasms or feline inductive odontogenic tumors (**Fig. 2.12**), both of which are different and distinct entities.<sup>92</sup>



Figure 2.12 Feline inductive odontogenic tumor with c-shaped arch of odontogenic epithelium.

Because of the discrepancy in the number of cases between the previous studies and the current findings as well as potential difficulties that arise in the histological differentiation of keratinizing ameloblastoma and SCC, additional research is likely warranted.

### 3.5 Conclusion.

This study is unique in several ways. It is the largest study by the sample size of surgical biopsies and the first retrospective study of feline oral neoplasia and neoplasia like lesions reported in Canada. The most common oral cavity malignancy in cats is squamous cell carcinoma which is in line with previous publications. Major differences were found among detected neoplasia types and their rates in contrast with previous surveys.

For example, conventional (keratinizing) ameloblastoma (CA) was more commonly diagnosed in the current study when compared to the previous Pennsylvania survey.

Differentiating between CA and, SCC can still represent a diagnostic challenge even for seasoned pathologists based on histomorphology alone and there are currently no reported ancillary testing methods to differentiate these two neoplasms.

FSA was almost 3-times less likely to be diagnosed in this study compared to the previous North American study.

Therefore, additional research is likely warranted. For instance, there might be a potential to develop additional tests (IHC, PCR, ISH etc.) that could help differentiate keratinized SCC from keratinized CA. In addition to this, an IHC panel could be recommended/routinely employed to help differentiate FSA from other types of neoplasia (amelanotic melanoma, POF etc.).

The current survey updates the data and documents the high degree of malignancy of feline oral neoplasms and raises some important questions of diagnostic challenges of squamous cell carcinomas, conventional (keratinizing) ameloblastoma and fibrosarcoma.

# CHAPTER 3 EXPRESSION OF AMELOGENIN, Ki67 AND AMELOBLASTIN IN FELINE CONVENTIONAL (KERATINIZING) AMELOBLASTOMA AND ORAL SQUAMOUS CELL CARCINOMA.

### 3.6 Abstract

Oral squamous cell carcinoma (OSCC) and feline conventional ameloblastoma (CA) represent two epithelium-derived neoplasms that affect the oral cavity of cats. The expression of amelogenin and ameloblastin has been previously established in the feline tooth bud and canine and human odontogenic tumors. The aim of this study was to characterize the amelogenin and ameloblastin expression profile of OSCC in comparison to CA. Samples from 15 OSCC and 15 CA cases were examined. Amelogenin expression was intranuclear of mild to moderate intensity in 15 OSCC cases, with all cases demonstrating high staining intensity. For CA, 14 of 15 cases demonstrated mild-moderate intranuclear staining intensity. Neither CA nor SCC expressed ameloblastin. Ki67 stained SCC samples had proliferation index 29.80% and CA had proliferation index 16.51%.

The difference in staining pattern and intensity of amelogenin and ameloblastin along with proliferation index of Ki76 in OSCC and CA did not help distinguish between the two neoplasia types.

### 3.7 Introduction.

Oral neoplasia in the cat accounted for 5.3% and 7.3% of all feline neoplasms in recent publications.<sup>22,23</sup> Most of these are malignant with squamous cell carcinoma (SCC) being by far the most common.<sup>1</sup> The previous chapter survey of feline oral neoplasms was in an agreement that malignant neoplasms are the most common with SCC being by far the most common malignancy. Cats with oral neoplasms are often presented with poor appetite and condition, dysphagia, halitosis, lethargy, and reduced grooming, often in pain. Therefore, it is important to provide accurate and timely diagnosis.

In our survey of feline oral malignancies, an increased number of neoplasms were diagnosed as conventional (keratinizing) ameloblastoma (CA) compared with previous surveys.<sup>1</sup> These are rare, benign, but locally invasive neoplasms<sup>20</sup>. CA, especially the keratinized ones can pose a diagnostic challenge by overlapping in some diagnostic features with SCC.<sup>9,93</sup> Because CA can have a better prognosis for the animal than SCC the correct diagnosis is of importance.<sup>9,93</sup> CA

are derived from the epithelium of the dental lamina while SCC are derived from the oral mucosa. In humans, certain cytokeratins are highly specific and allow to distinguish between these two types of epithelia, oral mucosa (CK19) and dental laminal epithelium (CK7,14).<sup>94</sup> Unfortunately, that is not the case in animals.<sup>11,95</sup> For instance, canine acanthomatous ameloblastoma (CAA) and OSCC both expressed uniform and strong labeling for CK14 and CK19 and lacked labeling for CK7.<sup>96</sup> Additional studies in cats and other species are needed to confirm the specificity of these or other antibodies in the investigation of odontogenic neoplasms.

Several studies have shown some promising results using several IHC markers in differentiating odontogenic epithelium from normal or neoplastic oral mucosa in humans<sup>97</sup>, felines<sup>11,98</sup> and canines<sup>99–101</sup>. Some of those markers were a range of different cytokeratins (AE1/AE3, CK 5/6, 8,9,10,14), amelogenin, ameloblastin, calretinin, collagen IV, laminin, p53, p65, EGFR, Ki67 etc.<sup>98–101</sup>

Three of these markers are particularly appealing as potentials markers to differentiate SCC and CA. These are amelogenin, ameloblastin and KI-67. Ameloblastoma and neoplastic odontogenic epithelium are both reported to have increased expression of amelogenin and ameloblastin among other IHC markers.<sup>11,97</sup>

Amelogenin is tooth matrix protein with a key role in amelogenesis<sup>102</sup> and ameloblastin is a cell adhesion molecule that plays a role in maintaining the differentiation state of ameloblasts.<sup>103</sup> Both are produced by ameloblasts and are promising markers specific to odontogenic epithelium .<sup>104</sup> Ameloblastin is the second most abundant protein during amelogenesis however its exact role during amelogenesis is not entirely elucidated, although mutation of ameloblastin coding gene AMBN was related to amelogenesis imperfecta occurance<sup>105</sup>, and it also appears to modulate osteoclastogenesis.<sup>106</sup>

Ki67 is a nuclear protein expressed only by cells active in the cell cycle and it is widely used for labeling human and canine neoplasms for diagnostic and prognostic purposes.<sup>100,107</sup> A more recent study noted a marked decrease of Ki67 labeling index in canine acanthomatous ameloblastoma compared with oral SCC.<sup>100</sup>

In this pilot project, we hypothesized that the odontogenic neoplastic epithelium of CA would widely express amelogenin, ameloblastin compared to low or lack of such expression in oral SCC and furthermore Ki67 labeling index (LI) will be higher in SCC.

### 3.8 Materials and methods.

Histologic evaluation and classification.

Archived formalin-fixed and paraffin wax-embedded tissues obtained for this study consisted of 30 feline oral neoplasms (15 CA and 15 OSCC). These were retrieved from the archive of the Department of Pathology Western College of Veterinary Medicine University of Saskatchewan, Canada and PDS. Bony samples underwent decalcification using 15% formic acid prior to routine tissue processing. Five micrometer sections from formalin-fixed and paraffinembedded tissues were stained with hematoxylin and eosin (HE) and assessed histologically by veterinary pathologists using previously established criteria that are characteristic of odontogenic epithelium (i.e. palisading, antibasilar nuclei, basilar clear zone, presence of the stellate reticulum like cells) with realization that not all of those (or even any) can be present in pathologic odontogenic epithelium. <sup>9,108</sup> The original histopathological diagnosis was reviewed and validated by a group of veterinary pathologists (BW, HP, SC) using a multiheaded microscope. Samples that were not confirmed as CA were excluded. Only samples that had sufficient tissue for processing were included. Cases of OSCC that were not grossly associated with gingival mucosa (i.e., palatal, buccal mucosal, oropharyngeal, or tonsillar) were excluded based on information obtained from submission forms, medical records, and imaging that were available. The histological grade of SCC cases was noted as either well differentiated, moderately or poorly differentiated. Clinical and demographical data such as age, sex, breed, location, invasiveness was noted. The anatomical location the neoplasia was recorded as maxillary or mandibular. When available, results of diagnostic staging procedures (i.e., draining lymph nodes aspirates, thoracic imaging) were reviewed to determine the presence of metastasis.

Immunohistochemical staining was performed on serial 4- µm sections conducted at Prairie Diagnostic Services, Saskatoon, SK using an automated slide stainer<sup>a</sup>. Following deparaffinization, and gradual hydration to 70% ethanol, inactivation of endogenous peroxidase was done by immersion in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes at room temperature. Epitope retrieval was performed in a Tris/EDTA pH 9 buffer at 97°C for 20 minutes.

Antibody	Source	Manufacturer	Catalog No.	Dilution
Ameloblastin (H-2)	Mouse	Santa Cruz <sup>1</sup>	sc-271012	1:100
	monoclonal			
Ameloblastin	Rabbit	Thermo Fisher Scientific <sup>2</sup>	PA5-113434	1:500
	polyclonal*			
Amelogenin (F-11)	Mouse	Santa Cruz <sup>1</sup>	sc-365284	1:400
	monoclonal			
Amelogenin (AMLX)	Rabbit	Thermo Fisher Scientific <sup>2</sup>	PA5-114845	1:1000
	polyclonal*			
Ki67 (MIB1)	Mouse	Agilent Dako <sup>3</sup>	GA62661-2	1:75; 1:100
	monoclonal			

 Table 3.3 Antibodies for immunohistochemistry.

<sup>1</sup>Santa Cruz Biotechnology, Inc, Santa Cruz, CA.

<sup>2</sup>Thermo Fisher Scientific, Waltham, MA

<sup>3</sup>Agilent Technologies Canada Inc., Mississauga, ON

\*After consultation with the committee members, it was decided to order polyclonal amelogenin and ameloblastin from a different manufacturer (Thermo Fisher Scientific).

After pretreating, nonspecific antibody interactions were then blocked by immersion in 4% normal goat serum for 20 min. Slides were drained and the following primary antibodies: Ameloblastin, Amelogenin and Ki67 were applied for 30 minutes at 1:75 to 1:1000 dilution range (see Table 3.1 for details). Binding of the primary antibodies was detected using an HRP-labelled polymer detection reagent and the staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB)<sup>c</sup> as the chromogen. Slides were counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO, USA). Negative controls were prepared by omitting the primary antibody and substituting an immunoglobulin (Ig) G correlate for each experiment. Positive control tissues were as follows: tooth germ (developing teeth) from 5 feline fetuses of roughly 55 days of gestation (crown to rump length 11.5; 11.9; 12.0; 11.5; 11.6 cm). In each assay run, a positive control reference tissue was included as a separate section/slide. In addition, pancreas, Amelogenin and kidney and, FIOT for Ameloblastin were used as per the manufacture's datasheets. Positive expression was interpreted as nuclear and granular cytoplasmic uptake (Amelogenin) or cytoplasmic uptake (Ameloblastin). Feline lymph node was used as positive control for Ki67.

The specimens were assessed and graded for the intensity of expression of Amelogenin and ameloblastin as previously described. The grading system was: –, no labelling; + weak or intermittent labelling; ++ moderate labelling and +++ strong labeling. All CA and OSCC were/not labeled for Amelogenin and Ameloblastin.

<sup>a</sup> Autostainer Plus, Agilent Technologies Canada Inc., Mississauga, ON

<sup>b</sup> EnVision+ System - HRP Labelled Polymer, Agilent Technologies Canada Inc., Mississauga, ON

<sup>c</sup> Dako Liquid DAB+ Substrate Chromogen System, Agilent Technologies Canada Inc., Mississauga, ON

Ki67 scoring.

Three representative high-power fields with characteristic histological features (2.37 mm<sup>2</sup>) of each Ki67 slide were photographed and areas of epithelial neoplastic nests were outlined to remove stromal cells and areas of heavy inflammation from the count. All positive and negative cells were counted twice – automatically using smart segmentation feature and manually using tagging both times utilizing Image-Pro Software (Media Cybernetics, Inc., USA). The corresponding cell counts in each field were tabulated (Microsoft Excel Worksheet). The Ki67 labeling index was determined by dividing the total number of positively labeled epithelial neoplastic cells counted in all three fields by the total number of neoplastic cells counted in all three fields by the percentage (PI - proliferation index).

Statistical analysis

No statistical analysis was performed, as there were no visually significant differences found between samples.

### 3.9 Results

The reported age of CA affected cats ranged from 12 to 17 years with the exception of one cat which was 22 months old. Of 15 cats with CA, eight were spayed females, and seven neutered males. Seven CA arose from mandibular gingiva, six from maxillary gingiva, one from unspecified gingival location and in one case the location was not provided. Six had lytic bone lesions, and two reported distortions of the underlying bone. Ages at the time of diagnosis of cats with SCC ranged from 10 to 17 years old, and the mean was 13 years. Of the 15 cats with oral SCC, 12 were neutered males and three were spayed females. Four SCC arose from the mandible, eight from the maxilla, two were sublingual and one was from an unspecified location. Nine cases of SCC reported lytic bone lesion and one sublingual case noted invasion of the tongue.

The qualitative analysis of the immunohistochemical labelling profiles of the tissues is provided in Table 3.2.

**Table 3.4** Immunohistochemical profiling of feline conventional ameloblastoma (CA) and feline oral squamous cell carcinoma (FOSCC).

Target	CA	FOSCC	
Anti-Ameloblastin monoclonal	Cytoplasmic patchy	Cytoplasmic patchy	
Anti-Ameloblastin polyclonal	Not detected	Not detected	
Anti-Amelogenin monoclonal	Intranuclear diffuse	Intranuclear diffuse	
Anti-Amelogenin polyclonal	Nuclear diffuse	Nuclear diffuse	
Anti-Ki67 labeling index (LI)	16.51	29.80	



Figure 3.13 Conventional ameloblastoma, ameloblastin monoclonal antibodies (20x;400x)

All positive and negative controls showed appropriate localization of the immunohistochemical stain for amelogenin and ameloblastin in suitable control tissues. The initial run was done with 12 randomly selected samples (six of SCC and six of CA) with monoclonal ameloblastin antibodies from Santa Cruz and no positive staining was detected (Figures 3.1 and 3.2).



Figure 3.14 Squamous cell carcinoma, ameloblastin monoclonal antibodies (20x; 400x)

All SCC (Figure 3.3) and all but one specimen of CA (Figure 3.4) stained with monoclonal anti-amelogenin antibody showed some degree of staining. This staining was both intranuclear and intracytoplasmic which after consulting with the study pathologists was deemed as not

diagnostically different. Polyclonal ameloblastin showed staining that was interpreted as nonspecific background staining in 12 out of 12 specimens (not shown).



Figure 3.15 Squamous cell carcinoma, amelogenin monoclonal (20x; 400x)



Figure 3.16 Conventional ameloblastoma, amelogenin monoclonal antibodies(20x;400x)

Since the polyclonal antibodies showed large amount of non-specific staining, higher dilutions of antibodies were applied. There was distinct granular intracytoplasmic staining within ameloblasts and enamel of fetal teeth (positive control). In SCC 15/15 cases (Figure 3.5) and CA 14/15 cases (a single case from 2003 was negative) (Figure 3.6) had variable expression of polyclonal amelogenin which was both intranuclear and intracytoplasmic without any particular specificity. The granular intracytoplasmic staining seen in positive control tissue was not observed. In a blind test a board-certified pathologist (BW) could not distinguish CA from SCC based on staining pattern alone. Ameloblastin only exhibited variable nonspecific background staining.



Figure 3.17 Squamous cell carcinoma, amelogenin polyclonal antibodies (20x; 400x)



Figure 3.18 Conventional ameloblastoma, amelogenin polyclonal antibodies (20x; 400x)

A single case from 2003 that yielded negative results with both monoclonal and polyclonal antibodies was re stained with the same negative result. It is thought this result is due to poor preservation of specimen.

The 7 samples of CA and 7 samples of OSCC were labeled with Ki67. Staining intensity of CA (Figure 3.7) and SCC (Figure 3.8) was prominent and ranged from patchy to diffuse intranuclear staining throughout the epithelium. An average of 524 cells (range 306 - 940) was counted per field for each sample of CA and 671 cells (range 491- 979) for each SCC sample. SCC samples had proliferation index 29.80% and CA had proliferation index of 16.51%.



Figure 3.19 Conventional ameloblastoma, Ki67 (100x)



Figure 3.20 Squamous cell carcinoma, Ki67 (100x)

### 3.10 Discussion.

The age, sex and breed of the study cohort of feline OSCC was similar to demographics reported before.<sup>1,22,24</sup> Patients were predominantly non pedigree older cats, and neutered males were more frequently affected (p-value is .030597) These results could be incidental however, given the small size of the sampled group.

The demographics of cats with CA have not been reviewed previously, likely due to the low prevalence of the disease. Our data suggest that CA, similar to feline OSCC, affects older cats, without a breed or sex predisposition, and similarly can invade bone and cause lytic bone lesions.

No convincing difference was seen between expression of amelogenin and ameloblastin in conventional ameloblastoma and oral squamous cell carcinoma in this study, and the Ki67 labelling index was comparable in both. Therefore, a further examination of different markers might be warranted.

In this study, amelogenin was expressed in all examined tissues from CA and OSCC. Human amelogenin and ameloblastin were expressed in 20-50% of neoplastic cells in feline amyloid producing odontogenic tumors (APOT) and porcine amelogenin and rat ameloblastin were expressed in 3-20% of neoplastic cells in a previous study.<sup>98</sup> This discrepancy could be the result of differences in the antibodies used. The polyclonal antibodies used in the previous studies have been discontinued by the manufacturer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and are no longer available. It is possible that the inconclusiveness and lack of specific staining was contributed to this factor.

Therefore, we used monoclonal antibodies from the same company expecting comparable results. The results we obtained were unsatisfactory and we believe this may have been due to the change to a monoclonal antibody. In order to see if this was the case, polyclonal antibodies from a different manufacture were used; unfortunately, the results of this were also inconclusive.

Examination of KI-67 labelling showed that the SCCs examined had an average proliferation index of 29.80% and CAs had an average proliferation index of 16.51%. This result differs from two previous studies, one had the average Ki67 labeling index (LI) of canine acanthomatous ameloblastoma as 2.2 and the average Ki67 LI of canine oral SCC as 23, and the other had 52.7% for feline OSCC.<sup>61,100</sup>Contrary to the previous studies this pilot project did not find solid evidence that feline ameloblastoma have a diagnostically significant different Ki67 LI compared to SCC.

### 3.11 Conclusions.

It is difficult to explain the unexpected negative results of this pilot project. It is possible that feline ameloblastomas might simply have a different IHC profile compared to human and canine ameloblastomas. The reported IHC staining profiles can also vary considerable between studies For example, a 2012 study in dogs found almost complete lack of calretinin expression in CAA, but positive calretinin staining in oral SCC<sup>96</sup>. While a 2018 publication described the opposite with negative to only focal calretinin immunoreactivity reported in conventional SCC and diffuse calretinin immunoreactivity was detected in CAA<sup>101</sup>.

Another possibility could be an existence of a subset of SCC in cats that mimics histomorphologically CA. Human SCCs and dogs SCCs are categorized into subsets based on morphological features and clinical behavior.<sup>109</sup> Papillary SCC in dogs especially in the deeper layers can present a diagnostic challenge and be misdiagnosed as benign or malignant odontogenic neoplasms.<sup>93,101</sup> It is therefore possible that what appeared morphologically to be CA may in fact be a variant form of SCC and not a neoplasm of odontogenic origin.

To our knowledge, this is the first study to examine the amelogenin, Ki67 and ameloblastin staining profile of CA in cats. Despite showing promising results in human and canine studies on SCC and ameloblastoma, our results lacked specificity to differentiate these two neoplasms.

### **CHAPTER 4 SUMMARY AND FUTURE RESEARCH**

### Summary.

Feline oral neoplasia is an under researched field compared to canines and humans as even a simple search of the published works in online data bases will confirm. Therefore, it was decided to review our diagnostic lab archives to contribute to and update the available information. This review was also warranted because the last large scale North American study was done in the late eighties.<sup>1</sup>

The first study was to conduct an extensive search and review of twenty years' worth of data. It found over five hundred cases of feline oral neoplasms encompassing over twenty different benign and malignant types of cancer. In comparison, a retrospective study from the eighties done at the University of Pennsylvania reviewed 371 cases<sup>1</sup> and a more recent publication from Portugal had 110 cases.<sup>110</sup> Reviewing PDS database, a subset of neoplasia diagnosed as CA was found. The number of CA cases exceeded more than tenfold previous findings<sup>1,110</sup> and CA wasn't even reported in a more recent small scale study from Europe.<sup>2</sup> This posed the question is it possible that ameloblastoma might be more common than previously thought? Based on this finding the next stage was trying to explain this result. Some information suggests that in dogs a subtype of SCC classified as papillary can mimic odontogenic cancer and present a diagnostic challenge.<sup>101</sup> It appears that some part of this neoplasia, especially deep portions of canine oral papillary SCC can have a feature of canine acanthomatous ameloblastoma.<sup>93</sup> Could this explain the higher numbers of CA? During the literature review it was noted that several studies of feline oral neoplasms revealed that amyloid producing ameloblastoma (aka APOT) can show different immunological phenotype compared to non-neoplastic mucosa.<sup>11,98</sup> Based on this information we hypothesized that it might be possible to distinguish normal odontogenic epithelium from odontogenic neoplastic epithelia and potentially from neoplasia of non-odontogenic origin. One of the options was to select a few target antigens that lack expression in normal odontogenic epithelium, but have been shown to have exaggerated immunoreactivity in neoplastic odontogenic epithelia.<sup>11,97,98</sup> Ameloblastin and amolegenin were selected for this purpose. In addition to this, several studies reported increased labeling index of Ki67 in more aggressive OSCC compared to locally invasive but benign ameloblastoma.<sup>61,100</sup> In our pilot project we compared the labeling index of feline OSCC and ameloblastoma and no significant difference in expression between aggressive SCC and locally invasive ameloblastoma was noted.

Unfortunately, the antibodies against epitopes of interest i.e., polyclonal amelogenin and ameloblastin used in previous studies were no longer available from the manufacturer (Santa Cruz). The company representee stated that monoclonal amelogenin and monoclonal amelogenin should work in a similar fashion. Nevertheless, obtained results were inconclusive and committee members agreed to make another order of polyclonal antibodies of the same class from a different company. After reviewing slides the expected immunoreactivity was not observed in either. Therefore, it was concluded that feline ameloblastoma either does not have an APOT-like odontogenic epithelia profile or there is a rare subtype of feline OSCC that might be morphologically similar to ameloblastoma.

### 3.12 Future research.

A primarily goal in our work was to concentrate on antibodies that were previously used in felines on similar type of neoplasms. Although in addition to feline and canine amyloid producing ameloblastoma (aka APOT) having immunoreactivity to ameloblastin, and amelogenin antibodies, a third marker sheathlin, was also reported by the same investigator to be expressed in canine APOT.<sup>99</sup> Because amelogenin and ameloblastin antibodies did not differentiate feline OSCC from CA in our pilot project, it is possible that sheathlin may have some value, but this would need to be investigated. Somewhat convoluted results were obtained by another investigator of APOT in cats, wherein neoplastic odontogenic epithelium showed immunoreactivity to ameloblastin, CK AE1/AE3, CK14 in over 75% of cells, but no amelogenin expression was observed.<sup>11</sup> It might also be worthwhile in the future to examine cytokeratin AE1/AE3 and CK14 expression in feline ameloblastoma compared to feline OSCC. Especially given that AE1/AE3 along with 34bE12, p63 showed promising results in distinguishing CAA from canine OSCC.<sup>101</sup>

A few alternate potential approaches might also be borrowed from canine and human research fields.

Firstly, Ki67 has been used to assist with differentiation of CAA from OSCC in dogs.<sup>100</sup> Unfortunately, no comparable study was done in cats. The only study that was done in cats showed that more aggressive and malignant canine SCC had significantly higher Ki67 LI.<sup>100</sup> We have tried this antibody on several specimens but results were less convincing compared to referred papers and further investigation was deemed unwarranted at this point. However, it might be worth revisiting with a larger number of specimens in the future.

A recent publication examined the expression of several markers including rabbit polyclonal amelogenin in CAA and oral SCC. There was no amelogenin expression in either neoplasm.<sup>101</sup> This further supports our results. However, the same publication demonstrated some promising results allowing to discriminate CAA from oral SCC using several markers, a few worth noting such as AE1/AE3 were expressed in SCC but not expressed in CAA<sup>101</sup> (this marker was expressed in odontogenic epithelium in a previous canine study<sup>11,98</sup>) and diffuse nuclear labeling for p63 in odontogenic carcinoma.<sup>93</sup> The already mentioned AE1/AE3, calretinin and p63 (a transcription factor of the p53 gene family) in particular seem interesting and might warrant further investigation in feline counterparts. Slightly different results were obtained by another research team where feline cutaneous spindle cell SCCs expressed CK5/6 (17/18, 94%), and AE1/AE3 (15/18, 83%), similar to canine OSCC. But expression of p63 protein was also found (18/18, 100%), which was not expressed in canine OSCC and there was no immunolabeling for CK8/18.<sup>111</sup> Also opposite results were obtained in a 2012 study with calretinin expression in canine oral SCC and CAA.<sup>96</sup>

Human oral pathology is a much more researched field when it comes to oral neoplasia. An impressive number of antibodies was tested in a publication from Korea. The study compared IHC profiles of peripheral ameloblastoma (PA) and oral basal cell carcinoma (OBCC). PA expressed ameloblastin, KL1, p63, carcinoembryonic antigen, focal adhesion kinase, and cathepsin K, and was slightly positive for amelogenin, Krox-25, E-cadherin, and PTCH1, OBCC expressed EpCam, matrix metalloprotease (MMP)-1,  $\alpha$ 1-antitrypsin, cytokeratin-7, p53, survivin, pAKT1, transforming growth factor- $\beta$ 1, NRAS, TGase-1, and tumor nescrosis factor- $\alpha$ , and consistently positive for  $\beta$ -catenin, MMP-2, cathepsin G, TGase-2, SOS-1, sonic hedgehog, and the  $\beta$  defensins-1, -2, -3. <sup>97</sup> It would be interesting to do a similar range of antibodies on feline counterparts, although it might be cost prohibitive for some institutions.

Other possible project could be to do immunohistochemistry with primary antibodies for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) on a group of feline OSCC and ameloblastoma. According to a 2016 study, cancer associated fibroblasts positive for  $\alpha$ -SMA were more numerous in aggressive neoplasms and increased numbers were associated with a worse prognosis.<sup>67</sup> Hypothetically ameloblastoma as a less aggressive, benign lesion should have relatively low numbers or absence

of cancer associated fibroblasts. If proven correctly, it might become an easily accessible, simple, and inexpensive ancillary diagnostic tool for veterinary pathologists.

Lastly, an identified group of undifferentiated malignant round cells neoplasms could be used as a case series project to further define the neoplasia type, perhaps with a use of several IHC markers as an IHC panel. Melanocytic neoplasia, soft tissue sarcoma, or recently identified oral histiocytic sarcoma could be among the differential diagnoses.<sup>74,112,113</sup>

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