Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
POSTGRADUATE DENTAL COLLEGE
NAVAL POSTGRADUATE DENTAL SCHOOL
8955 WOOD ROAD
BETHESDA, MARYLAND 20889

THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: Immunohistochemical marker expresson in the lateral peridontal cyst and its variants

Name of Candidate: Adam R. Ochsner
Master of Science Degree
June 01, 2021

THESIS/MANUSCRIPT APPROVED: DATE:

6/10/21

Bradley E. Jones
ORAL & MAXILLOFACIAL PATHOLOGY DEPARTMENT, NAVAL POSTGRADUATE DENTAL SCHOOL
Committee Chairperson

6/10/21

Kerry B. Baumann
ORAL & MAXILLOFACIAL PATHOLOGY DEPARTMENT, NAVAL POSTGRADUATE DENTAL SCHOOL
Committee Member

6/10/21

Glen M. Imamura
RESEARCH DEPARTMENT, NAVAL POSTGRADUATE DENTAL SCHOOL
Committee Member

IMMUNOHISTOCHEMICAL MARKER EXPRESSION IN THE LATERAL PERIODONTAL CYST AND ITS VARIANTS

by

Adam R. Ochsner
LTC, Dental Corps
United States Army

A thesis submitted to the Faculty of the
Oral & Maxillofacial Pathology Graduate Program
Naval Postgraduate Dental School
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science
in Oral Biology
June 2021

ACKNOWLEDGMENTS

Thank you Dr. Robert Redman for organizing this study and providing the cell count data and photomicrographs. Thank you Lyvouch Filkoski for performing the immunohistochemistry.

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

ABSTRACT

IMMUNOHISTOCHEMICAL MARKER EXPRESSION IN THE LATERAL PERIODONTAL CYST AND ITS VARIANTS

ADAM R. OCHSNER, DDS

Directed by: Dr. Bradley Jones DDS, MS - Program Director, Oral & Maxillofacial Pathology Residency, Naval Postgraduate Dental School

Introduction: The lateral periodontal cyst (LPC), gingival cyst of the adult (GCA) and botryoid odontogenic cyst (BOC) are developmental odontogenic cysts. BOC is unique among the three because of its more aggressive behavior and higher recurrence rate. A pilot study by Redman et al (2017) evaluated a BOC, GCAs and LPCs using Ki-67, p53, BCL-2 and caspase-3 stains. The single BOC sample showed elevated BCL-2 and p53 levels when compared to the other variants. **Objectives**: This follow-up study evaluated if there were significant differences in proliferative activity and apoptosis of cells within the epithelial linings of GCA, LPC and BOC. Additionally, it explored Ki-67, p53, BCL-2 and caspase-3 activity in a larger cohort of specimens. **Methods**: Archived formalin fixed paraffin embedded tissue blocks of BOC (15 specimens), LPC (6 specimens), GCA (6 specimens) and 3 odontogenic keratocysts (OKCs) were prepared for immunohistochemical evaluation. Diagnoses of individual specimens were confirmed using hematoxylin and eosin. The specimens were then stained with Ki-67, p53, BCL2 and caspase-3. A labeling index (LI) was determined for each specimen by counting the number of positively labeled epithelial cells per 1000 epithelial cells. A mean LI + standard deviation (SD) was then established for each immunohistochemical marker and cyst type in order to perform statistical analysis ($p<0.05$). GCAs, LPCs and BOCs served as test samples and the three OKCs as control. **Results**: A statistically significant

difference in mean LI was found in BCL-2 stained BOCs when compared to BCL-2 stained GCAs and LPCs. p53 staining was not elevated in BOCs when compared to GCAs and LPCs. **Conclusions:** Within the limitations of this study, a certain subset of BOCs may demonstrate an extremely elevated level of expression of the anti-apoptotic marker BCL-2 when compared to GCAs and LPCs. Factors associated with apoptosis could play a role in the more aggressive behavior of BOC.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATONS

BCOMFP	Board Certified Oral & Maxillofacial Pathologist
BOC	Botryoid Odontogenic Cyst
CA	California
DAB	Diaminobenzidine
DC	District of Columbia
DCVAMC	Department of Veteran Affairs Medical Center
EDTA	Ethylenediamine Tetraacetic Acid
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
IAW	In Accordance With
Ig	Immunoglobulin
IHC	Immunohistochemical
IRB	Institutional Review Board
LI	Labeling Index
LPC	Lateral Periodontal Cyst
MD	Maryland
NH	New Hampshire
OKC	Odontogenic Keratocyst
SD	Standard deviation
TX	Texas
WHO	World Health Organization

CHAPTER 1: Introduction

The gingival cyst of the adult (GCA), lateral periodontal cyst (LPC) and botryoid odontogenic cyst (BOC) are considered closely related developmental odontogenic cysts based on a common site of predilection and nearly identical histologic features. Placement of these three cysts into the same nosologic-group termed "cysts of periodontal tissues" has been proposed because of these similarities (Menditti et al. 2018). The common site of predilection is in the mandible, positioned anteriorly to the first molars in the premolar-canine region in an interproximal location between two adjacent teeth. Shared histologic features of these cysts include a thin, non-keratinizing stratified squamous epithelial lining that is one to five cell layers thick with occasional epithelial whorls containing slightly clear cytoplasm but no mucous cells, a flat interface to the cyst wall and minimal to no inflammation (Betz 158). Clear cells, that upon close examination may be found in the epithelial lining of all three cysts, have been shown to contain PAS positive diastase digestible material consistent with the presence of glycogen (Siponen et al 2011, Greer 1988, Kar 2020, DeCarvalho 2010, Phelan 1988).

Despite many similarities, each of these cysts has unique features. GCAs are found in gingival soft tissue and can present with superficial bone erosion from pressure resorption that may result in a radiolucency (Giunta 2002). LPC and BOC are found in the alveolar bone, often appearing incidentally as well demarcated and often corticated radiolucent lesions with possible expansion of bone (El Naggar et al. 2017). The proposed histogenesis of GCA is from remnants of the dental lamina in the gingival or alveolar soft tissues (rests of Serres) while LPC and BOC may develop either from the rests of dental lamina, reduced enamel epithelium or rests of Malassez (El Naggar et al.

2017, Giunta 2002). GCA occurs with a slight female predilection while LPC and BOC show evidence of being equally distributed between sexes (El Naggar et al. 2017, Chrcanovic 2019).

BOC is extremely rare and is considered to be a multicystic variant of the more commonly encountered LPC. It presents with a multilocular radiographic appearance much more frequently than LPC and therefore should be included in the differential diagnosis of a long list of more common multilocular lesions of the jaw including ameloblastoma, odontogenic myxoma, central giant cell lesion, central hemangioma, odontogenic keratocyst (OKC) and glandular odontogenic cyst.

BOC often has a unique gross presentation when compared to GCA and LPC. In fact, the term 'botryoid' is derived from the Greek word "botrios", which literally means "bunch of grapes", and appropriately describes the BOC's characteristic multilocular gross appearance (deCarvalho 2010). The only consistent microscopic difference between BOC and LPC is the multicystic compartmentalization of the BOC compared to the unicystic LPC. This histologic distinction is important with regard to the treatment aspect and follow up period as LPC can be treated by surgical enucleation with a very low recurrence rate while BOC should be treated with careful surgical excision and requires close clinical follow up to due a significantly higher recurrence rate (Anuradha 2014).

A recent comprehensive literature review by Chrcanovic and Gomez focused on identifying clinical features that distinguish BOC from LPC. They found that BOCs were larger in size, had a higher propensity to occur in the mandible than maxilla, presented at

an older age, had a more frequent multilocular radiographic presentation and recurred at a higher rate compared to LPC (Chrcanovic 2019).

Controversy has existed regarding the true recurrence rate of BOC with some arguing for it being overreported and others for it being underreported. Currently, the Word Health Organization (WHO) states recurrence is documented in as many as 20% of cases (El-Naggar et al 2017). Initially, this may seem a conservative estimate if we factor in historic and recent literature of higher recurrence rates including Anuradha's estimate of 34% and estimates as high as 40% noted by both Santos and Liu. However, in light of the largest systematic review of BOCs of its kind involving 96 BOC cases compiled by Chrcanovic et al which recorded a recurrence rate of 21.7%, the WHO's estimate may be appropriate. By comparison, in the same systematic review, LPC and GCA each demonstrated a recurrence rate of less than 3% with sample sizes of 213 and 157 respectively (Chrcanovic 2019).

Reasons for the higher recurrence of BOC compared to LPC has been investigated, but a definitive answer remains unknown. The WHO Classification of Head and Neck Tumors, 4th edition suggests that recurrences are probably due to the multicystic nature of the lesion (El-Naggar et al 2017). Size and multilocular radiographic appearance are two clinical features that have also been proposed as main factors for recurrence (Mendez 598). According to Greer, the frequent multilocular radiographic and gross appearance supports the concept that it may be a problematic lesion for surgeons to remove. Similarly, Kaugars suggests a surgeon will face more difficulty in removing an entire multicompartamentalized BOC lesion because it is incompletely divided by bony septa. He presumes that any remnants left behind after

surgery may act as a nidus for future recurrence. Liu, citing Mendez, also recently stated that a high recurrence can be explained by difficulty in removing the entire multilocular lesion (Liu 2020). We take the stance of Kaugars and others who presume that any remnants left behind after surgery may act as a nidus for future recurrence.

BOC and odontogenic keratocyst (OKC) have both been designated as aggressive jaw cysts requiring special attention due to aggressive behavior and propensity for recurrence (Stoelinga 2). Unfortunately, due to its extreme rarity, very limited immunohistochemical (IHC) studies have been performed on the cystic lining of BOC. Conversely, IHC studies investigating markers of proliferation, apoptosis and cell cycle regulation in the epithelial linings of OKC are extensive.

A brief review and simple understanding of prevailing OKC staining patterns referenced from the literature is important. First, Ki-67, a marker of proliferation, is the most extensively studied and demonstrates a higher proliferation rate in OKC compared to other cyst types (Woo 2017). Specifically, expression of Ki-67 above the basal cell layer is characteristic in OKC (Hunter 2017). Next, p53, a marker of a protein product of the p53 suppressor gene that plays an important role in apoptosis, cell cycle and genetic stability, should stain basal and suprabasal cells in OKC (Fatemeh 2017, Woo 2017). Specifically, in a study by Tenorio, p53 was expressed in the cytoplasm of the basal cell layer and sometimes in the parabasal layer of OKC. Additionally, complete absence of expression was noted in the upper epithelial layers in all cases and in the basal layer in some cases (Tenorio 2018). For BCL-2, an anti-apoptotic marker, staining patterns in OKC are not as well defined. According to Kaczmarzyk, BCL-2 will demonstrate membranous-cytoplasmic staining in basal and suprabasal layers of the epithelial lining

of OKC (Kaczmarzyk 2018). On the contrary, Kichi and Diniz argued BCL-2 positive cells are recognized exclusively in the basal cell layer of OKCs with Kichi specifying that BCL-2 stained 96% of the OKC basal cells in their study. Similar to Kichi and Diniz, Tenorio's previously mentioned study found that BCL-2 was mainly observed in the basal cell layer in their study involving 20 OKC samples (Tenorio 2018). Lastly, da Costa found that caspase-3, a marker of cells actively undergoing apoptosis, shows cytoplasmic and nuclear expression in selected neoplastic cells of all epithelial layers of OKC and expression appeared less intense in the basal layer (da Costa 2018).

In a recent exploratory pilot study, Redman et al. identified unique immunohistochemical staining patterns in the epithelial lining of one BOC sample when compared to that of several LPC and GCA samples. Most notably, a higher expression of p53 and remarkably higher expression of BCL2 was found in the BOC sample (Redman 2017). Because these differences offer possible reasons for the more aggressive behavior of BOC compared to LPC and GCA, the primary goal of this study was to perform a similar study using a larger sample size to see the extent to which the unique staining pattern of BOC holds true. In this retrospective study, we will compare staining expression of Ki-67, p53, BCL-2 and caspase-3 among GCA, LPC and BOC samples. Three OKC samples will be used as a quality control to assure that the staining patterns for each marker are similar to that found in the previously mentioned literature.

CHAPTER 2: Materials and Methods

APPROVALS, PERMISSIONS AND LOGISTICS

The protocol for this study was approved by the Walter Reed National Military Medical Center Department of Research Programs and determined to be exempt from IRB review according to IAW 32 CFR 219.101 (b)(4). In this retrospective study, 30 formalin fixed paraffin embedded tissue blocks including 15 cases of BOC, six cases of LPC, six cases of GCA and three cases of OKC were retrieved from the archives of the Oral and Maxillofacial Pathology Department of the National Naval Postgraduate Dental School, Walter Reed-National Naval Medical Center, Bethesda, MD, and the Department of Oncology and Diagnostic Sciences, University of Maryland Dental School, Baltimore, MD. Permission for use of the tissue samples was obtained from the Institutional Review Boards of the participating institutions. Laboratory procedures were performed in the Histology Section of the Laboratory and Pathology Service of the Washington, DC Department of Veteran Affairs Medical Center (DCVAMC).

LABORATORY PROCEDURES

Multiple 4 micron thick sections were cut from each of the 30 tissue blocks and mounted on Superfrost Plus™ slides (Erie Scientific Co., Portsmouth, NH). One slide fabricated from each block was then stained with hematoxylin and eosin to reconfirm a diagnosis of either OKC, GCA, LPC or BOC and also to ensure the tissue was suitable and adequate for the study. Immunohistochemistry was performed on the remaining slides obtained from the blocks as follows:

For all immunostaining, The EnVision™ system was used in conjunction with dual link HRP in a Dako (Carpenteria, CA) autostainer. All reactions were enhanced by antigen retrieval in appropriate EnVision™ Flex Target Retrieval solutions (see below) in a 97° C water bath. The slides were then incubated with one of the following antibodies:

- Pre-diluted monoclonal mouse anti-human Ki-67 antigen clone MIB-1 (Agilent Dako Technologies, Santa Clara, CA) reactions enhanced by antigen retrieval in pH 6.1 citrate
- Monoclonal mouse anti-human isotype IgG_{2a} (kappa light chain) anti-caspase-3 antibody (E-8, sc7272) (Santa Cruz Biotechnologies, Dallas, TX) provided at 200 μg/ml (a 1:300 dilution was determined optimal for results using the control tissue); reactions enhanced by antigen retrieval in pH 6.1 citrate and further enhanced using a mouse linker.
- Prediluted monoclonal mouse anti-human p53 protein clone DO-7 (Agilent Dako Technologies, Santa Clara, CA); reactions enhanced by antigen retrieval in pH 9 Tris/EDTA
- Prediluted monoclonal mouse anti-human BCL2 oncoprotein clone 124 (Agilent Dako Technologies, Santa Clara, CA) reactions enhanced by antigen retrieval in pH 9 Tris/EDTA

Sections were localized as a brown precipitate after reacting with 3% H_20_2 and 3,3 diaminobenzidine (DAB). Native peroxidase was quenched using 3% H_20_2. Lastly, all slides were counterstained with hematoxylin, dehydrated, and coverslipped for analysis.

Tonsil was used as a positive control for Ki-67, Bcl-2 and caspase-3 stains and an adenocarcinoma of the colon was used as positive control for p53 stains. Negative controls were established by replacing the primary antibodies with a cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies (Basile 2020).

CELL COUNTING

A labeling index (LI) was determined for each specimen by counting the number of positive staining epithelial cells per 1000 epithelial cells at high power magnification (40X) and multiplying by 100. Counts were independently performed at separate times by two Board Certified Oral & Maxillofacial Pathologists (BCOMFPs) and then averaged together. If a specimen was found for which there was more than a 2-fold difference in initial counts, a third count was performed by another BCOMFP and the three counts were averaged together. The mean LI + standard deviation (SD) was then determined for each immunohistochemical marker and cyst type in order to perform statistical analysis (Basile 2020).

STATISTICAL ANALYSIS

A Kruskal-Wallis test was performed to investigate differences in LIs between GCA, LPC and BOC. The Wilcoxon rank-sum test was performed to investigate differences between the LI of BOC and the combined LI of LPC and GCA (LPC+GCA). Differences were considered statistically significant at $p \leq 0.05$ for both tests. OKC samples, serving as a validating control in this study, are not part of the statistical analysis.

Subjective Analysis

Additionally, a subjective analysis of differences in the staining patterns by cyst type and immunohistochemical marker was performed.

CHAPTER 3: Results

STATISTICAL ANALYSIS

A large range in LIs for the BCL2 staining of BOCs (0-100) and LPCs (0.3-50), the p53 staining of BOCs (0-70) and LPCs (0-50) and the caspase-3 staining of BOCs (0-80) and LPCs (0-90) was noted. Conversely, a much smaller range in LIs for Ki-67 (0-5) was demonstrated across the three cyst types. GCA demonstrated the lowest range in LIs of the three cysts we compared.

Results for the Kruskal-Wallis test are shown in Table 1. Data collection for caspase-3 was insufficient to be included in the Kruskal-Wallis test.

Table 1. Kruskal-Wallis test

	Ki-67	p53	BCL-2
BOC	(0.54±1.0)	(6.83±12.78)	(23.95±36.10)
LPC	(0.78±1.31)	(20.57±16.80)	(11.99±6.80)
GCA	(0.21±0.33)	(0±0)	(1.12±1.50)
p value	0.87	0.17	0.02
significant?	no	no	yes

( ) = mean LI±SD

A statistically significant difference in mean LI was found in BCL-2 staining of BOC when compared to BCL-2 staining of LPC and BCL-2 staining of GCA. The mean LI hierarchy for both Ki-67 and p53 was LPC>BOC>GCA while the mean LI hierarchy for BCL-2 was BOC>LPC>GCA.

The results for the Wilcoxon Rank Sum Test are shown in Table 2. Although differences between mean LI for BCL-2 staining of BOCs and mean LI for BCL-2 staining of LPC+GCA approached significance, we ultimately found no significant differences between mean LI staining of BOC and LPC+GCA for any of the markers. The mean LI hierarchy for Ki-67, p53 and caspase-3 were each LPC+GCA>BOC while the mean LI hierarchy for BCL-2 was BOC>LPC+GCA.

Table 2. Wilcoxon Rank Sum Test using 2 tailed hypothesis.

	Ki-67	p53	BCL-2	caspase-3
BOC	(0.54±1.0)	(6.83±12.78)	(23.95±36.10)	(9.57±8.28)
LPC+GCA	(0.55±1.08)	(11.43±16.17)	(6.59±7.31)	(12.50±12.23)
p value	0.99	0.41	0.11	0.52
significant?	no	no	no	no

( ) = mean LI±SD

SUBJECTIVE ANALYSIS (STAINING PATTERNS)

Staining patterns of the quality control OKC specimens for Ki-67, p53, BCL-2 and caspase-3 were similar to findings reported in the previously mentioned literature. Ki-67 stains revealed strong nuclear labeling of scattered cells predominately in the suprabasilar layer cells with some focal weak staining of scattered basal cells (Fig 1a). p53 demonstrated strong nuclear staining of scattered cells in both basal and suprabasilar layers with predominance of staining in the basal layer (Fig 1b). BCL-2 staining was strong in cells of the basal layer with some light staining of one or two suprabasilar cell layers, sparing the remainder of the epithelium (Fig 1c). Finally, despite uncommon staining of caspase-3 overall in the OKC samples, a diffuse, light cytoplasmic staining pattern involving the intermediate to upper epithelial layers and sparing the basal layer was observed when staining was present (Fig 1d).

The mean LI+SD of the three quality control OKC samples are shown in Table 3. No statistical comparison against the tested cysts (GCA, LPC, BOC) was performed.

Table 3. Mean LI±SD of the three quality control OKC samples.

	Ki-67	p53	BCL-2	caspase-3
OKC	1.51±1.39	1.85±1.73	17.5±0	9.44±8.20

Importantly, there were distinct trends in the staining patterns within the epithelial cystic linings of the tested cysts (GCA vs. LPC vs. BOC) for each immunohistochemical marker that we tested (Ki-67, p53, BCL-2, and caspase-3). First, Ki-67 labeled mostly basal cells in all three cyst types with some focal staining in plaque-like thickened areas (Fig 2 a, b & c). The hierarchy in density of staining for Ki-67 was LPC>BOC>GCA (p value, 0.87).

Next, p53 labeled scattered cells throughout all cell layers especially in LPC and BOC while staining occurred less common in GCA to the point of being negligible (Fig 3 a, b &c) (Basile 2020). The hierarchy in density of staining for p53 was LPC>BOC>GCA (p value, 0.17).

For BCL-2, all three cysts stained throughout all layers of epithelial lining (Fig 4 a,b,c). The hierarchy in density of staining for BCL-2 was BOC>LPC>GCA and was significantly different ($p<0.05$) between cysts. Notably, Fig 4c shows one of the two 'outlier' BOC samples in which the entire epithelium is almost completely stained by BCL-2 sparing only the most superficial layer of cells. This "outlier" pattern was identical to the pattern seen in the sole BOC sample in our initial pilot study. The majority of the BOC samples (13/15) did not stain anywhere near as extensively as these outliers. It is evident that the BOC displayed a less predictable staining pattern with BCL-2 than either LPC and GCA in this study.

Finally, staining of caspase-3 was uncommon in all three cyst types. Moderate to strong labeling was mostly in basal cells in all three cyst types when present (Fig 5 a, b & c). Note that Fig 5b shows a sample with complete absence of staining (Basile 2020). A

hierarchy in density of staining for caspase-3 was not clearly evident due to limited sampling.

CHAPTER 4: Discussion

The staining of OKC samples served as a useful quality control in our study. Importantly, the Ki-67, p53, BCL-2 and caspase-3 staining patterns for our three OKC samples closely mirrored those found in the OKC literature. Considering this fact, along with all positive and negative controls reacting appropriately throughout the laboratory procedure, we feel this to be a valid study during which the potential for introduction of procedural error was sufficiently minimized and all IHCs were appropriately validated.

One unexpected finding in our study was a low mean LI for Ki-67 in our three OKC control samples (1.51±1.39). By comparison, in recent studies, Dandena et al reported a Ki-67 labeling index of 7.4±0.54 among 20 OKC samples, Brito-Mendoza reported a Ki-67 labeling index of 17.71±6.29 among 22 OKC samples and Modi reported a Ki-67 labeling index of 12.76±4.78 among 15 OKC samples. Reasons for low Ki-67 proliferative index for our OKC samples are unclear but we note that according to Feng et al. factors that lead to poor reproducibility of Ki-67 scoring results may include type of biopsy, time to fixative, type of antibody, method of reading and area of reading. (Feng et al. 2020).

Degree of inflammation was a variable considered in preparation of our OKC control samples because a recent study revealed significant variability in the amount of p53 staining in OKC based on the degree of inflammation present. Specifically, inflamed OKCs demonstrated significantly higher LI (17.3±7.1) compared to non-inflamed OKCs (5.4±4.8) among 18 inflamed OKCs and 16 non-inflamed OKCs (Fatimeh 2017). With this in mind, we intentionally chose OKC samples that contained traditional morphology and negligible to no inflammation, and, as a result, selected against inflammation as a

possible confounding factor which could artificially inflate p53 expression in our control samples. As mentioned in our introduction, GCA, LPC and BOC by definition will have minimal to no inflammation, and therefore assessing the degree of inflammation when selecting test cysts in preparation for this study was deemed unnecessary.

From a statistical standpoint, the mean LI of only three samples is of little value in making comparisons, although, as one would expect, we did find that the Ki-67 mean labeling index was higher in the OKC control samples than that of GCA, LPC and BOC samples. However, as mentioned, it was still lower than expected when we use prior studies as a benchmark for comparison. The primary purpose in our staining of OKCs was to demonstrate that staining patterns for the markers we used were in keeping with that found in the OKC literature. Because all stains were successfully validated against the three OKC control samples we were able to make comparisons of GCA, LPC and BOC with the hope of achieving the primary objective of this study.

Inherent weaknesses in our study design may limit the strength of our findings. First, our sample sizes, although larger than the initial pilot study that included only one BOC sample along with four LPCs and four GCAs, should ideally be larger for all cyst types. Also, due to limited tissue in some instances, there was some difficulty in obtaining definitive cell counts 'per 1000 cells' for every sample. This was especially true for GCA samples that were stained with caspase-3 and ultimately resulted in exclusion from the Kruskal-Wallis analysis. A larger sample size with more samples to select from would have alleviated concerns regarding a possible limitation of data collection to the point of losing statistical relevance by ensuring immediate replacement of any samples demonstrating a scant amount of tissue or of questionable quality. Next,

data for our cell counts was obtained from only two BCOMFPs for most samples, and, in the best-case scenario, it was obtained from the average of three BCOMFPs. Counts from a larger number of BCOMFPs would theoretically reduce random error in measurements by averaging over a larger number of observations, increase the precision of measurements, and reduce the likelihood of researcher bias. Lastly, transferring images to a grid via a semiquantitative method as described by Bologna-Molina et al or using an image analysis program could have facilitated cell counting, making the process more efficient and possibly improving the accuracy of cell counts by reducing human error. Neither of these methods were incorporated into our study.

We recognize that a degree of uncertainty is factored into our results because there was a considerable range in the mean LIs for p53, BCL-2 and caspase-3 staining of LPC and BOC samples. Specifically, the large range in LIs resulted in high standard deviations. Most notably, the highest standard deviation was found in the BCL-2 stained BOCs (SD=36.10), the results of which are central to achieving this study's primary goal. Importantly, we understand that because standard deviation is commonly used to measure the confidence in statistical conclusions and may serve as a measure of uncertainty, any conclusions we draw will naturally carry along with them this level of mathematical uncertainty. Additionally, the unexpected low mean LI for Ki-67 staining of our OKC samples adds some degree of uncertainty in our study. With these samples serving as our control, it is feasible that our calculated mean LI of Ki-67 for all cyst types studied (BOC, LPC, and GCA) may be an underestimate of their "true" or "actual" Ki-67 mean LI. That being said, the relative proliferative indices we found between these cysts are more than likely accurate due to the validation of our stains.

Reasons for our two BOC BCL-2 “outliers”, which assuredly contributed to high mean LI and standard deviation values, are unclear and we can only speculate as to why some BOC samples stained with an extremely high LI for BCL-2 and others did not. We deduce that this was not an “artificially high” staining pattern resulting from randomly introduced error because the sole BOC sample in our prior pilot study stained in an identical fashion and our BCL-2 IHCs were appropriately validated using OKC, the pattern and labeling index of which matched that expected from the literature.

We are unable to draw absolute conclusions regarding these “outliers” other than that they are a possibility in BOCs when immunolabeled with BCL-2. The clinical significance of this staining in regards to correlation with more aggressive behavior and higher recurrence potential, likewise, can only be speculated until a larger collection of samples is analyzed along with clinical correlation. It is our hope that future studies may further investigate the unpredictable and seemingly bipolar staining pattern in BOCs stained with BCL-2. Additionally, we envision future studies improving upon our other mentioned weaknesses, keeping in mind that a study with a larger sample size would be a difficult undertaking due to the rarity of BOC.

CHAPTER 5: Conclusions

Within the limitations of this retrospective study, a certain subset of BOC may demonstrate an extremely elevated level of expression of the anti-apoptotic marker BCL-2 when compared to the cystic linings in the closely related LPC and GCA. The cystic linings of GCA, LPC and BOC show similar levels and patterns of expression of Ki-67. p53 staining of GCA is negligible compared to either LPC or BOC but not statistically significant. The staining patterns of Ki-67, p53, BCL-2 and caspase-3 are predictable in both GCA and LPC and the staining patterns of Ki-67, p53 and caspase-3 are predictable in BOC. Conversely, the staining pattern and level expression of BCL-2 is less predictable in BOC than either LPC or GCA.

Conclusively, the extremely elevated BCL-2 LI of the sole BOC in our initial pilot study may simply represent an outlier staining pattern which is found in only a small percentage of BOCs. The elevated p53 LI of the sole BOC in our pilot study was not evident in this follow up study involving a larger sample size. Future studies may elucidate the clinical significance, if any, of our observations.

FIGURES

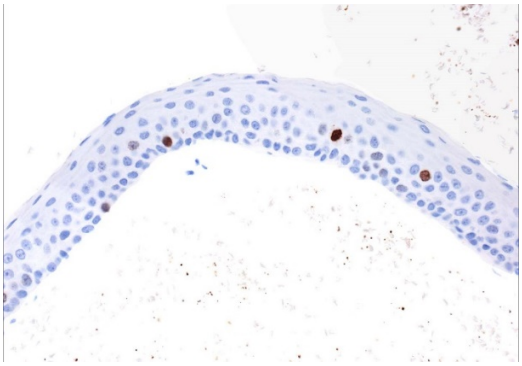

Fig1a. OKC - Ki-67 immunostain

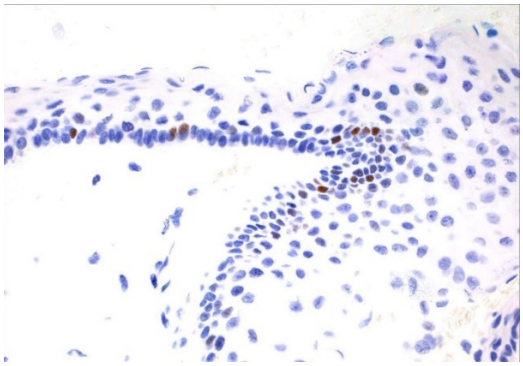

Fig 1b. OKC - p53 immunostain

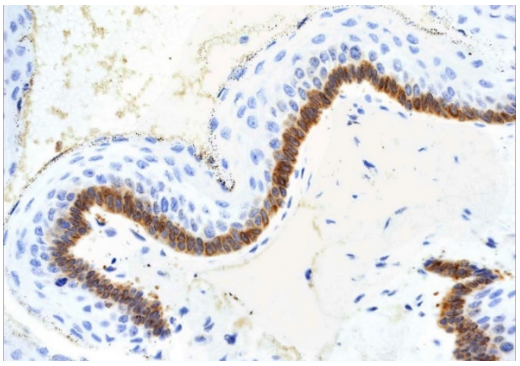

Fig 1c. OKC – BCL-2 immunostain

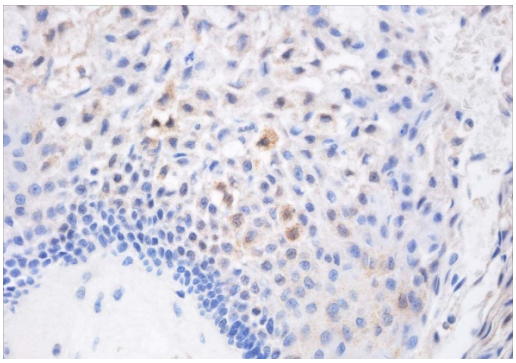

Fig 1d. OKC – caspase-3 immunostain

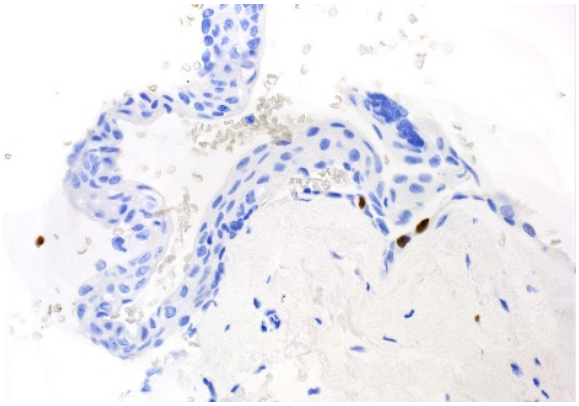

Fig 2a. GCA – Ki-67 immunostain.

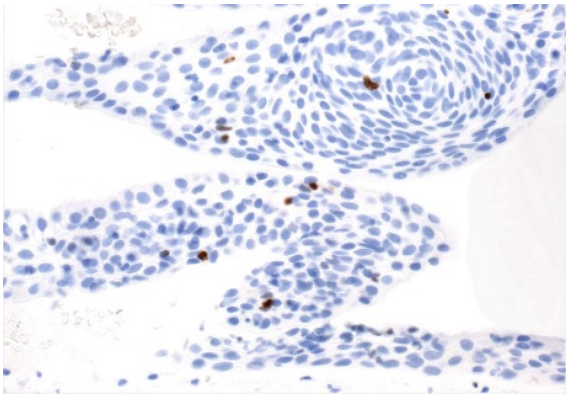

Fig 2b. LPC – Ki-67 immunostain.

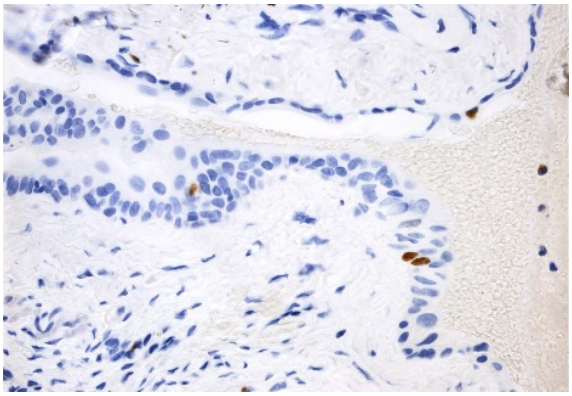

Fig 2c. BOC – Ki-67 immunostain

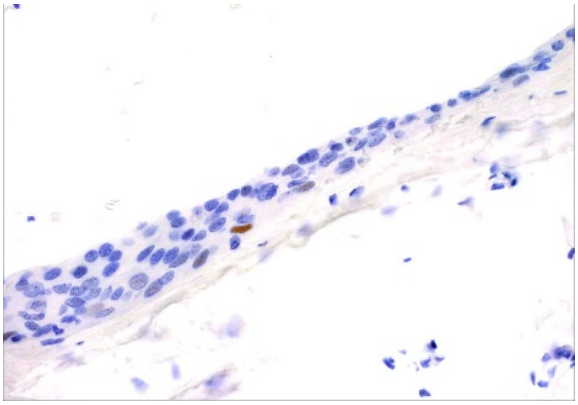

Fig 3a. GCA-p53 immunostain.

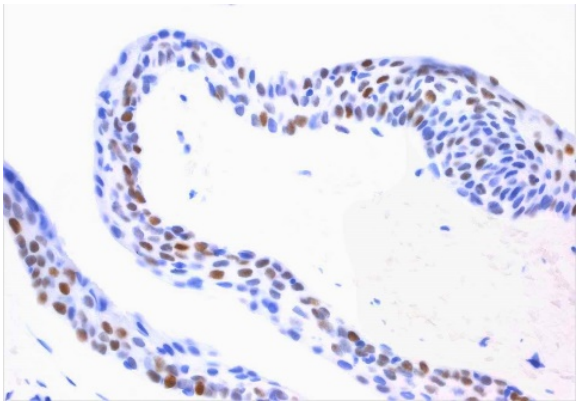

Fig 3b. LPC – p53 immunostain

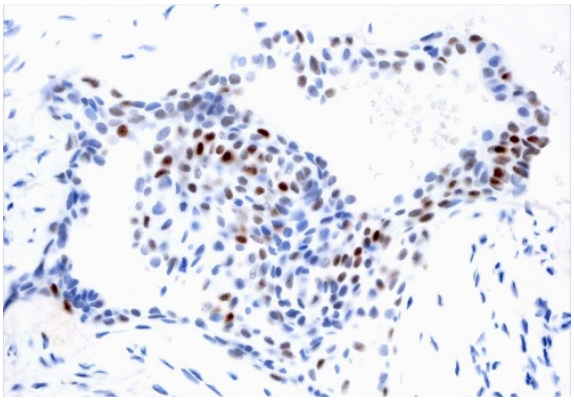

Fig 3c. BOC – p53 immunostain

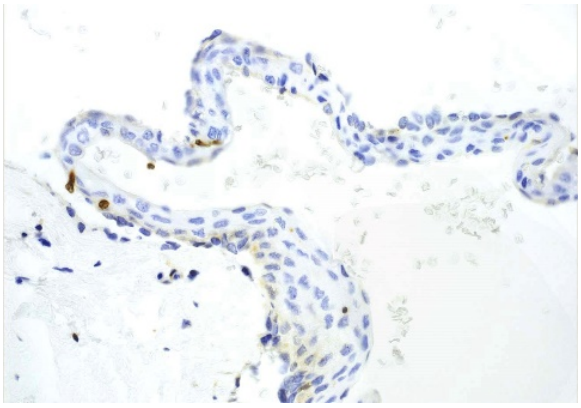

Fig 4a. GCA – BCL-2 immunostain.

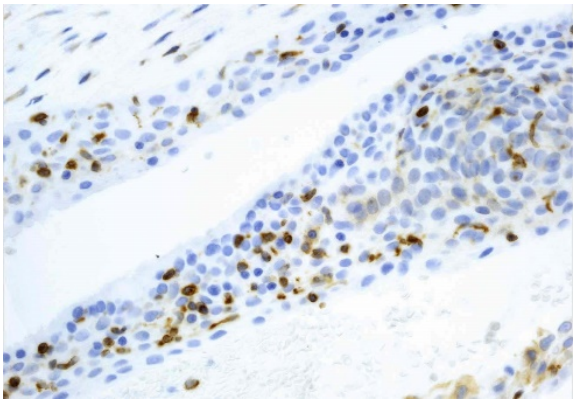

Fig 4b. LPC – BCL-2 immunostain

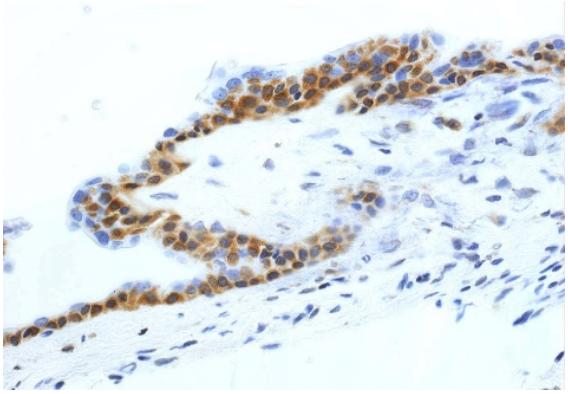

Fig 4c. BOC – BCL2- immunostain

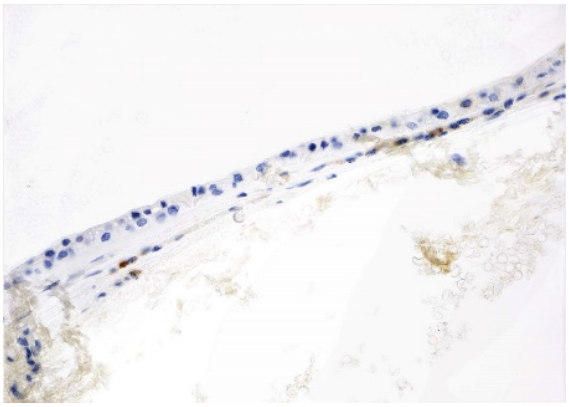

Fig 5a. GCA-caspase-3 immunostain

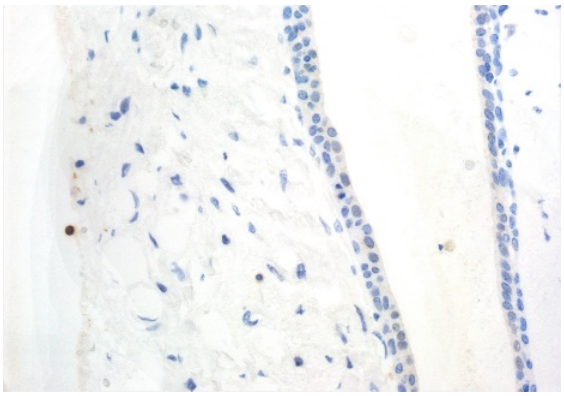

Fig 5b. LPC – caspase-3 immunostain

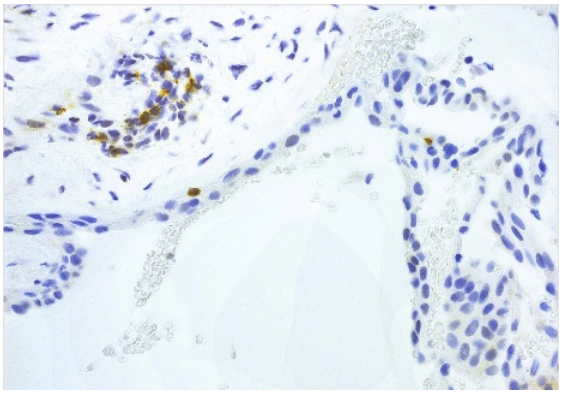

Fig 5c. BOC – caspase-3 immunostain

REFERENCES

Anuradha A, Urmila U, Vijay Sriniva G, Deviramisetty S, Puneeth HK. Botryoid odontogenic cyst: a diagnostic chaos. *Journal of Clinical and Diagnostic Research*. 2014 Dec, Vol-8(12): ZD11-ZD13. DOI: 10.7860/JCDR/2014/10136.5284.

Basile JR, Castle JT, Redman RS. Immunohistochemical profile of the anti-apoptosis, apoptosis and proliferation markers, BCL-2, caspase-3, p53, and Ki-67, in botryoid odontogenic cysts compared to lateral periodontal cysts and gingival cysts in the adult. *Biotech & Histochemistry*. 2020 Jul 9; 1-6.doi 10.1080/10520295.2020.1790660.

Betz SJ, Padilla RJ. 2019. Jaw and Bones of the Head and Face in Practical Head and Neck Pathology, *Practical Anatomic Pathology*, D. Elliot Range, X. "Sara" Jiang (eds.), 158. Switzerland: Springer Nature.

Bologna-Molina R, Damian-Matsumura P, Molina-Frechero N. An easy cell counting method for immunohistochemistry that does not use an image analysis program. *Histopathology*, vol. 59, no. 4, pp. 801-803, 2011.

Brito-Mendoza L, Bologna-Molina R, Irigoyen-Camacho E, Martinez G, Sánchez-Romero, Mosqueda-Taylor A. A Comparison of Ki67, Syndecan-1 (CD138), and Molecular RANK, RANKL, and OPG Triad Expression in Odontogenic Keratocysts, Unicystic Ameloblastoma, and Dentigerous Cysts. *Disease Markers*. Volume 2018, Article ID 7048531, 7 pages.

Chrcanovic BR, Gomez RS. Gingival cyst of the adult, lateral periodontal cyst, and botryoid odontogenic cyst: An updated systematic review. *Oral Dis*. 2019; 25:26-33. https://doi.org/10.1111/odi.12808

da Costa, N.M.M., de Siqueira, A.S., Ribeiro, A.L.R. *et al.* Role of HIF-1α and CASPASE-3 in cystogenesis of odontogenic cysts and tumors. *Clin Oral Invest* 22**,** 141–149 (2018). https://doi.org/10.1007/s00784-017-2090-6

Dandena VK, Thimmaiah SY, Kiresur, MA, Hunsigi P, Toy S, Rashmi M. A comparative study of odontogenic keratocyst and orthokeratinized cyst using Ki67 and smooth muscle actin. *J Oral Maxillofac Pathol*. 2017 Sep-Dec; 21 (3): 458-459.

De Carvalho LF, Lima CF, Cabral LA, Brandao AA, Almeida JD. Lateral PeriodontalCyst: a Case Report and Literature Review. *J Oral Maxillofac Res* 2010; 1 (4): e5. DOI: 10.5037/jomr.2010.1405.

Diniz MG, Gomes CC, de Castro WH, Guimaraes AL, De Paula AM, Amm H, RenC, MacDougall, Gomez RS. miR-15a/16-1 influences BCL2 expression in keratocystic odontogenic tumors. *Cell. Oncol*. 35: 285-291 (2012).

El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ., Eds. (2017) World Health Organization Classification of Head and Neck Tumors, 4th ed.., vol 9. IARC Press, Lyon, France. p 236-268.

Fatemeh M, Sepideh A, Sara BS, Nazanin M. p53 protein expression in dental follicle, dentigerous cyst, odontogenic keratocyst, and inflammatory subtypes of cysts: an immunohistochemical study. *Oman Medical Journal* (2017), Vol 32, No. 3: 227-232.

Feng, M., Deng, Y., Yang, L. *et al.* Automated quantitative analysis of Ki-67 staining and HE images recognition and registration based on whole tissue sections in breast carcinoma. *Diagn Pathol* 15**,** 65 (2020). https://doi.org/10.1186/s13000-020-00957-5

Giunta JL. Gingival cysts in the adult. *J. Periodontal*. 73: 827-831 (2002).

Greer RO Jr., Johnson M. Botryoid odontogenic cyst Clinicopathologic analysis of ten cases with three recurrences. *J. Oral Maxillofac. Surg*. 46:574-579 (1988).

Hunter KD, Speight PM. The diagnostic usefulness of immunohistochemistry for odontogenic lesions. *Head and Neck Pathol* (2014) 8:392-399.

Kaczmarzyk T, Kisielowski K, Koszowski R, Rynkiewicz M, Gawelek E, Babiuch K, Bednarczyk A, Drozdzowska B. Investigation of clinicopathological parameters and expression of COX-2, bcl-2, PCNA, and p53 in primary and recurrent sporadic odontogenic keratocysts. *Clincal Oral Investigations* (2018) 22: 3097-3106.

Kar A, Pattnaik K, Kar T, Biswal P, Mishra C, Guru L. Clear cell lesions in pathology: Histomorphologic approach to diagnosis *J Pathol Microbiol* 2020: 63: 177-87\

Kaugars, GE. Botryoid odontogenic cyst. *Oral Surg. Oral Med. Oral Pathol*. 1986: Vol 62, No 5: 555-559,ISSN 0030-4220, https://doi.org/10.1016/0030-4220(86)90320-8.

Kichi E. Enokiya Y, Muramatsu T, Hashimoto S, Inoue T, Abiko Y, Shimono M. Cell proliferation, apoptosis and apoptosis-related factors in odontogenic keratocysts and dentigerous cysts. *J. Oral Pathol*. Med. 34: 280-286 (2005).

Liu C, Samani M, Kwok J, Sproat C. Conservative management of botryoid odontogenic cysts using Carnoy's solution. Letters to the editor/*British Journal of Oral and Maxillofacial Surgery* 2020; 58: 245-247.

Mendez P, Junquera L, Gallego L, Baladron J. Botryoid odontogenic cyst: clinical and pathological analysis in relation to recurrence. *Med. Oral Patol. Oral. Cir. Bucal*. 12: 594-598 (2007).

Menditti D, Laino L, Domenico MD, Troiano G, Guglielmotti M, Sava S, Mezzogiorno A, Baldi A. Cysts and Pseudocysts of the Oral Cavity: Revision of the Literature and a New Proposed Classification. *In Vivo* 2018; 32: 999-1007.

Modi TG, Chalishazar M, Kumar M. Expression of Ki-67 in odontogenic cysts: A comparative study between odontogenic keratocysts, radicular cysts and dentigerous

cysts. *J Oral Maxillofac Pathol*. 2018 Jan-Apr; 22 (1): 146. DOI: 10.4103/jomfp.JOMFP_94_16.

Phelan JA, Kritchman D, Fusco-Ramer M, Freedman PD, Lumerman H. Recurrent botryoid odontogenic cyst (lateral periodontal cyst). *Oral Surg. Oral Med. Oral Pathol*. 66: 345-348 (1988).

Redman RS, Paal E, Chauhan S, Avers R, Bayley N. (2017) Botryoid odontogenic cyst. Exploration of proliferative activity, apotosis and expression of TP53 and BCL2 compared to the histologically identical periodontal and gingival cysts. *Biotech. Histochem*. 92:569-576.

Santos PPRde A, Freitas VS, Freitas Rde A, Pinto LP, Souza LB. Botryoid odontogenic cyst: A clinicopathologic study of 10 cases. *Ann. Diag. Pathol*. 15:221-224 (2011).

Siponen M, Neville BW, Damm DD, Allen CM. Multifocal lateral periodontal cysts: a report of 4 cases and review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011; 111: 225-233.

Stoelinga PJW. The Management of Aggressive Cysts of the Jaws. *J. Maxillofac Oral Surg*. (Jan-Mar 2012) 11(1):2-12.

Tenório J-dR, Santana T, Queiroz S-I-M-L, de Oliveira D-H-I-P, Queiroz L-M-G. Apoptosis and cell cycle aberrations in epithelial odontogenic lesions: An evidence by the expression of p53, Bcl-2 and Bax. *Med Oral Patol Oral Cir Bucal*. 2018 Mar 1; 23 (2) e120-5.

Woo, S-B. Oral pathology: a comprehensive atlas and text. 2nd Edition Philadelphia, PA: Elsevier, 370-371; 379-381 (2017).