The Expression and Potential Significance of Human Kallikreins 6, 7, 8, 10, 13, and 14 in the Epithelium of Selected Odontogenic Cysts and Tumors of Variably Aggressive Biological Behaviour

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THE EXPRESSION AND POTENTIAL SIGNIFICANCE OF HUMAN KALLIKREINS 6, 7, 8, 10, 13, AND 14 IN THE EPITHELIUM OF SELECTED ODONTOGENIC CYSTS AND TUMORS OF VARIABLY AGGRESSIVE BIOLOGICAL BEHAVIOUR.

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Pathology

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This study analyzed the immunohistochemical staining, localization and relative concentrations of human kallikreins (KLKs) 6,7,8,10,13,14 to determine if there is a predilection for odontogenic epithelium and if differential staining could predict biological behavior of selected odontogenic lesions.

We found that all KLKs studied were present in the epithelium of all lesions examined. However, there were significant differences in location of intra-epithelial staining and staining scores between the lesions. There was no significant, unexplainable difference in staining between odontogenic cysts and the control cyst indicating no specific role in odontogenic cystogenesis. However, the odontogenic neoplasms showed statistically significant increased expression of KLK13, supported by increases in KLK10, suggesting a role in odontogenic neoplasia. Our data indicate that this is a secondary role, not related to cell cycling, but likely related to differentiation. We suggest that KLKs 13 and 10 may be useful as in-situ markers on tissue sections to screen for aggressiveness of odontogenic tumors.

KEYWORDS: Odontogenic, Cysts, Odontogenic Tumors, Human Tissue Kallikreins, Tumor Biomarkers.
DEDICATION

I dedicate my thesis to my late grandparents, Mercedes and George Woodford,
my living and loving grandparents, Margaret and Morgan Hinchey, my
wonderful parents, Anne and Rex Woodford, and especially, my supportive
fiancée, Jordan Dyke and my darling niece, Abigaile.
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# Table of Contents

Abstract........................................................................................................................................ ii  
Dedication .................................................................................................................................. iii  
Acknowledgements ................................................................................................................. iv  
Table of Contents ...................................................................................................................... v  
List of Tables ................................................................................................................................ ix  
List of Figures ................................................................................................................................ ix  
List of Abbreviations .............................................................................................................. xi

Chapter 1: Introduction and Literature Review ............................................................ 1  
1.1 Tooth Development ............................................................................................................. 1  
   1.1.1 Tooth Function ............................................................................................................. 1  
   1.1.2 Tooth Crown Development ......................................................................................... 1  
   1.1.3 Amelogenesis ............................................................................................................. 3  
1.2 Odontogenic Cysts and Tumors ..................................................................................... 4  
   1.2.1 Odontogenic Cysts ...................................................................................................... 4  
      1.2.1.1 Lateral Periodontal Cyst ....................................................................................... 5  
          1.2.1.1.1 Clinical Features ......................................................................................... 5  
          1.2.1.1.2 Histopathologic Features ........................................................................... 5  
          1.2.1.1.3 Treatment and Prognosis ........................................................................... 5  
      1.2.1.2 Dentigerous Cyst ................................................................................................. 6  
          1.2.1.2.1 Clinical Features ......................................................................................... 6  
          1.2.1.2.2 Histopathologic Features ........................................................................... 6  
          1.2.1.2.3 Treatment and Prognosis ........................................................................... 7  
      1.2.1.3 Keratocystic Odontogenic Tumor (KOT) ................................................................. 7  
          1.2.1.3.1 Etiology ...................................................................................................... 7  
          1.2.1.3.2 Clinical Features ......................................................................................... 8  
          1.2.1.3.3 Radiographic Features ............................................................................... 8  
          1.2.1.3.4 Histopathologic Features ........................................................................... 9  
          1.2.1.3.5 Treatment and Prognosis ........................................................................... 9
1.2.2 Odontogenic Tumors .................................................................10
  1.2.2.1 Ameloblastoma .................................................................10
    1.2.2.1.1 Etiology ................................................................10
      1.2.2.1.1.1 Conventional Solid or Multicystic Ameloblastoma ..........11
        1.2.2.1.1.1.1 Clinical Features ..................................11
        1.2.2.1.1.1.2 Radiographic Features ..................11
        1.2.2.1.1.1.3 Histopathologic Features ...............11
        1.2.2.1.1.1.4 Treatment and Prognosis .............12
  1.3 Controls ..................................................................................13
    1.3.1 Odontoma (Non-neoplastic odontogenic control) ..............13
      1.3.1.1 Etiology ................................................................13
      1.3.1.2 Clinical Features ..................................................13
      1.3.1.3 Radiographic Features ........................................14
      1.3.1.4 Histopathologic Features ................................14
      1.3.1.5 Treatment and Prognosis ................................14
    1.3.2 Nasopalatine Duct Cyst (Non-odontogenic control cyst) ....15
      1.3.2.1 Etiology ................................................................15
      1.3.2.2 Clinical Features ..................................................15
      1.3.2.3 Radiographic Features ........................................15
      1.3.2.4 Histopathologic Features ................................15
      1.3.2.5 Treatment and Prognosis ................................16
  1.4 Human Kallikreins (KLKs) ............................................................16
    1.4.1 History of Kallikreins ......................................................16
    1.4.2 Human Kallikrein Locus Organization ..............................17
    1.4.3 Structure of Human Kallikrein Genes ................................17
    1.4.4 Kallikrein Protein Structures ..........................................18
    1.4.5 Proteolytic Functions of Kallikreins ................................20
    1.4.6 Kallikrein Expression in Specific Tissues .........................22
    1.4.7 Role of Kallikreins as Biomarkers ...................................23
    1.4.8 Kallikreins in Odontogenesis and Amelogenesis ............24
List of Tables

Table 3.1 Positive Controls Used for KLKs 6,7,8,10,13, and 14 ........................................42
Table 3.2 Antibody Titres and Incubation Times and Conditions for KLKs
6,7,8,10,13, and 14 ........................................................................................................46
Table 3.3 Scores for Estimated Average of Staining Intensity in Stained Cells
........................................................................................................................................47
Table 3.4 Scores for Estimated Percentage of Cells Stained ........................................47
Table 4.1 Clinical Parameters of the Odontogenic and Non-Odontogenic Cysts
and Tumors ......................................................................................................................49
Table 4.2 Mean Values for Odontogenic Cysts/Tumor and Controls versus
KLKs ..................................................................................................................................65
Table 4.3 Median Values for Odontogenic Cysts/Tumor and Controls versus
KLKs ..................................................................................................................................65
Table 4.4 Statistically Significant Correlations as Determined by Spearman’s
Rank Correlation Test ....................................................................................................73

List of Figures

Figure 4.1 Lateral Periodontal Cyst Immunostaining and Localization by
Antibodies to KLKs 6,7,8,10,13, and 14 ..................................................................53
Figure 4.2 Dentigerous Cyst Immunostaining and Localization by Antibodies to
KLKs 6,7,8,10,13, and 14 ............................................................................................54
Figure 4.3 Keratocystic Odontogenic Tumor Immunostaining and Localization
by Antibodies to KLKs 6,7,8,10,13, and 14 .............................................................55
Figure 4.4 Ameloblastoma Immunostaining and Localization by Antibodies to
KLKs 6,7,8,10,13, and 14 ............................................................................................56
Figure 4.5 Nasopalatine Duct Cyst Immunostaining and Localization by
Antibodies to KLKs 6,7,8,10,13, and 14 ..................................................................57
Figure 4.6 Odontoma Immunostaining and Localization by Antibodies to KLKs
6,7,8,10,13, and 14 .......................................................................................................58
Figure 4.7 KLK6 Immunostaining of the Cysts and Tumors Studied .................59
Figure 4.8 KLK7 Immunostaining of the Cysts and Tumors Studied .................60
Figure 4.9 KLK8 Immunostaining of the Cysts and Tumors Studied .................61
Figure 4.10 KLK10 Immunostaining of the Cysts and Tumors Studied ..........62
Figure 4.11 KLK13 Immunostaining of the Cysts and Tumors Studied ..........63
List of Abbreviations

Amelo – Ameloblastoma
CNS – Central Nervous System
CSF – Cerebrospinal Fluid
DCyst – Dentigerous Cyst
DAB - Diaminobenzidine
HS – Horse Serum
IRS – Immunoreactive score
KLK – Kallikrein protein
KLK – Kallikrein gene locus
KOT – Keratocystic Odontogenic Tumor
LPC – Lateral Periodontal Cyst
LP cyst – Lateral Periodontal Cyst
MMP – Matrix Metalloproteinase
NPDC – Nasopalatine Duct Cyst
NPD cyst – Nasopalatine Duct Cyst
PBS – Phosphate buffered saline
RT – Room temperature
1.1 Tooth Development

1.1.1 Tooth Function

Teeth are important in everyday functions, such as mastication, providing a stable occlusion, as well as providing an esthetic and appropriate facial height and balance. However, cysts and tumors may develop from the tissues that form teeth.

1.1.2 Tooth Crown Development

The first sign of tooth development in humans, histologically, occurs in the 6th to 7th weeks of embryogenesis when thickening of the stomatodeal epithelium of the first branchial arch occurs in the areas where tooth formation will take place. Ectomesenchyme and epithelial interactions are necessary for tooth formation. If odontogenic ectomesenchyme is combined with any epithelium, the epithelium induces tooth forming gene expression in the mesenchyme and tooth structures develop. However, if odontogenic epithelium is added to any mesenchyme, the epithelium loses its dental characteristics and assumes the epithelial characteristics of the origin of the mesenchyme.

Tooth development occurs in 3 continuous stages: 1. Bud stage, 2. Cap stage, and 3. Bell stage. During the bud stage, the first epithelial infiltration into the ectomesenchyme of the jaw occurs forming the dental lamina, with bud-like enlargements where the teeth will develop. The epithelial cells show little to no
change in their shape or function. The supporting ectomesenchymal cells then congregate below and about the epithelial bud. The epithelial bud continues to proliferate around the ectomesenchyme, forming a cap-like structure (cap stage), bringing along with it a portion of the dental lamina. At this point it is possible to differentiate the formative elements of the tooth and its supporting tissues. The cap of epithelium, designated the enamel organ, will eventually form the enamel of the tooth. The ball of ectomesenchyme, designated the dental papilla, will form the pulp and dentin of the tooth. The surrounding tissues of the condensed ball of ectomesenchyme, designated the dental follicle, will form the periodontal ligament and alveolar bone. The combination of the enamel organ, the dental papilla, and the dental follicle, form what is called the dental organ or tooth germ. Lastly, the bell stage occurs as the crown of the tooth assumes its final shape. Ameloblasts and odontoblasts, which are cells that eventually produce the hard tissues of the crown, undergo histodifferentiation. Outer enamel epithelium surrounds the boundaries of the enamel organ, and inner enamel epithelium is composed of the cells of the enamel organ that lie adjacent to the dental papilla. The outer and inner epithelia meet at a point, which is named the cervical loop. Cells here continue to divide until the tooth’s crown form has reached its complete size. Once the crown form has been completed, the cells of the cervical loop will continue to divide to form Hertwig’s root sheath which migrates apically and induces the formation of the tooth root. In the crown a layer of cells, which are found between the inner enamel epithelium and stellate reticulum,
differentiates to form the stratum intermedium, which produces large amounts of alkaline phosphatase.\(^{(Nanci, 2003)}\)

The dental lamina breaks up into residual islands of epithelium, providing a separation of the developing tooth from the oral epithelium.\(^{(Nanci, 2003)}\) These islands of epithelium usually involute but some may persist as dental lamina cell rests, which have been thought to give rise to some odontogenic cysts and tumors.\(^{(Nanci, 2003)}\) The inner enamel epithelium produces enamel matrix and odontoblasts form pre-dentin.\(^{(Nanci, 2003)}\)

### 1.1.3 Amelogenesis

Enamel formation, also known as amelogenesis, has three phases:

1. Secretory, 2. transition, and 3. maturation.\(^{(Simmer & Hu, 2002)}\) The secretory phase occurs with the secretion of enamel matrix proteins as well as proteases by ameloblasts.\(^{(Simmer & Hu, 2002)}\) Uncleaved enamel proteins are found on the surface/outer enamel layer, whereas cleaved products are found in the deeper/inner enamel layer.\(^{(Simmer & Hu, 2002)}\) Proteins such as amelogenin, ameloblastin and enamelin are the significant proteins involved in the laying down of enamel crystals.\(^{(Simmer & Hu, 2002)}\) Once these proteins are secreted, they are cleaved by enzymes and are degraded or reabsorbed by ameloblasts.\(^{(Simmer & Hu, 2002)}\) This secretory phase involves growth of the crystals in length, causing expansion of the enamel layer.\(^{(Simmer & Hu, 2002)}\) The crystallite lengthening occurs at a mineralization barrier which is close in proximity to the secretory surfaces of ameloblasts.\(^{(Simmer & Hu, 2002)}\) Mineral is deposited on the apices and sides of the crystallite.\(^{(Simmer & Hu, 2002)}\)
The transition phase involves ameloblast reorganization of intracellular components, once crystallites have attained their final length. (Kallenbach, 1974) Approximately one quarter of ameloblasts undergo apoptosis. (Simmer & Hu, 2002) It has been shown in rats that ameloblasts begin to express KLK4 at this point in time. (Simmer & Hu, 2002) KLK4 acts to destroy the proteins in enamel that sustain cell adhesion to the mineral layer. (Simmer & Hu, 2002)

With the removal of enamel proteins, the maturation phase occurs with mineral being deposited only on the sides of the crystallites, causing widening and thickening of the crystallites, until contact with adjacent crystallites occurs, impeding growth. (Simmer & Hu, 2002) This allows hardening of the enamel layer. (Simmer & Hu, 2002) The final enamel is comprised of approximately 85% mineral crystals. (Smith, 1998)

1.2 Odontogenic Cysts and Tumors

1.2.1 Odontogenic Cysts

As a group, odontogenic cysts are quite common, whereas odontogenic tumors are relatively uncommon. (Neville, Damm, Allen, & Bouquot, 2002) Most of these lesions are found within the jaw bones, but some may occasionally be found in the gingiva or alveolar mucosa. (Neville et al., 2002) Odontogenic cysts can be classified as either developmental or inflammatory. It is currently unknown why or how developmental odontogenic cysts occur. (Neville et al., 2002)
Some examples of developmental odontogenic cysts are: lateral periodontal cyst, dentigerous cyst, orthokeratinized odontogenic cyst, and glandular odontogenic cyst.

1.2.1.1 Lateral Periodontal Cyst

1.2.1.1.1 Clinical Features

Lateral periodontal cysts are typically found between the roots of adjacent vital teeth on routine radiographs, especially in the maxilla between the lateral incisor and cuspid teeth and in the mandible between the bicuspid teeth. They tend to present in the 5th-7th decade and are very rare in individuals less than 30 years of age.

1.2.1.1.2 Histopathologic Features

The lateral periodontal cyst consists of a non-inflamed, thin, fibrous wall, and a thin non-keratinized epithelial lining of flat squamous epithelial cells, or cuboidal cells, one to four layers thick. Glycogen-rich cells with clear cytoplasm may be present sometimes in clusters called “plaques.”

1.2.1.1.3 Treatment and Prognosis

The typical treatment of lateral periodontal cysts is conservative enucleation. Recurrence is very rare.
1.2.1.2 Dentigerous Cyst

Dentigerous cysts envelop the crowns of impacted or unerupted teeth and are the most common developmental odontogenic cyst. (Neville et al., 2002)

1.2.1.2.1 Clinical Features

Dentigerous cysts may be associated with any unerupted tooth, but most commonly occur around the crowns of mandibular third molars. (Fonseca et al., 2009; Neville et al., 2002) These cysts can present in patients of all ages, but most develop in patients between the ages of 10-30 yrs old. (Neville et al., 2002) Dentigerous cysts have a slight male predilection, and present more commonly in white than in black populations. (Neville et al., 2002)

Dentigerous cysts may increase in size and present as large lesions associated with asymptomatic expansion of bone and cause displacement of the associated tooth. (Fonseca et al., 2009; Neville et al., 2002) A large dentigerous cyst may cause displacement of a mandibular third molar superiorly into the mandibular ramus or inferiorly to the inferior border of the mandible. (Neville et al., 2002) A large cyst may also cause displacement of maxillary anterior teeth into the nose or of maxillary posterior teeth into the maxillary sinus. (Neville et al., 2002)

On images, the cyst appears as a well defined, unilocular radiolucency which surrounds the crown of an impacted/unerupted tooth. (Neville et al., 2002)

1.2.1.2.2 Histopathologic Features

The typical non-inflamed dentigerous cyst presents with a loose fibrous connective tissue wall with variable amounts of glycosaminoglycans and odontogenic epithelial rests, which may present as islands or strands. (Fonseca et al., 2009;
Non-keratinized stratified squamous epithelium of variable thickness with cuboidal basal cells and a flat connective tissue interface comprises the epithelial lining of the cyst. ([Fonseca et al., 2009; Neville et al., 2002])

1.2.1.2.3 Treatment and Prognosis

The typical treatment of a dentigerous cyst involves the enucleation of the cyst as well as extraction of the associated impacted/unerupted tooth. ([Fonseca et al., 2009; Neville et al., 2002]) For large dentigerous cysts, marsupialization may be performed in order to allow the cyst to shrink and minimize the surgical bony defect after eventual surgical curettage. ([Neville et al., 2002]) Dentigerous cysts have a good prognosis and have a low recurrence rate after complete excision.

1.2.1.3 Keratocystic Odontogenic Tumor (KOT) (previously known as the Odontogenic Keratocyst (OKC))

1.2.1.3.1 Etiology

Although it is now considered a neoplasm, it will be described in the section of odontogenic cysts since much of the literature regarding KOTs was written when it was considered a cyst.

The KOT exhibits intrinsic proliferation of the epithelial lining. ([Neville et al., 2002]) Further, some sporadic KOTs and those associated with the nevoid basal cell carcinoma (Gorlin-Goltz) syndrome have been shown to have mutations in the PTCH tumor suppressor gene, and overexpression of bcl-1 and TP53, due to allelic loss, at two or more loci, of 9q22. ([Barnes, Eveson, Reichart, & Sidransky, 2005])
Organization (WHO) defines the KOT as a “benign uni- or multicystic, intraosseous tumor of odontogenic origin, with a characteristic lining of parakeratinized stratified squamous epithelium and potential for aggressive, infiltrative behavior.” (Barnes et al., 2005)

1.2.1.3.2 Clinical Features

KOTs occur over a wide age range but 60% present in individuals between the ages of 10 and 40 years old, and with a slight male predilection. (Barnes et al., 2005; Neville et al., 2002) Roughly 60-80% occur in the mandible, with most presenting in the posterior body and ramus of the mandible. (Barnes et al., 2005; Neville et al., 2002) Larger KOTs may be locally destructive and symptomatic, presenting with pain, swelling, and/or drainage. (Barnes et al., 2005; Neville et al., 2002) KOTs usually expand in an anteroposterior direction within the medullary region of bone and rarely expand the cortical surface of the jaw. (Neville et al., 2002) This allows differentiation from dentigerous cysts and radicular cysts as these lesions, when large, will cause bony expansion. (Neville et al., 2002)

The nevoid basal cell carcinoma (Gorlin-Goltz) syndrome is associated with multiple KOTs of the jaws. (Neville et al., 2002) On clinical exam, the cyst lumen is described as containing a diagnostic “cream of wheat” fluid, or a cheesy material (keratinaceous debris). (Neville et al., 2002)

1.2.1.3.3 Radiographic Features

KOTs may present as well defined unilocular or multilocular lesions and may have a scalloped border. (Barnes et al., 2005; Neville et al., 2002) Approximately 25-40% of cases are associated with an unerupted/impacted tooth, They are thought to arise from dental lamina rests surrounding the tooth. (Neville et al., 2002)
1.2.1.3.4 Histopathological Features

The histopathological features of KOTs are diagnostic. The wall of a KOT is lined by a 6-8 cell layer thick stratified squamous epithelium, often devoid of inflammatory cells.\textsuperscript{(Barnes et al., 2005; Neville et al., 2002)} The boundary between the epithelium and connective tissue is flat.\textsuperscript{(Neville et al., 2002)} The epithelial surface consists of parakeratin that is uniform and wavy/corrugated.\textsuperscript{(Barnes et al., 2005; Neville et al., 2002)} Desquamation of the parakeratin is often seen.\textsuperscript{(Barnes et al., 2005)} KOTs contain a palisaded layer of cuboidal to columnar basal epithelial cells that have hyperchromatic nuclei, which may be displaced away from the basement membrane.\textsuperscript{(Barnes et al., 2005; Neville et al., 2002)} In 7-26\% of KOTs the fibrous wall may contain small islands or strings of odontogenic epithelium.\textsuperscript{(Neville et al., 2002)}

1.2.1.3.5 Treatment and Prognosis

The typical treatment of a KOT involves enucleation and curettage of the lesion.\textsuperscript{(Neville et al., 2002)} Overall, larger studies have shown a recurrence rate of roughly 30\%.\textsuperscript{(Neville et al., 2002)} Typically, the majority of recurring KOTs will do so within the first 5 years after surgical removal, yet they may recur for up to 10 years.\textsuperscript{(Neville et al., 2002)}

Despite the high rate of recurrence, KOTs have a good prognosis.\textsuperscript{(Neville et al., 2002)} Rarely, a KOT may expand significantly, invade the base of the skull, or become aggressive.\textsuperscript{(Neville et al., 2002)}
1.2.2 Odontogenic Tumors

Odontogenic tumors are a large group of lesions with different clinical and prognostic features. (Neville et al., 2002) Odontogenic tumors range in aggressiveness from hamartomas to malignant neoplasms. (Neville et al., 2002) Included in this group are tumors of odontogenic epithelium, odontogenic ectomesenchyme, and both odontogenic epithelium and ectomesenchyme simultaneously (mixed odontogenic tumors which may contain dental hard tissues). (Barnes et al., 2005; Neville et al., 2002) Details of these tumors can be found in widely used publications. (Barnes et al., 2005; Neville et al., 2002) The ameloblastoma and the odontoma are studied in this thesis.

1.2.2.1 Ameloblastoma

1.2.2.1.1 Etiology

Of all of the epithelial odontogenic tumors, ameloblastomas are considered to be the most significant and most common. (Neville et al., 2002) Although ameloblastomas are classified as benign neoplasms, they are locally invasive. (Neville et al., 2002) The dysregulation of multiple genes required for tooth formation may be involved in the formation of ameloblastomas. (Barnes et al., 2005) There are three different clinical types of ameloblastomas that have differing frequencies, clinical and radiographic features, and different treatment regimens and prognosis. In this thesis, only the conventional/solid/multicystic intra-bony type was studied.
1.2.2.1.1.1 Conventional, Solid or Multicystic Ameloblastoma

1.2.2.1.1.1.1 Clinical Features

This type of ameloblastoma can occur in any age, but rarely occurs in those under 20 years of age.\textsuperscript{1} There is a predilection for the 3\textsuperscript{rd} to 7\textsuperscript{th} decades of life.\textsuperscript{2} They occur in equal frequency in males and females with a slight tendency to occur more often in black patients.\textsuperscript{3} They most commonly (80-85\%) occur in the molar region or ascending ramus of the mandible, with only 15\% occurring in the maxilla, usually posteriorly.\textsuperscript{4} They may present as a painless swelling of the jaw.\textsuperscript{5} Ameloblastomas have the potential to grow to a large size and cause facial deformity.\textsuperscript{6}

1.2.2.1.1.1.2 Radiographic Features

On radiographic examination ameloblastomas may have a “soap-bubble” appearance, composed of large loculations, or a “honeycomb” pattern, composed of smaller loculations, in an overall multilocular radiolucent tumor.\textsuperscript{7} These lesions may expand the jaw bone and frequently cause root resorption of nearby teeth.\textsuperscript{8} Ameloblastomas have been found around impacted third molars, and can mimic a cyst when they appear unilocular.\textsuperscript{9}

1.2.2.1.1.1.3 Histopathologic Features

This type of ameloblastoma may contain both cystic and solid areas.\textsuperscript{10} There are different microscopic variants classified according to their histopathologic features. These are: 1. Follicular, 2. Plexiform, 3. Acanthomatous, 4.
Granular Cell, 5. Desmoplastic, and 6. Basal Cell (Neville et al., 2002) There is no significant difference in prognosis for the different types.

The classical follicular subtype is the easiest to diagnose and is also the most common pattern seen (Neville et al., 2002). It occurs in a mature fibrous stroma as epithelial islands, which have loose angular cells resembling stellate reticulum in the center and are surrounded by a basal layer of palisaded cuboidal to columnar ameloblast-like cells that exhibit nuclear hyperchromasia and reversed polarity (Barnes et al., 2005; Neville et al., 2002).

The plexiform subtype does not have islands of epithelium, but rather narrow, interconnected strands or ropes of epithelium consisting of ameloblast-like basal cells enclosing loose epithelial stellate reticulum-like cells (Neville et al., 2002). A loose, vascular stroma surrounds the epithelium (Neville et al., 2002).

1.2.2.1.1.4 Treatment and Prognosis

Treatment options vary from simple enucleation and curettage to en bloc resection of the jaw (Neville et al., 2002). Because ameloblastomas are aggressive, they infiltrate through marrow spaces beyond clinical/surgical or radiographic margins (Barnes et al., 2005; Neville et al., 2002). Therefore, curettage does not provide complete removal of the tumor and is associated with high recurrence rates of 50-90% (Neville et al., 2002). Marginal resection, with 1cm margins beyond the radiographic boundaries, is the usual treatment and has an associated 15% recurrence rate (Neville et al., 2002). Long-term follow-up is important as these lesions may recur even more than 5-10 years later (Barnes et al., 2005; Neville et al., 2002). Ameloblastomas are rarely fatal (usually
from direct extension into the cranium from maxillary lesions) and rarely undergo malignant transformation. (Neville et al., 2002)

1.3 Controls

1.3.1 Odontoma (Non-neoplastic odontogenic control)

1.3.1.1 Etiology

Odontomas are hamartomas of odontogenic tissues. (Barnes et al., 2005; Neville et al., 2002) Odontomas contain all of the tissues of a normal tooth (enamel, dentin, cementum, and pulp tissue), but do not form proper tooth morphology or size. (Neville et al., 2002) Proliferating odontogenic epithelium and mesenchyme are present in the early stages, which lead to the production of dental hard tissues. (Neville et al., 2002) There are two types of odontomas: the compound odontoma, which is a focal collection of toothlets, and the complex odontoma, which is an irregular gnarled mass of dental hard and soft tissues. (Neville et al., 2002) Complex odontomas are less common than compound odontomas. (Neville et al., 2002)

1.3.1.2 Clinical Features

Odontomas commonly present during the first two decades of life, with a mean age of occurrence of 14 years. (Barnes et al., 2005; Neville et al., 2002) They are often discovered when a radiograph is taken to determine why a tooth has failed to erupt into the oral cavity. (Barnes et al., 2005; Neville et al., 2002) Odontomas may vary in size, and large ones may cause expansion of the jaw bone. (Neville et al., 2002) They may present in either jaw, but are more frequently diagnosed in the maxilla. (Neville et al., 2002) The
The compound odontoma has a predilection for the anterior maxilla, and the complex odontoma has a predilection for the posterior maxilla or mandible.\textsuperscript{1} Gender predilection is not present.\textsuperscript{1}

1.3.1.3 Radiographic Features

A compound odontoma is a collection of structures that resemble small, misshapen teeth surrounded by a radiolucent rim.\textsuperscript{1} A complex odontoma is also surrounded by a radiolucent rim, but contains a radiopaque mass that does not resemble tooth structure.\textsuperscript{1}

1.3.1.4 Histopathologic Features

The compound odontoma is composed of tooth-like structures that are present in a loose fibrous stroma.\textsuperscript{1} Enamel matrix, dentin, and pulp tissue, are found on microscopic examination, even if mature enamel areas are lost during slide decalcification.\textsuperscript{1} Tissues similar to tooth germs in normal teeth are seen in early lesions.\textsuperscript{1} The complex odontoma demonstrates mature tubular dentin which encloses spaces where mature enamel was present prior to decalcification (enamel matrix may be seen here).\textsuperscript{1} Follicular fibrous tissue is also observed surrounding the odontoma.

1.3.1.5 Treatment and Prognosis

Odontomas are treated by curettage or local excision.\textsuperscript{1} Odontomas are associated with an excellent prognosis, and recurrence is extremely rare.\textsuperscript{1} Not all odontomas require removal.
1.3.2 Nasopalatine Duct Cyst (Non-Odontogenic Control Cyst)

1.3.2.1 Etiology

The nasopalatine duct cyst is the most common non-odontogenic cyst of the oral and maxillofacial region, with an incidence of 1% \cite{fonseca2009, neville2002}. It is believed to arise from the embryonic nasopalatine duct. In this study, nasopalatine duct cysts were used as a control.

1.3.2.2 Clinical Features

Nasopalatine duct cysts can present at any age, but are usually observed in the 4th-6th decades of life, and are more common in males \cite{fonseca2009, neville2002}. The common symptoms that a patient presents with are swelling of the anterior palate, drainage, pain, and long duration of symptoms as they tend to be intermittent \cite{neville2002}. On the other hand, many may be asymptomatic and are found incidentally on routine radiographs \cite{fonseca2009, neville2002}.

1.3.2.3 Radiographic Features

On radiographic examination, the nasopalatine duct cyst appears as a well-circumscribed radiolucency between the roots of the maxillary central incisors (midline of the anterior hard palate) \cite{fonseca2009, neville2002}. It often appears as a heart-shaped radiolucency because of radiographic superimposition of the anterior nasal spine \cite{fonseca2009, neville2002}.

1.3.2.4 Histopathologic Features

Nasopalatine duct cysts may be lined by non-keratinized stratified squamous epithelium (most common), pseudostratified columnar epithelium, simple columnar
epithelium (+/- cilia and goblet cells), simple cuboidal epithelium, or any combination of these epithelial types. (Fonseca et al., 2009; Neville et al., 2002)

1.3.2.5 Treatment and Prognosis

The typical treatment of nasopalatine duct cysts is surgical enucleation. (Neville et al., 2002) Recurrence is very rare. (Fonseca et al., 2009)

1.4 Human Kallikreins (KLKs)

1.4.1 History of Kallikreins

The year 2009 marked the 100th year since kallikrein research began. (Lawrence, Lai, & Clements, 2010) Werle and colleagues first described a high protein isolate in the pancreas in the 1930's, which they named “kallikrein,” after the Greek word for pancreas, but this protein had been previously noted in urine by Abelous and Bardier. (Borgono & Diamandis, 2004; Borgono, Michael, & Diamandis, 2004; Lawrence et al., 2010; Lundwall et al., 2006; Yousef & Diamandis, 2001) KLK1 was the first kallikrein described, and was originally found to release vasoactive kinin peptides from kininogens, resulting in hypotension. (Lawrence et al., 2010) As knowledge of the structure and purpose of the kallikrein-related peptidase locus increased, the understanding of kallikreins advanced. (Lawrence et al., 2010) KLK1 was recognized to be a part of a multigene family. (Lawrence et al., 2010) Subsequently, KLK2 and KLK3 were described. (Lawrence et al., 2010) By the 1990's the human kallikrein family expanded as more serine peptidase encoding genes were discovered and linked to the kallikrein locus. (Lawrence et al., 2010) Eventually, the family expanded to 15 kallikreins, with KLK1, KLK2, and KLK3 being regarded as the classical kallikreins. (Lawrence et al., 2010) KLK2 and KLK3 have a greater
amino acid similarity with KLK1 than do the remaining KLKs.\footnote{Harvey et al., 2000; Lawrence et al., 2010} The kallirein proteins are written in standard font (ex: KLK3) while the corresponding gene is written in italics (ex: \textit{KLK3}).\footnote{Lundwall et al., 2006}

1.4.2 Human Kallikrein Locus Organization

The human kallikrein locus is present at chromosome 19q13.3-13.4 and is the longest consecutive arrangement of peptidases found in humans, approximately 265 kb in length.\footnote{Lawrence et al., 2010} The distance between individual genes ranges from approximately 1.5 kb between KLK1 and KLK15 to 32.5 kb between KLK4 and KLK5.\footnote{Lawrence et al., 2010} This arrangement, however, may be disrupted in tumor cells, including copy number gains of the locus in ovarian, breast, and bladder cancer cell lines.\footnote{Bayani et al., 2008; Lawrence et al., 2010} The human kallikrein locus organization is complex and is not entirely understood at this point.

1.4.3 Structure of Human Kallikrein Genes

Not only are human kallikrein genes located on the same chromosome, but they are also similar in function and form.\footnote{Lawrence et al., 2010} All KLKs consist of five coding exons, with exon 1 encompassing the 5′UTR and start codon, and exon 5 encompassing the termination codon and 3′UTR.\footnote{Lawrence et al., 2010} Exons 2, 3, and 5 contain the catalytic triad of histidine, aspartate, and serine residues.\footnote{Lawrence et al., 2010} All of the genes have the same intron phases and splice sequences, except for a variation in KLK10.\footnote{L. Luo et al., 1998; Yousef & Diamandis, 2001} The noncoding portions of the human kallikrein genes, however, vary greatly.\footnote{Lawrence et al., 2010}
1.4.4 Kallikrein Protein Structures

Kallikreins must become proteolytically active through cleavage as they are produced as preproenzymes. As kallikreins are produced, they must go to the endoplasmic reticulum via an amino acid signal peptide and be secreted. Once they are secreted, the signal peptide is cleaved off and the kallikrein is now active. This allows for a change in the binding site of the kallikrein, allowing the kallikrein to bind and cleave various substrates. The cleavage is brought about by peptidases, including other kallikreins, that are similar to trypsin, as they cleave the signal peptide at residues such as arginine or lysine. The exception is KLK4, which is cleaved by a matrix metallopeptidase or a cysteine peptidase and has a glutamine residue. Those kallikreins with an arginine residue, KLKs 1,2,3,5,9, and 11, are more easily activated by other kallikreins than those with a lysine residue. Some kallikreins can activate themselves, such as KLKs 2,5,11,12, and 14. Regarding the thrombostasis axis, several involved peptidases also cleave specific kallikreins.

The catalytic triad of histidine, aspartate, and serine is what makes human kallikreins active. Serine is responsible for initiating the proteolytic activity of kallikreins by targeting a carboxyl group within a substrate. The substrate binding region of kallikreins at residue 189 is what determines which specific substrates a particular kallikrein will bind.
have different residues at site 189, with KLKs 1,2,4,5,6 and 10-14 having an aspartate residue, KLK3 having a serine residue, KLK7 having an asparagine residue, KLK 9 having a glycine residue, and KLK15 having a glutamate residue at this site.\cite{Lawrence2010} Also, around the opening of the active site of the kallikrein there are eight loops of residues and exosites on the protein's surface, that further define the substrate specificity of each kallikrein.\cite{Debela2008}

In roughly 50\% of the human kallikreins, almost 50\% of the amino acid residues are preserved.\cite{Clements2004} The kallikreins contain the same 39 amino acids, of which 37 are also present in trypsin.\cite{Lawrence2010} The particular amino acids that are important for proteolysis and folding of the proteins are similar between kallikreins, whereas the amino acids that determine specificity for substrates vary between kallikreins.\cite{Lawrence2010} The residues that are identical between kallikreins are 10 disulfide bridge-forming cysteine residues, as well as those residues in the catalytic triad.\cite{Lawrence2010} Also, Glycine\textsubscript{193} is found in all human kallikreins, with the exception of KLK10, which has a glutamate residue at this site.\cite{Lawrence2010} This variation of KLK10 may be the reason for KLK10's absence of proteolysis of the usual kallkrein substrates.\cite{Zhang2006} The region where the most diversity exists between kallikreins is the eight loops of the active site.\cite{Lawrence2010} Although these external loops vary between kallikreins, the core structure is identical, thus indicating that various kallikreins can use the same mechanism to cleave different substrates.\cite{Lawrence2010}
1.4.5 Proteolytic Functions of Kallikreins

Kallikreins can be created and be active in a local region, or they can be secreted into bodily fluids. Kallikreins have various functions and they fall into various groups, such as: extracellular matrix proteins, growth factors, cell adhesion proteins, signaling molecules, and cell surface receptors. Kallikrein 1 (KLK1) cleaves kininogen, a low molecular weight substrate, at two sites, creating kallidin (lys-bradykinin), which can be further cleaved by other peptidases to produce des-Arg -kallidin. These two end-products then bind to bradykinin receptors 1 and 2, carrying out the functions of KLK1. This then leads to the creation of nitric oxide, prostaglandins, as well as other mediators that ultimately lead to smooth muscle contraction and relaxation, inflammation, vasodilation, and pain. This creates a protective function in medical conditions such as ischemic stroke, renal disease, and cardiovascular disease. On the other hand, it worsens inflammatory conditions, such as asthma.

Kallikreins 2,5,8, and 14 cleave low and high molecular weight kininogen. KLK2 also generates kinin, but much less efficiently than KLK1. Other kallikreins function by activating and/or inactivating other kallikreins; KLKs 2,5,12, and 14 activate other kallikreins while KLK5 and KLK14 first activate then inactivate KLK3. Kallikreins also take part in enzyme cascades as KLK1 can activate matrix metallopeptidases 2 and 9, and KLKs 2,4, and 8 can activate urokinase-type plasminogen activator, thus
amplifying or altering the reaction to a particular stimulus.\cite{Beaufort2006, Rajapakse2005, Takayama1997, Takayama2001, Tschesche1989}

Kallikreins have been shown to have a role in skin desquamation as KLK1 and KLKs 4-14 are all evident in skin tissue.\cite{Brattsand1999, Hansson1994, Komatsu2005, Komatsu2003, Lundwall2008, Pampalakis2007, Stefansson2006} They cause skin deqauamation by breaking down various desmosomal adhesion proteins found in the outer layer of skin.\cite{Borgono2007, Caubet2004} Activation of cathelicidins, which are antimicrobial, may be brought about by KLK5 and KLK7, aiding innate immunity in the skin.\cite{Yamasaki2006} When KLK7 is overexpressed in the skin of mouse models, inflammation and itching occurs, and when KLK8 is knocked out, there is prolonged recovery of ultraviolet B-irradiated skin.\cite{Hansson2002, Kirihara2003} Thus, kallikreins may be possible targets for therapy to treat skin diseas.\cite{Lawrence2010}

Kallikreins also have a role in reproduction via cleavage of semenogelins I and II, which are the main gel-forming proteins in semen.\cite{Jonsson2006} Kallikreins 2, 3, and 11 are the most common kallikreins found in semen, although all KLKs are present in semen.\cite{Emami2009, Shaw2007} Kallikreins 2,3,5, and 14 cause fluidity of semen by cleaving semenogelins, thus allowing sperm to move.\cite{deLamirande2007, Deperthes1996, Emami2008, Lilja1985, Michael2006} KLK8 is involved in semen liquefaction.\cite{Pampalakis2007} Other kallikreins may cleave semenogelins, fibronectin, or other
1.4.6 Kallikrein Expression in Specific Tissues

Kallikrein expression has been observed in many different body tissues, and overlap has been noted. For example, the prostate contains high concentrations of KLK2 and KLK3. KLK1 has been observed in high levels in the pancreas, kidney, and salivary glands. KLK6 is found in high levels in the central nervous system. KLK8 has been found in high levels in tissues from the esophagus, skin, and fetal tissues of ureter and tonsil and in lower levels in tissues from testis, fallopian tubes, breasts, tonsils, salivary glands, and lymph nodes. KLK8 has also been shown to be highly expressed in various fluids, such as, breast milk, amniotic fluid, seminal fluid, follicular fluid, cerebrospinal fluid (CSF), and serum. Despite higher concentrations in specific tissues, no kallikrein is entirely specific to a particular tissue. Salivary glands contain almost all of the kallikrein family. The lowest level kallikreins in the body are KLK14 and KLK15.
Genes adjacent to one another on the gene locus often have alike expression. As an example, \( KLK2, 3 \) and \( 4 \) are adjacent to one another in the locus, and their proteins are all present in prostate tissue. As well, \( KLK5, 6, 7 \) and \( 8 \) are adjacent genes and their proteins are found in ovarian cancer. Kallikreins \( 2,3,4,14, \) and \( 15 \), are expressed in the luminal epithelial cells in the prostate, which supports the fact that they are also found in seminal plasma. Further, glandular epithelial cells in visceral organs express \( KLK2,3,5,7,8,10,13,14 \) and \( 15 \). KLK4 has a role in tooth mineralization and KLK8 has a role in skin desquamation.

1.4.7 Role of Kallikreins as Biomarkers

Kallikreins may have a role as biomarkers due to their conserved expression profile in particular tissues. KLK3, also known as prostate-specific antigen (PSA), is the most researched and most understood of the kallikrein family. PSA has been used as a tissue and serum biomarker for prostate cancer for many years. However, KLK3 is also found in increased levels in benign prostatic hyperplasia (BPH).

There have been studies done to find other kallikreins, such as KLK2, to aid PSA (KLK3) as a biomarker. Kallikreins have not only been researched as biomarkers for prostate cancer, but also for conditions of the breast, ovaries, lung, skin, and the nervous system.
et al., 2010) KLK8 has been shown to be increased in ovarian cancer, and has been proposed as a novel biomarker for ovarian cancer, as well as an independent and favourable prognostic marker for ovarian cancer. (Darling et al., 2008; Kishi et al., 2003; Magklara et al., 2001; Yousef, Polymeris, et al., 2003) KLK8 has been found to inhibit cell invasion and motility in non-small cell lung carcinoma. (Sher et al., 2006) KLK8 has also been shown to be overexpressed in cervical carcinoma, and has been thought to be a potential diagnostic tool for determining therapy response, to detect early recurrence post-therapy, and to function as a target antigen for treatment in those cervical carcinomas that are resistant to standard therapy. (Cane et al., 2004; Darling et al., 2008) As well, KLK8 overexpression may be a potential biomarker for the diagnosis of endometrial cancer. (Jin et al., 2006) Hashem et al. showed that KLK14 may serve as a biomarker of salivary gland tumors. (N. N. Hashem et al., 2010) KLK13 has also been shown to be increased in several salivary gland tumors. (Darling, Jackson-Boeters, Daley, & Diamandis, 2006b)

Studies have shown that it is beneficial to measure multiple kallikreins in a disease as biomarkers as the combination of kallikreins has greater specificity than individual kallikrein expression. (Lawrence et al., 2010) Therefore, it is important to have an understanding of the variable kallikrein expression in specific diseases, as well as, more importantly, the combination of kallikrein expression in conditions in order to fully understand their significant potential as biomarkers.

1.4.8 Kallikreins in Odontogenesis and Amelogenesis

KLK4 is initially secreted during the transition phase and plays a role during the maturation phase of amelogenesis, during which the final enamel crystal
formation occurs. In the maturation phase, KLK4 allows crystallites to achieve their ideal dimensions and greatest mineralization by removing extracellular matrix proteins in the developing enamel. KLK4 is the principal enzyme involved in removal of enamel matrix proteins, including amelogenin, and destruction of junctions between ameloblasts. KLK4 has been designated “enamel matrix serine proteinase 1 (EMSP1).” MMP-20 (matrix metalloproteinase 20) is an enzyme that is considered the predominant processing enzyme during the secretory phase of amelogenesis, while KLK4 is the predominant enzyme that degrades enamel matrix proteins, and allows the enamel layer to harden, in the later phases. Studies have been done in mice, pigs, and humans.

When KLK4 is mutated, crystalline growth of the enamel is inhibited during the maturation stage of tooth/enamel development and protein retention occurs resulting in some forms of amelogenesis imperfecta. However, the thickness of the enamel, as well as the orientation of crystallites and prism architecture (which is developed by ameloblasts), are not affected. KLK4 mutation results in a yellow-brown discoloration of the enamel in affected individuals, as seen in patients with the hypomutation pigmented type of amelogenesis imperfecta. Studies have also shown that KLK4 may have a role in junctional epithelium and periodontal maintenance after tooth eruption. Also of note, KLK4 has been shown to be expressed by odontoblasts, and therefore may also play a role in cleavage of
dentin proteins.\cite{Hu2002} The role of other KLKs or a KLK cascade in enamel mineralization has not been previously investigated.

### 1.4.9 Kallikrein Role in Cancer, Invasion, and Metastasis

Studies show that kallikreins play an important role in various cancers.\cite{Borgono2004} For example, kallikreins may have roles in different stages of metastasis, including KLK3 activation of the TGF-β complex, which is involved in activation of epithelial-mesenchymal transformation (EMT).\cite{Derynck2001,Killian1993} As well, KLKs are involved in the destruction of various extracellular matrix proteins, including proteoglycans, collagens, laminins, and fibronectin.\cite{Borgono2004,Borgono2004a,Emami2007a,Emami2007b} For example, KLK8 exhibits trypsin-like characteristics and can break down casein, collagen type IV, fibronectin, high-molecular weight kininogen, and gelatin.\cite{Darling2008,Rajapakse2005} Another important factor in the destruction of extracellular matrix is plasmin, which is released via the uPA-uPAR (urokinase plasminogen activator- urokinase plasminogen activator receptor) signaling pathway and the induction of matrix metalloproteinases (MMPs), which can be activated by KLKs.\cite{Borgono2004,Emami2007a,Emami2007b,Giusti2005,Sidenius2003} This destruction of extracellular matrix proteins allows the cancer cells to pierce barriers and thus invade and metastasize.\cite{Borgono2004,Emami2007a,Emami2007b} Tumor cell invasion is enhanced \textit{in vitro} with KLKs 1,3-10, and 13.\cite{Shinoda2007} Also, KLK6 increases E-cadherin shedding and promotes cell proliferation, migration, and invasion.\cite{Klucky2007}
Kallikreins may also cause proliferation of tumor cells. Insulin-like growth factor binding proteins (IGFBPs) are cleaved by kallikreins 2-5, 11, and 14.\cite{Borgono, Michael, Shaw, et al., 2007; Cohen et al., 1992; Koistinen et al., 2002; Matsumura et al., 2005; Michael et al., 2006; Plymate et al., 1996; Rajapakse & Takahashi, 2007; Rehault et al., 2001; Sano et al., 2007} These kallikreins, therefore, may increase unbound IGF-I as its affinity for cleaved IGFBPs is decreased, resulting in mitogenesis and antiapoptosis.\cite{Fielder et al., 1994} Specific cell lines may be increased in vitro by KLKs 3, 4, and 6.\cite{Klokk et al., 2007; Klucky et al., 2007; Niu et al., 2008; Pampalakis et al., 2009; Veveris-Lowe et al., 2005}

The functions of the different kallikreins vary even within the same tumor.\cite{Lawrence et al., 2010} Kallikreins cannot be deemed tumor activators or suppressors because, depending on the tumor type, the particular kallikrein, such as KLK6 and KLK10, can either enhance or suppress the tumor’s progression.\cite{Klucky et al., 2007; Lawrence et al., 2010; Pampalakis et al., 2009; Ruckert et al., 2008; Zhang et al., 2006} Overall, KLK overexpression in malignant tumors has, paradoxically, been shown to be associated with both favourable and poor prognosis.\cite{Borgono & Diamandis, 2004; Darling et al., 2008; N. N. Hashem et al., 2010}

1.4.10 Summary of Known Functions and Locations of KLKs Used in this Study

1.4.10.1 KLK6:

**Functions:**
- involved in tumor invasion, E-cadherin shedding, and promotion of cell proliferation and migration,\cite{Klucky et al., 2007; Lawrence et al., 2010} in various cancers including those of the breast, salivary glands, prostate and colon.\cite{Darling et al., 2006b; N. N. Hashem et al., 2011; Lin et al., 2002; L. Y. Luo, Grass, &
Diamandis, 2000; Palmer et al., 2003; Pampalakis & Sotiropoulou, 2006; Shaw & Diamandis, 2008; Ting, Bao, Reeder, Messing, & Lee, 2007; Yousef, Chang, & Diamandis, 2000; Yousef & Diamandis, 2000; Yousef, Luo, Scherer, Sotiropoulou, & Diamandis, 1999; Yousef, Magklara, & Diamandis, 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002)

- has a role in multiple sclerosis and immune mediated demyelination diseases in the central nervous system (CNS), in Alzheimer’s disease and in Parkinson’s disease (Clements et al., 2004)
- has also been implicated as a tumor suppressor in breast cancer (Klucky et al., 2007; Lawrence et al., 2010; Pampalakis et al., 2009; Ruckert et al., 2008; Zhang et al., 2006)
- causes pro-hormone activation of insulin, glucagon, somatostatin, and pancreatic polypeptide with KLK1, KLK10, and KLK13 (N.N. Hashem, 2008)
- involved in skin desquamation, myelination and synaptogenesis (N.N. Hashem, 2008)

**Locations:**

- present in all placental mammals (Lawrence et al., 2010)
- found in increased levels in the central nervous system (Lawrence et al., 2010) and in several salivary gland tumors (Darling et al., 2006b; N.N. Hashem et al., 2011) but appears to be downregulated in comparison with normal salivary gland tissue (Darling, Jackson-Boeters, Daley, & Diamandis, 2006a) It is increased in ovarian cancer (Yousef, Polymeris, et al., 2003) in some breast carcinoma (L. Y. Luo et al., 2000; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000; Yousef & Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002) in cervico-vaginal secretion during
the secretory phase of the menstrual cycle, (Shaw, Petraki, Watson, Bocking, & Diamandis, 2008) in cancers of the head and neck, colon, and prostate, as well as in normal and altered skin keratinocytes in humans in response to vitamin D3, (Lin et al., 2002; Palmer et al., 2003; Ting et al., 2007) and in semen. (Emami et al., 2009; Shaw & Diamandis, 2007)

- found in decreased amounts in prostate cancer, (Lawrence et al., 2010) and in VK2 vaginal epithelial cells in response to estrogen. (Shaw et al., 2008)
- observed predominantly in the islets of Langerhans in the normal endocrine pancreas, foci of nesidioblastosis and insulin-, glucagon-, and somatostatin-producing tumors, along with KLKs 10 and 13.

The precise role in the pancreas is not yet known. (Clements et al., 2004)

1.4.10.2 KLK7:

Functions:

- involved in keratinization, stratum corneum formation, and turnover/desquamation of skin through the degradation of the cell adhesion glycoproteins corneodesmosin and plakoglobin. (Clements et al., 2004)
- has a role in salivary gland, ovarian, breast, lung, cervical, and endometrial cancers, and is involved in tumor invasion, activation of cathelicidins (aiding innate immunity), and antimicrobial function. (Berglund et al., 2008; Darling et al., 2006b; N.N. Hashem et al., 2011; Paliouras et al., 2007; Petraki et al., 2006; Shaw et al., 2008; Shinoda et al., 2007; Yamasaki et al., 2006; Yousef, Borgono, et al., 2004; Yousef & Diamandis, 2000; Yousef, Magklara, et al, 2000; Yousef, Polymeris, et al., 2003; Yousef, Scorilas, Magklara, Soosaipillai, & Diamandis, 2000)
Locations:

- present in all placental mammals, \( \text{(Lawrence et al., 2010)} \)
- shown to be increased in several salivary gland tumors, \( \text{(Darling et al., 2006b; N.N. Hashem et al., 2011)} \)
  - in ovarian cancer, \( \text{(Yousef, Polymeris, et al., 2003)} \)
  - in glandular epithelial cells in visceral organs, \( \text{(Berglund et al., 2008; Petraki et al., 2006)} \)
  - in cervico-vaginal secretion during the secretory phase of the menstrual cycle, \( \text{(Shaw et al., 2008)} \)
  - in breast carcinoma, \( \text{(Yousef, Borgono, et al., 2004; Yousef & Diamandis, 2000; Yousef, Magklara, et al., 2000; Yousef, Scorilas, Magklara, et al., 2000)} \)
  - in skin keratinocytes in response to vitamin D, \( \text{(Lu et al., 2005)} \)
  - and in semen, \( \text{(Emami et al., 2009; Shaw & Diamandis, 2007)} \)
- present in the cerebrospinal fluid (CSF) of patients with Alzheimer’s disease and dementia \( \text{(Clements et al., 2004)} \)

1.4.10.3 KLK8:

Functions:

- functions as a tumor suppressor gene in breast cancer, along with KLKs 6 and 10, \( \text{(Klucky et al., 2007; Lawrence et al., 2010; Pampalakis et al., 2009; Ruckert et al., 2008; Zhang et al., 2006)} \)
- implicated in tumor cell invasion and cancers, such as as salivary gland cancer, ovarian cancer, skin cancer, cervical cancer, endometrial cancer, breast cancer, and lung cancer \( \text{(Borgono et al., 2003; Cane et al., 2004; Darling et al., 2006b; Darling et al., 2008; N.N. Hashem et al., 2011; Inoue et al., 1998; Jin et al., 2006; Kishi et al., 2003; Kuwae et al., 2002; L.Y. Luo et al., 2000; Magklara et al., 2001; Paliouras et al., 2007; Paliouras & Diamandis, 2007, 2008; Shaw & Diamandis, 2008; Sher et al., 2006; Yousef, Chang, et al., 2000; Yousef &} \)
Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Polymeris, et al., 2003; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002) (in non-small cell lung cancer, KLK8 seems to inhibit cancer cell invasion and motility (Sher et al., 2006))

- exhibits trypsin-like characteristics and can break down casein, collagen type IV, fibronectin, high-molecular weight kininogen, and gelatin, thus aiding in the destruction of the extracellular matrix (Darling et al., 2008; Rajapakse et al., 2005)

- involved in skin desquamation, myelination and synaptogenesis (N.N. Hashem, 2008) cleavage of low and high molecular weight kininogen, activation of a urokinase-type plasminogen activator, amplifying or altering the reaction to a particular stimulus (Beaufort et al., 2006; Rajapakse et al., 2005; Takayama et al., 1997; Takayama et al., 2001; Tschesche et al., 1989) and involved in semen liquefaction (Pampalakis & Sotiropoulou, 2007)

- has a role in multiple sclerosis, immune mediated demyelination diseases in the CNS, in Alzheimer’s disease and in Parkinson’s disease (Clements et al., 2004)

Locations:

- present in all placental mammals (Lawrence et al., 2010)

- found in increased levels in the esophagus and skin, in fetal tissues of ureter and tonsil (Darling et al., 2008; Kishi et al., 2003) in various fluids, such as, breast milk, amniotic fluid, seminal fluid, follicular fluid, cerebrospinal fluid (CSF), and serum (Darling et al., 2008; Kishi et al., 2003) as well as in several
Salivary gland tumors, (Darling et al., 2006b; N.N. Hashem et al., 2011) in ovarian cancer, (Yousef, Polymeris, et al., 2003) in glandular epithelial cells in visceral organs, (Berglund et al., 2008; Petraki et al., 2006) in brain tissue, (Chen et al., 1995; Mitsui, Tsuruoka, Yamashiro, Nakazato, & Yamaguchi, 1999) in normal and pathological skin, (Inoue et al., 1998; Kuwae et al., 2002) in cervical carcinoma, (Cane et al., 2004; Darling et al., 2008) in the endometrium and endometrial cancer, (Jin et al., 2006) in breast carcinoma, (L. Y. Luo et al., 2000; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000; Yousef & Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002) and in the prostate, (Kishi et al., 2003; Shaw & Diamandis, 2008; Yousef, Magklara, et al., 2000; Yousef, Scorilas, Jung, Ashworth, & Diamandis, 2001)

- found in decreased levels in tissues from the testis, fallopian tubes, breasts, tonsils, salivary glands, and lymph nodes, (Darling et al., 2008; Kishi et al., 2003) as well as in prostate cancer, (Lawrence et al., 2010) and breast carcinoma. (Darling et al., 2008; Yousef, Yacoub, et al., 2004)

### 1.4.10.4 KLK10:

**Functions:**

- has a role in skin desquamation (Brattsand & Egelrud, 1999; Hansson et al., 1994; Komatsu et al., 2005; Komatsu et al., 2003; Lundwall & Brattsand, 2008; Pampalakis & Sotiropoulou, 2007; Stefansson et al., 2006)

- is implicated in ovarian cancer, breast cancer, and tumor cell invasion (Borgono et al., 2003; Hsieh et al., 1997; L. Y. Luo et al., 2000; L. Y. Luo, Grass, & Diamandis, 2003; Magklara, Grass, & Diamandis, 2000; Paliouras et al., 2007; Paliouras & Diamandis, 2007, 2008;
Shaw & Diamandis, 2008; Shinoda et al., 2007; Yousef, Chang, et al., 2000; Yousef & Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002

- KLK6 and KLK10 can either enhance or suppress tumor progression. (Klucky et al., 2007; Lawrence et al., 2010; Pampalakis et al., 2009; Ruckert et al., 2008; Zhang et al., 2006) KLK10 has been shown to be a tumor suppressor in cancers such as breast, prostate, and testicular cancer. (N.N. Hashem, 2008)
- also involved in pro-hormone activation of insulin, glucagon, somatostatin, and pancreatic polypeptide (N.N. Hashem, 2008)

Locations:
- present in all placental mammals. (Lawrence et al., 2010)
- found in increased levels in glandular epithelial cells in visceral organs, (Berglund et al., 2008; Petraki et al., 2006) in ovarian cancer, (Paliouras et al., 2007) in breast carcinoma (L. Y. Luo et al., 2000; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000; Yousef & Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002) (but also has been long known as a tumor suppressor in breast cancer), (Anisowicz, Sotiropoulou, Stenman, Mok, & Sager, 1996; Liu, Wazer, Watanabe, & Band, 1996; Pampalakis et al., 2009; Zhang et al., 2006) and in skin keratinocytes. (Lu et al., 2005)
- found in decreased levels in prostate cancer, (Lawrence et al., 2010) and in VK2 vaginal epithelial cells in response to estrogen. (Shaw et al., 2008)
- present in the CSF of patients with Alzheimer’s disease and dementia (Clements et al., 2004)
observed predominantly in the islets of Langerhans in the normal endocrine pancreas, foci of nesidioblastosis and insulin-, glucagon-, and somatostatin-producing tumors, along with KLKs 6 and 13. The precise role in the pancreas is not yet known. (Clements et al., 2004)

1.4.10.5 KLK13:

Functions:

- has a role in skin desquamation (Brattsand & Egelrud, 1999; Hansson et al., 1994; Komatsu et al., 2005; Komatsu et al., 2003; Lundwall & Brattsand, 2008; Pampalakis & Sotiropoulou, 2007; Stefansson et al., 2006)
- is involved in salivary gland, ovarian, and breast cancers, and tumor cell invasion (Borgono et al., 2003; Darling et al., 2006b; N.N. Hashem et al., 2011; L. Y. Luo et al., 2000; Palouras et al., 2007; Palouras & Diamandis, 2007, 2008; Shaw & Diamandis, 2008; Shinoda et al., 2007; Yousef, Chang, et al., 2000; Yousef & Diamandis, 2000; Yousef, Fracchioli, et al., 2003; Yousef et al., 1999; Yousef, Magklara, et al., 2000; Yousef, Scorilas, & Diamandis, 2000; Yousef et al., 2002)
- also involved in pro-hormone activation of insulin, glucagon, somatostatin, and pancreatic polypeptide. Other KLKs involved are: KLK1, KLK6, and KLK10 (N.N. Hashem, 2008)

Locations:

- present in all placental mammals (Lawrence et al., 2010)
- found in increased levels in several salivary gland tumors (Darling et al., 2006b; N.N. Hashem et al., 2011) in ovarian cancer (Palouras et al., 2007) in glandular epithelial cells in visceral organs (Berglund et al., 2008; Petraki et al., 2006) in breast carcinoma (L. Y. Luo et al., 2000; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000;
and in skin keratinocytes in response to vitamin D. (Lu et al., 2005)

- observed predominantly in the islets of Langerhans in the normal endocrine pancreas, foci of nesidioblastosis and insulin-, glucagon-, and somatostatin-producing tumors, along with KLKs 6 and 10. The precise role in the pancreas is not yet known. (Clements et al., 2004)

### 1.4.10.6 KLK14:

**Functions:**

- plays a role in salivary gland tumors, (Darling et al., 2006b; N. N. Hashem et al., 2011) ovarian cancers, (Paliouras et al., 2007) and breast cancers, (Borgono et al., 2003; L. Y. Luo et al., 2000; Paliouras & Diamandis, 2007, 2008; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000; Yousef et al., 1999) as well as tumor invasion, (Briot et al., 2009; Gao, Chao, & Chao, 2010; Klucky et al., 2007; Mize, Wang, & Takayama, 2008; Oikonomopoulou, Hansen, Saifeddie, Tea, et al., 2006; Oikonomopoulou, Hansen, Saifeddine, Vergnolle, et al., 2006; Ramsay, Dong, et al., 2008; Ramsay, Reid, et al., 2008; Stephenson, Verity, Ashworth, & Clements, 1999) and skin desquamation. (Brattsand & Egelrud, 1999; Hansson et al., 1994; Komatsu et al., 2005; Komatsu et al., 2003; Lundwall & Brattsand, 2008; Pampilakis & Sotiropoulou, 2007; Stefansson et al., 2006)

- also cleaves low and high molecular weight kininogen, and has a role in causing fluidity of semen by cleaving semenogelins, (de Lamirande, 2007; Deperthes et al., 1996; Emami et al., 2008; Lilja, 1985; Michael et al., 2006)
along with KLKs 2, 5, and 12, is known to activate other kallikreins while KLK5 and KLK14 first activate, then inactivate, KLK3. (Emami & Diamandis, 2008; Michael et al., 2006; Yoon et al., 2009; Yoon et al., 2008)

also cleaves insulin-like growth factor binding proteins (IGFBPs). (Borgono, Michael, Shaw, et al., 2007; Cohen et al., 1992; Koistinen et al., 2002; Matsumura et al., 2005; Michael et al., 2006; Plymate et al., 1996; Rajapakse & Takahashi, 2007; Rehault et al., 2001; Sano et al., 2007)

Locations:

- present in all placental mammals. (Lawrence et al., 2010)

- found in increased levels in breast and prostate cancer, (Lawrence et al., 2010; Yousef, Bharaj, Yu, Poulopoulos, & Diamandis, 2001) in the luminal epithelial cells in the prostate, (Veveris-Lowe et al., 2007) in glandular epithelial cells in visceral organs, (Berglund et al., 2008; Petraki et al., 2006) in salivary gland tumors, (N. N. Hashem et al., 2010) in ovarian cancer, (Paliouras et al., 2007) and in breast carcinoma. (Borgono et al., 2003; L. Y. Lao et al., 2000; Paliouras & Diamandis, 2007, 2008; Shaw & Diamandis, 2008; Yousef, Chang, et al., 2000; Yousef et al., 1999)

1.4.11 Common Known Functions of the KLKs Studied:

Skin desquamation cascade: involves KLK5, KLK6, KLK7, KLK8, KLK10, KLK13 and KLK14.
Antimicrobial function: involves KLK7 (Note: KLK5 also involved)

Semen liquefaction: involves KLK6, KLK7, KLK8, KLK14 (Note: KLK3 also involved)

Myelination and Synaptogenesis: involves KLK6 and KLK8

Pro-hormone activation of insulin, glucagon, somatostatin, and pancreatic polypeptide: involves KLK1, KLK6, KLK10, KLK13 (Note: KLK1 also involved)

Carcinogenesis: involves all 15 KLKs

Amelogenesis: Not previously known in the KLKs we studied, however KLK4 is known to be involved.

Chapter 2

HYPOTHESIS AND RATIONALE
2.1 Hypothesis

In this study, it is hypothesized that the selected odontogenic cysts and tumors will show altered expression of the KLKs 6, 7, 8, 10, 13, and 14, compared to control tissues and that these alterations will be valuable as potential biomarkers for the diagnosis of odontogenic cysts and tumors. In addition, it is hypothesized that alterations in kallikrein expression profiles will help differentiate non-neoplastic odontogenic cysts from low-grade cystic odontogenic neoplasms and an aggressive odontogenic neoplasm. We hypothesize that differences will be present that will provide insights into cystogenesis and neoplasia of odontogenic epithelium.

2.2 Rationale

There is a spectrum of clinical behaviour in developmental cysts and neoplasms of odontogenic epithelium. The lateral periodontal cyst has limited growth potential and does not recur after enucleation; the dentigerous cyst may be aggressive and cause considerable bone destruction; the odontogenic keratocyst is now believed to be a cystic neoplasm (the keratocystic odontogenic tumor (KOT)) because of its destructive behavior, association with a known mutation of the PTCH tumor suppressor gene, and high recurrence rate; and the generic ameloblastoma is considered to be an aggressive, locally invasive neoplasm that requires treatment by en-bloc or segmental resection. There is little information to help explain this behavioural cascade and thus KLKs may help us better understand the molecular biology of cystogenesis and neoplasia of odontogenic epithelium.
2.3 Aims and Objectives

1. To determine immunohistochemically if KLKs 6,7,8,10,13, and 14 are expressed in the epithelium of lateral periodontal cysts, dentigerous cysts, KOTs, ameloblastomas, and in control tissues - nasopalatine duct cysts and odontomas.

2. If staining is present, to determine the epithelial localization of KLKs 6,7,8,10,13, and 14 in the above listed lesions.

3. To determine if cystic odontogenic epithelium preferentially expresses any or all of the studied KLKs by comparing the studied odontogenic cysts to the non-odontogenic nasopalatine duct cyst.

4. To determine if the KLK expression is different in the odontogenic hamartoma (odontoma) compared to the aggressive odontogenic tumor (ameloblastoma).

5. To determine if the differences in the relative expression profiles of these KLKs are useful as diagnostic biomarkers among these lesions.

6. Given objectives 3,4, and 5 above, to determine if KLKs are able to provide insights into cystogenesis and neoplasia of odontogenic epithelium.

Chapter 3

Materials and Methods

3.1 Tissue Specimens
Tissue sections and blocks of formalin-fixed, paraffin-embedded cases from the archives of the Oral Pathology Diagnostic Service, Western University, London, Ontario were obtained. Ten (10) lateral periodontal cysts, 10 dentigerous cysts, 11 KOTs, 10 ameloblastomas, 10 nasopalatine duct cysts, and 10 odontomas (8 Complex, 2 Compound), were each stained and analyzed for KLKs 6,7,8,10,13, and 14. H&E tissues sections were reviewed to confirm the diagnoses, based on the criteria published by the WHO (2005). (Barnes et al., 2005)

Although our plan was to have 10-11 specimens for each of the odontogenic lesions and controls, when tissue sections were cut, some of the specimens lacked sufficient tissue for analysis because the blocks were cut through, resulting in less than 10 data points for some of the lesions for some of the KLKs.

3.1.1 Criteria for Selection of Odontogenic Tumors, Cysts, and Control Tissues

Selected odontogenic cysts and tumors were chosen to represent the different degrees of aggressiveness of developmental lesions of odontogenic epithelium. The odontoma, an odontogenic hamartoma, was chosen as a control tissue because it most closely mimics the tissues and cells of a normal developing tooth. Within our frame of reference, ethics approval was not granted to allow us to use developing teeth from aborted human fetuses as controls. The nasopalatine duct cyst was used as a non-odontogenic cyst of the jaws to assess the influence of odontogenic epithelium on KLK expression. The various cells examined in these specimens included: basal layer cells, clear cells, preameloblasts, stellate reticulum-like cells, ghost cells, squamous layer cells, and odontogenic parakeratin.
3.2 Assay Methods

3.2.1 Immunohistochemistry

In this study, a standard immunoperoxidase staining technique was performed to localize the immunohistochemical expression of human tissue KLKs 6,7,8,10,13, and 14 in the lesions selected. To ensure the sensitivity of the reactions, immunoreactive tissues with known expression of specific KLKs were used as positive controls. Table 3.1 lists the positive control tissues for each KLK. Tissue sections from the control groups and the odontogenic cysts and tumor groups, treated identically, but with the primary antibodies omitted, were used as negative controls in the experiments. Initial pilot experiments had been carried out to determine the ideal antibody titre for each KLK.

<table>
<thead>
<tr>
<th>KLK</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Positive Controls Used for KLKs 6,7,8,10,13, and 14
<table>
<thead>
<tr>
<th>KLK6</th>
<th>Hyperplastic Dental Follicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK7</td>
<td>Adenoid Cystic Carcinoma</td>
</tr>
<tr>
<td>KLK8</td>
<td>Skin</td>
</tr>
<tr>
<td>KLK10</td>
<td>Salivary Gland</td>
</tr>
<tr>
<td>KLK13</td>
<td>Salivary Gland and Skin</td>
</tr>
<tr>
<td>KLK14</td>
<td>Skin</td>
</tr>
</tbody>
</table>

### 3.2.2 Antibody Selection

The primary antibodies that were used in this research study were provided by Dr. E.P. Diamandis of the Department of Laboratory Medicine and Pathobiology at the University of Toronto, Toronto, Ontario. All of the antibodies were rabbit polyclonal antibodies raised against the kallikreins being investigated (KLKs 6, 7, 8, 10, 13, and 14), and were produced via recombination in a mammalian stable cell line system. The KLKs were created and purified by high pressure liquid chromatography (HPLC) as previously reported by Diamandis et al. Previous studies, which used Western Blotting analysis, have shown these antibodies to be highly specific and to lack cross-reactivity with other KLKs.

### 3.2.3 Paraffin Sections
For each formalin-fixed and paraffin-embedded tissue block, 5-µm thick serial sections were cut using a microtome (Microm HM 325; GMI Inc., Ramsey, MN) and were then transferred to a water bath at 45°C. Sections were mounted on positively-charged microscopic glass slides and dried by incubating in an oven at 37°C overnight. Sections had the paraffin removed and were rehydrated by immersion in solutions as follows:

1. Xylene solution immersion for 5 minutes. This is repeated for 5 minutes, then 3 minutes in 3 different containers.
2. Absolute alcohol (100% ethanol) immersion for 2 minutes, then for 1 minute in a different container.
3. 95% alcohol immersion for 2 minutes, then for 1 minute in a different container.
4. 70% alcohol immersion for 1 minute.
5. 100% distilled water immersion for 2 minutes.
6. Slides were then quenched with fresh 3% hydrogen peroxide in methanol for 5 minutes.
7. Slides were then rinsed with distilled water for 5 minutes and then placed in phosphate buffered saline (PBS) on a shaker for 5 minutes.

3.2.4 Staining Procedure
The staining procedure took place as follows:

1. Antigen retrieval was performed in citrate buffer (pH 6.0) in a de-cloaking chamber and then slides were rinsed in running tap water and in PBS for 5 minutes.

2. Slides were blocked in 10% horse serum for 30 minutes at room temperature (RT) in a humidified chamber, then the blocking serum was drained onto a paper towel.

3. Slides were then incubated in a humidified chamber with the primary polyclonal antibodies (rabbit) for the respective kallikreins investigated at the appropriate pre-determined dilution (Table 3.2). Slides which were tested for KLK6 and KLK13 were incubated with antibodies for 1 hour at RT whereas those for KLK7, KLK8, KLK10, and KLK14 were incubated at 4°C overnight (Table 3.2).

4. Slides were then rinsed in PBS for 5 minutes on the shaker to remove excess antibodies.

5. Slides were incubated with ImmPRESS® kits (ImmPRESS® Reagent Kit; Vector Laboratories, Burlingame, CA) anti-rabbit horse-radish peroxidase micro-polymer solution for 30 minutes at RT in a humidified chamber.

6. Slides were then rinsed in PBS for 5 minutes on the shaker to remove excess immunoglobulins.

7. The enzymatic reaction was developed in a freshly prepared solution of 3,3’-diaminobenzidine tetrahydrochloride (DAB Substrate Kit for Peroxidase; Vector Laboratories, Burlingame, CA) which was prepared while antibodies were being
rinsed as follows: to 5 ml dH$_2$O add 2 drops of buffer, 4 drops of DAB, then 2 drops of H$_2$O$_2$ in that order, with vortexing after each step.

8. Slides were then incubated with DAB for 10 minutes at RT and were then drained into a waste container using distilled water (to stop the reaction).

9. Slides were then counterstained in Harris Hematoxylin for 1 minute, then rinsed in running tap water.

10. Slides were blued by being immersed 2-3 times in Ammonium Alcohol (2% Ammonium Hydroxide in 70% Alcohol) then rinsed in running tap water.

11. Slides were then dehydrated by immersion in the following reagents in the following order:

   a. 70% Alcohol for 1 minute
   b. 95% Alcohol for 1 minute
   c. 95% Alcohol for 1 minute
   d. Absolute (100%) alcohol for 2 minutes
   e. Absolute alcohol (100%) for 1 minute
   f. Xylene for 5 minutes
   g. Xylene for 3 minutes

12. Slides were then mounted and cover-slipped in Cytoseal® permount (VWR; Mississauga, ON).
Table 3.2 Antibody Titres and Incubation Times and Conditions for KLKs 6, 7, 8, 10, 13, and 14

<table>
<thead>
<tr>
<th>Kallikrein</th>
<th>Antibody Titre ((\mu\text{L antibody/}\mu\text{L of HS}))</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK6</td>
<td>1/300</td>
<td>1 hour at RT</td>
</tr>
<tr>
<td>KLK7</td>
<td>1/1600</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>KLK8</td>
<td>1/400</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>KLK10</td>
<td>1/400</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>KLK13</td>
<td>1/200</td>
<td>1 hour at RT</td>
</tr>
<tr>
<td>KLK14</td>
<td>1/800</td>
<td>Overnight at 4°C</td>
</tr>
</tbody>
</table>

3.3 Data Collection and Analysis

3.3.1 Scoring Criteria

Staining was viewed under light microscopy and assessed in a semi-quantitative manner by two experienced pathologists (TD, MRD) and a trained graduate student (RW) using a previously described method, (Allred et al., 1993; Darling et al., 2006a; Tuck et al., 1998) which involves assigning a score for intensity of staining (Table 3.3) and the estimated percentage of cells stained in the specimen (Tables 3.4). The sum of these scores is the Immunoreactive Score (IRS) (also reported as the Overall Staining Score (OSS)), (range: 0 for negative staining and 2-8 for positive staining). This scoring system is more valid than solely examining the staining intensity as it takes into account the percentage of cells stained, therefore giving a more accurate assessment of the expression of the KLKs.
The average IRS for each of the KLKs in the control groups and study groups were compared. All cell types were not present in each tissue examined, but were calculated when they were found. The average KLK staining scores were calculated for each individual case and then the final IRS was calculated for each KLK in each of the different control and study groups. Although nuclear staining was seen it was unclear whether this was caused by cytoplasmic overlap. Therefore, it was elected to assess cytoplasmic and cytoplasmic membrane staining only to ensure accuracy.

**Table 3.3 Scores for Estimated Average of Staining Intensity in Stained Cells**

<table>
<thead>
<tr>
<th>Score</th>
<th>Staining Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Weak</td>
</tr>
<tr>
<td>2</td>
<td>Moderate/Intermediate</td>
</tr>
<tr>
<td>3</td>
<td>Strong</td>
</tr>
</tbody>
</table>

**Table 3.4 Scores for Estimated Percentage of Cells Stained**

<table>
<thead>
<tr>
<th>Score</th>
<th>Percentage of Cells Stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>2</td>
<td>1-10%</td>
</tr>
<tr>
<td>3</td>
<td>10-30%</td>
</tr>
<tr>
<td>4</td>
<td>30-60%</td>
</tr>
<tr>
<td>5</td>
<td>&gt;60%</td>
</tr>
</tbody>
</table>
3.3.2 Statistical Analysis

The IRS of each KLK for each of the odontogenic cysts, tumors and controls were analysed and compared. Means and medians were determined and descriptive statistics based on means are reported. Initial tests for significance were done using the non-parametric Kruskall-Wallis Test (KW) followed by both the Dunn's Multiple Comparisons Test (Dunn's) and the Wilcoxon Comparison Test to ensure accuracy. Only results showing statistical significance by both post-tests were considered valid. The level of significance was P<0.05. The non-parametric Spearman's Rank Correlation Test was performed to determine correlation. Analyses were carried out using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA and SAS (Statistical Analysis System), SAS Institute, Cary, North Carolina, 2014.
Chapter 4

RESULTS

4.1 Patient Demographics

Clinical parameters including anonymized patients’ gender, age at diagnosis, and site of the lesions were obtained (Table 4.1).

Mean age and range, expressed in years were: lateral periodontal cyst: mean = 67.9 +/- 9.0, range = 58-84; dentigerous cyst: mean = 42.8 +/- 8.7, range = 26-55; KOT: mean = 46.0 +/- 18.2, range = 19-71; ameloblastoma: mean = 52.2 +/- 21.8, range = 15-82; nasopalatine duct cyst: mean = 47.7 +/- 26.1, range = 4-81; compound odontomas: mean = 14.0 +/- 4.2, range = 11-17; complex odontomas = 16.4 +/- 6.7, range = 7-29; odontomas overall: mean = 15.9 +/- 6.1, range = 7-29.

This study has been approved by the Review and Ethics Board of Western University. (Western University Ethics File #: 6851)

Table 4.1 Clinical Parameters of the Odontogenic and Non-Odontogenic Cysts and Tumors

Lateral Periodontal Cysts

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>Female</td>
<td>Anterior mandible</td>
</tr>
<tr>
<td>84</td>
<td>Female</td>
<td>Anterior mandible</td>
</tr>
<tr>
<td>75</td>
<td>Male</td>
<td>Left maxilla premolar area.</td>
</tr>
<tr>
<td>79</td>
<td>Female</td>
<td>11/21 area</td>
</tr>
<tr>
<td>69</td>
<td>Male</td>
<td>Right mandible</td>
</tr>
<tr>
<td>70</td>
<td>Male</td>
<td>Left mandible</td>
</tr>
<tr>
<td>62</td>
<td>Female</td>
<td>Left mandible 34 area</td>
</tr>
<tr>
<td>58</td>
<td>Male</td>
<td>Right mandible 43/44 area</td>
</tr>
<tr>
<td>59</td>
<td>Female</td>
<td>Left mandible</td>
</tr>
<tr>
<td>62</td>
<td>Female</td>
<td>Left maxilla 21 area</td>
</tr>
</tbody>
</table>
### Dentigerous Cysts

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>Male</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>40</td>
<td>Female</td>
<td>Impacted 38</td>
</tr>
<tr>
<td>40</td>
<td>Female</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>43</td>
<td>Male</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>55</td>
<td>Female</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>26</td>
<td>Female</td>
<td>Impacted 38</td>
</tr>
<tr>
<td>45</td>
<td>Male</td>
<td>Impacted 38</td>
</tr>
<tr>
<td>49</td>
<td>Male</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>50</td>
<td>Male</td>
<td>Impacted 48</td>
</tr>
<tr>
<td>32</td>
<td>Male</td>
<td>Impacted 38</td>
</tr>
</tbody>
</table>

### Keratocystic Odontogenic Tumors (KOTs)

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>Male</td>
<td>Right mandible 48 area</td>
</tr>
<tr>
<td>32</td>
<td>Female</td>
<td>Left posterior mandible.</td>
</tr>
<tr>
<td>71</td>
<td>Male</td>
<td>Mandible 33 and 35 area</td>
</tr>
<tr>
<td>19</td>
<td>Female</td>
<td>Left ramus</td>
</tr>
<tr>
<td>64</td>
<td>Male</td>
<td>Right mandible 46 area</td>
</tr>
<tr>
<td>44</td>
<td>Male</td>
<td>Right mandible</td>
</tr>
<tr>
<td>46</td>
<td>Male</td>
<td>Mucosa between 27 and 24</td>
</tr>
<tr>
<td>57</td>
<td>Male</td>
<td>Left body and ramus mandible</td>
</tr>
<tr>
<td>67</td>
<td>Male</td>
<td>Maxilla 24 area</td>
</tr>
<tr>
<td>20</td>
<td>Male</td>
<td>Left mandible 38 area</td>
</tr>
<tr>
<td>52</td>
<td>Male</td>
<td>Left maxilla 24/25 area</td>
</tr>
</tbody>
</table>

### Ameloblastomas

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>Female</td>
<td>Lingual to 31 to 42.</td>
</tr>
<tr>
<td>74</td>
<td>Male</td>
<td>Left anterior mandible</td>
</tr>
<tr>
<td>35</td>
<td>Female</td>
<td>Left posterior mandible</td>
</tr>
<tr>
<td>55</td>
<td>Male</td>
<td>Left ascending ramus</td>
</tr>
<tr>
<td>51</td>
<td>Female</td>
<td>Left mandible</td>
</tr>
<tr>
<td>15</td>
<td>Female</td>
<td>38 area</td>
</tr>
<tr>
<td>75</td>
<td>Female</td>
<td>Right maxillary sinus</td>
</tr>
<tr>
<td>52</td>
<td>Male</td>
<td>Right mandible 46 area</td>
</tr>
<tr>
<td>57</td>
<td>Male</td>
<td>32/33 area</td>
</tr>
<tr>
<td>26</td>
<td>Female</td>
<td>35 area</td>
</tr>
</tbody>
</table>
### Nasopalatine Duct Cysts

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>Male</td>
<td>Right maxilla 11 area</td>
</tr>
<tr>
<td>32</td>
<td>Female</td>
<td>11/21 area</td>
</tr>
<tr>
<td>78</td>
<td>Male</td>
<td>Incisive canal</td>
</tr>
<tr>
<td>53</td>
<td>Male</td>
<td>Anterior maxilla</td>
</tr>
<tr>
<td>12</td>
<td>Male</td>
<td>Anterior maxilla 11/21 area</td>
</tr>
<tr>
<td>53</td>
<td>Female</td>
<td>Right anterior maxilla</td>
</tr>
<tr>
<td>67</td>
<td>Female</td>
<td>Anterior maxilla</td>
</tr>
<tr>
<td>59</td>
<td>Male</td>
<td>Right anterior maxilla</td>
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<tr>
<td>4</td>
<td>Male</td>
<td>Hard palate</td>
</tr>
<tr>
<td>38</td>
<td>Female</td>
<td>Anterior maxilla</td>
</tr>
</tbody>
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### Odontomas

#### Complex Odontomas

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>Female</td>
<td>Maxilla 22/23 area</td>
</tr>
<tr>
<td>19</td>
<td>Female</td>
<td>Left posterior maxilla</td>
</tr>
<tr>
<td>18</td>
<td>Female</td>
<td>Mandible 38 area</td>
</tr>
<tr>
<td>18</td>
<td>Male</td>
<td>Maxilla 28 area</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>Maxilla 16 area</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>Right mandible 85 area</td>
</tr>
<tr>
<td>13</td>
<td>Male</td>
<td>Left mandible apical to 73</td>
</tr>
<tr>
<td>17</td>
<td>Female</td>
<td>Left maxillary tuberosity</td>
</tr>
</tbody>
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#### Compound Odontomas

<table>
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<th>Site</th>
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</thead>
<tbody>
<tr>
<td>17</td>
<td>Male</td>
<td>Left mandible 32/33 area</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>Right anterior maxilla</td>
</tr>
</tbody>
</table>
4.2 Experimental Results of Staining

The presence and localization of staining for each KLK in each of the studied cysts/tumors are illustrated in Figs. 4.1 to 4.6, and crossreferenced in Figs. 4.7 to 4.12. The mean and median values for the study and control groups versus KLKs are presented in Tables 4.2 and 4.3 respectively. Fig 4.13 is a composite of KLK immunostaining vs odontogenic cysts, tumors and controls. Figs 4.14 to 4.19 illustrate the median value of KLK immunostaining and the standard deviation for each odontogenic cyst, tumor and control. Figs 4.20 to 4.25 illustrate the median value of staining of the odontogenic cysts, tumors and controls for each KLK. Fig 4.26 illustrates the overall staining score of each KLK per lesions. Fig 4.27 illustrates the overall staining score of each lesion per KLK. Table 4.4 illustrates the statistically significant correlations as determined by the Spearman’s Rank Correlation Test.

Stated magnification in the figures below refers to the power of the objective lens when the image was taken.
Fig 4.1. Lateral Periodontal Cyst (LPC) immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.2. Dentigerous Cyst immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.3. Keratocystic Odontogenic Tumor (KOT) immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.4. Ameloblastoma immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.5. Nasopalatine Duct (NPD) Cyst immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.6. Odontoma immunostaining and localization by antibodies to KLKs 6,7,8,10,13,14.
Fig 4.7. KLK6 immunostaining of the cysts and tumors studied.
Fig 4.8. KLK7 immunostaining of the cysts and tumors studied.
Fig 4.9. KLK8 immunostaining of the cysts and tumors studied.

NPD Cyst – X20 magnification  
LPC – X10 magnification

Dentigerous Cyst – X10 magnification  
KOT – X10 magnification

Odontoma – X10 magnification  
Ameloblastoma – X10 magnification
Fig 4.10. KLK10 immunostaining of the cysts and tumors studied.

- NPD Cyst – X20 magnification
- LPC – X10 magnification
- Dentigerous Cyst – X10 magnification
- KOT – X10 magnification
- Odontoma – X10 magnification
- Ameloblastoma – X10 magnification
Fig 4.11. KLK13 immunostaining of the cysts and tumors studied.

NPD Cyst – X20 magnification
LPC – X10 magnification

Dentigerous Cyst – X10 magnification
KOT – X10 magnification

Odontoma – X10 magnification
Ameloblastoma – X10 magnification
Fig 4.12. KLK14 immunostaining of the cysts and tumors studied.
### Table 4.2. Mean Values for Odontogenic Cysts/Tumor and Controls vs KLKs

<table>
<thead>
<tr>
<th></th>
<th>Nasopalatine Duct Cyst</th>
<th>Lateral Periodontal Cyst</th>
<th>Dentigerous Cyst</th>
<th>KOT</th>
<th>Odontoma</th>
<th>Ameloblastoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK6</td>
<td>6.9 ± 1.05</td>
<td>7.3 ± 0.48</td>
<td>6.7 ± 0.46</td>
<td>6.6 ± 0.50</td>
<td>7.0 ± 0.00</td>
<td>7.8 ± 0.63</td>
</tr>
<tr>
<td>KLK7</td>
<td>5.6 ± 0.70</td>
<td>6.4 ± 0.97</td>
<td>5.8 ± 0.40</td>
<td>6.6 ± 0.50</td>
<td>6.2 ± 0.83</td>
<td>6.1 ± 0.87</td>
</tr>
<tr>
<td>KLK8</td>
<td>6.5 ± 0.71</td>
<td>6.8 ± 0.44</td>
<td>6.7 ± 0.46</td>
<td>6.2 ± 0.60</td>
<td>6.3 ± 0.70</td>
<td>6.9 ± 0.31</td>
</tr>
<tr>
<td>KLK10</td>
<td>6.3 ± 1.06</td>
<td>7.0 ± 0.67</td>
<td>6.6 ± 0.69</td>
<td>7.0 ± 0.00</td>
<td>6.2 ± 0.97</td>
<td>7.5 ± 0.70</td>
</tr>
<tr>
<td>KLK13</td>
<td>6.7 ± 0.67</td>
<td>6.1 ± 0.33</td>
<td>6.1 ± 0.30</td>
<td>7.0 ± 0.00</td>
<td>5.6 ± 2.21</td>
<td>7.7 ± 0.48</td>
</tr>
<tr>
<td>KLK14</td>
<td>5.7 ± 1.41</td>
<td>6.6 ± 1.24</td>
<td>6.4 ± 0.92</td>
<td>6.6 ± 0.67</td>
<td>5.5 ± 0.93</td>
<td>6.7 ± 0.48</td>
</tr>
</tbody>
</table>

Value ± Standard Deviation

### Table 4.3. Median Values for Odontogenic Cysts/Tumor and Controls vs KLKs

<table>
<thead>
<tr>
<th></th>
<th>Nasopalatine Duct Cyst</th>
<th>Lateral Periodontal Cyst</th>
<th>Dentigerous Cyst</th>
<th>KOT</th>
<th>Odontoma</th>
<th>Ameloblastoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK6</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td>KLK7</td>
<td>5.5</td>
<td>7.0</td>
<td>6.0</td>
<td>7.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>KLK8</td>
<td>6.0</td>
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<td>7.0</td>
<td>6.0</td>
<td>6.0</td>
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</tr>
<tr>
<td>KLK10</td>
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<td>7.0</td>
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<td>8.0</td>
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</table>
Odontogenic Cysts/Tumor and Controls vs KLKs

Fig 4.13 Composite of immunostaining mean values vs odontogenic cysts and tumors and controls

KLK expression in Nasopalatine Duct Cyst.

Fig 4.14 KLK expression in Nasopalatine Duct Cyst. Median values of overall staining score for each KLK in the nasopalatine duct cyst are displayed numerically adjacent to each dot plot.
Fig 4.15 KLK expression in Lateral Periodontal Cyst. Median values of overall staining score for each KLK in the lateral periodontal cyst are displayed numerically adjacent to each dot plot.

Fig 4.16 KLK expression in Dentigerous Cyst. Median values of overall staining score for each KLK in the dentigerous cyst are displayed numerically adjacent to each dot plot.
Fig 4.17 KLK expression in Keratocystic Odontogenic Tumor. Median values of overall staining score for each KLK in the keratocystic odontogenic tumor are displayed numerically adjacent to each dot plot.

Fig 4.18 KLK expression in Odontoma. Median values of overall staining score for each KLK in the odontoma are displayed numerically adjacent to each dot plot.
**Fig 4.19 KLK expression in Ameloblastoma.** Median values of overall staining score for each KLK in the ameloblastoma are displayed numerically adjacent to each dot plot.

**Fig 4.20 KLK6 expression in odontogenic cysts and tumors and controls.** Median values of overall staining score for KLK6 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.
Fig 4.21 KLK7 expression in odontogenic cysts and tumors and controls. Median values of overall staining score for KLK7 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.

Fig 4.22 KLK8 expression in odontogenic cysts and tumors and controls. Median values of overall staining score for KLK8 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.
Fig 4.23 KLK10 expression in odontogenic cysts and tumors and controls. Median values of overall staining score for KLK10 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.

Fig 4.24 KLK13 expression in odontogenic cysts and tumors and controls. Median values of overall staining score for KLK13 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.
**Fig 4.25** KLK14 expression in odontogenic cysts and tumors and controls. Median values of overall staining score for KLK14 in each odontogenic cyst and tumor and controls are displayed numerically adjacent to each dot plot.

**Immunostaining Profiles: Overall Staining Score of each KLK per Lesion**

**Fig 4.26.** Immunostaining profiles: overall staining score of each KLK per lesion (odontogenic cysts and tumors and controls)
Fig 4.27. Immunostaining profiles: overall staining score of each lesion (odontogenic cysts and tumors and controls) per KLK

Table 4.4 Statistically significant correlations as determined by Spearman’s Rank Correlation Test at P<0.05. “All” indicates the combined data from all lesions studied.
4.3 Statistical Analysis

Experimental findings indicated that KLK6 was expressed in the epithelium of all of the control and study groups but there was variation of median expression (KW p=0.0001). KLK6 was shown to be statistically significantly higher in ameloblastomas than in dentigerous cysts, KOTs, and odontomas with post-KW comparison tests p<0.001, p<0.001 and p<0.05 respectively for those groups.

KLK7 was expressed in the epithelium of all tissues, but variation of expression was found between the groups (KW p=0.0142). However, post-KW comparison tests showed a significant difference in expression only between KOTs and nasopalatine duct cysts (p<0.05).

KLK8 was expressed in the epithelium of all tissues. There was variable expression amongst controls, cysts and tumors (KW p=0.0108), with significantly higher expression in ameloblastomas than KOTs (post-KW comparison tests p<0.05).

KLK10 was also expressed throughout the epithelium in all tissues studied, but was expressed with greatest intensity in the parakeratin layer of KOTs. Variable expression of KLK10 was also seen amongst the different groups (KW p=0.0052), and expression was found to be significantly higher in ameloblastomas than in nasopalatine duct cysts and odontomas (post-KW comparison tests p<0.05 for both respectively).

KLK13 was expressed throughout the epithelium in all tissues studied. There was variable expression amongst controls, cysts, and tumors (KW p<0.0001). When comparing expression in ameloblastomas to the other groups using the post-KW
comparison tests, a statistically significant greater expression was evident in ameloblastomas compared to lateral periodontal cysts (p<0.001), dentigerous cysts (p<0.001), and odontomas (p<0.001). Also, again using the post-KW comparison tests, there was a significantly higher expression of KLK13 in KOTs when compared to lateral periodontal cysts (p<0.05), dentigerous cysts (p<0.05), and odontomas (p<0.05).

KLK14 was expressed throughout the epithelium in all tissues studied. Unlike the other KLKs, KLK14 did not show variable expression between the control, cyst, and tumor groups (KW p=0.1202), and no significant difference was found between any of the groups studied (post-KW comparison tests p>0.05 for all groups).

4.3.1 Summary of Statistical Analysis

Odontoma
- Variation between all KLKs (Kruskal-Wallis p=0.0168)
- KLK6 significantly higher than KLK14 (post-KW comparison tests p<0.01)

NPD Cyst
- Variation between all KLKs (KW p =0.0211)
- KLK6 is significantly higher than KLK7 (post-KW comparison tests p<0.05)
LP Cyst
- Variation between all KLKs (KW p= 0.0039)
- KLK6 is significantly higher than KLK13 (post-KW comparison tests p<0.01)

Dentigerous Cyst
- Variation between all KLKs (KW p=0.0006)
- KLK6 is significantly higher than KLK7 (post-KW comparison tests p<0.01)
- KLK8 is significantly higher than KLK7 (post-KW comparison tests p<0.01)

KOT
- Variation between all KLKs (KW p = 0.0008)
- KLK10 is significantly higher than KLK8 (post-KW comparison tests p<0.01)
- KLK13 is significantly higher than KLK8 (post-KW comparison tests p<0.01)

Ameloblastoma
- Variation between all KLKs (KW p< 0.0001)
- KLK6 is significantly higher than KLK7 (post-KW comparison tests p<0.001)
- KLK6 is significantly higher than KLK8 (post-KW comparison tests p<0.05)
- KLK6 is significantly higher than KLK14 (post-KW comparison tests p<0.01)
- KLK10 is significantly higher than KLK7 (post-KW comparison tests \( p<0.01 \))
- KLK13 is significantly higher than KLK7 (post-KW comparison tests \( p<0.001 \))
- KLK13 is significantly higher than KLK14 (post-KW comparison tests \( p<0.05 \))

**KLK6**
- Variation exists amongst controls, cysts and tumors (KW \( p=0.0001 \))
- Significantly higher in Ameloblastoma than Dentigerous Cyst (post-KW comparison tests \( p<0.001 \))
- Significantly higher in Ameloblastoma than KOT (post-KW comparison tests \( p<0.001 \))
- Significantly higher in Ameloblastoma than Odontoma (post-KW comparison tests \( p<0.05 \))

**KLK7**
- Variation exists amongst controls, cysts, and tumors (KW \( p=0.0142 \))
- Significantly higher in KOT than NPD cyst (post-KW comparison tests \( p<0.05 \))

**KLK8**
- Variation exists amongst controls, cysts and tumors (KW \( p=0.0108 \))
- Significantly higher in Ameloblastoma than KOT (post-KW comparison tests p<0.05)

**KLK10**
- Variation exists amongst controls, cysts, and tumors (KW p=0.0052)
- Significantly higher in Ameloblastoma than NPD cyst (post-KW comparison tests p<0.05)
- Significantly higher in Ameloblastoma than Odontoma (post-KW comparison tests p<0.05)

**KLK13**
- Variation exists amongst controls, cysts, and tumors (KW p<0.0001)
- Significantly higher in KOT than LP cyst (post-KW comparison tests p<0.05)
- Significantly higher in Ameloblastoma than LP cyst (post-KW comparison tests p<0.001)
- Significantly higher in KOT than Dentigerous cyst (post-KW comparison tests p<0.05)
- Significantly higher in Ameloblastoma than Dentigerous cyst (post-KW comparison tests p<0.001)
- Significantly higher in KOT than Odontoma (post-KW comparison tests p<0.05)
- Significantly higher in Ameloblastoma than Odontoma (post-KW comparison tests p<0.001)

**KLK14**
- NO variation exists amongst controls, cysts, and tumors (KW p = 0.1202)
- No significant difference found between controls, cysts, and tumors (post-KW comparison tests p>0.05)
4.4 KLK Staining of Specific Tissues

4.4.1 Enamel Matrix

Enamel matrix stained with most of the KLKs studied, although not with the same intensity. Staining was most intense with KLKs 8 and 13; moderately intense with KLKs 6, 7 and 10; and minimal (possibly background staining) with KLK14. Fig 4.28 illustrates KLK staining of enamel matrix in odontomas.
4.4.2 Epithelial Layers of Odontogenic Cysts

4.4.2.1 Non-Staining of Basal/Parabasal Layer Cells

There was a lack of staining of the basal and sometimes the parabasal layer cells in NPD cysts by KLKs 6,10; LP cysts by KLKs 6,7 (fig 4.29),10,13, and 14; and KOTs by KLKs 6,7,8,10, and 14.

Fig 4.29: Lack of staining in the basal and parabasal layer cells in an LP cyst with KLK7.
4.4.2.2 KLK Specific Staining of Parakeratin in KOTs

An interesting finding was the diversity of staining of the parakeratin layer of KOTs. Heavy staining of parakeratin was found for KLKs 7 and 10 (fig 4.30a), while the same layers were unstained or poorly stained by KLKs 8 (fig 4.30b), 13 and 14.

Fig 4.30a: There was heavy staining of the upper layers, especially the parakeratin layer by KLK10 in KOTs.
Fig 4.30b. KLK8 failed to stain the parakeratin layer (and only weak, inconsistent staining of the basal layer) in KOTs.
4.4.2.3 Desmosomal Staining

Cell membrane staining, interpreted to be desmosomal staining, was found in some examples of LP cysts by KLKs 8, 10, and 14; dentigerous cysts by KLKs 8, 10, and 14; odontomas by KLKs 8 and 14; and ameloblastomas by KLKs 7 and 8. (Fig 4.31)

Fig 4.31. KLK8 staining of desmosomes of ameloblasts in an odontoma.
Chapter 5

DISCUSSION

5.1 The Expression of KLKs 6,7,8,10,13, and 14 in Odontogenic Epithelium of Cysts and Tumors.

This study is the first documentation of the presence of KLKs 6,7,8,10,13 and 14 in odontogenic epithelium, whether normal or pathological.

We found that KLK6 was highly expressed in all of the control and study groups but variation of expression existed (KW p=0.0001), based largely on the very high expression in ameloblastomas. KLK6 was shown to be statistically significantly higher in ameloblastomas than in dentigerous cysts (post-KW correlation tests p<0.001), KOTs (post-KW correlation tests p<0.001), and odontomas (post-KW correlation tests p<0.05). Since ameloblastomas are the most aggressive tumor, this suggests that KLK6 is involved in some way in the behaviour of the more aggressive tumors.

We found that KLK7 expression was generally clustered, and less well expressed than the other KLKs with the exception of staining in the KOT. The KOT increased staining is likely related to the known function of KLK 7 in keratinization and desquamation.\(^\text{(Clements et al., 2004)}\) The expression of KLK7 was significantly higher in KOTs than in the control NPD cyst (post-KW correlation tests p<0.05), perhaps again related to the known role of KLK7 in keratinization and desquamation, but the possibility of specific activity related to odontogenic lesions cannot be dismissed. KLKs 6 and 7 showed statistically significant positive correlation (Spearman’s Rank Correlation Test p=0.0476) in KOTs.
Expression of KLK8 was relatively clustered for all lesions (fig 4.26) with the exception of statistically significantly greater expression in ameloblastomas than in KOTs (post-KW correlation tests p<0.05), related in part to the lack of staining by KLK8 in the basal and keratinized layers of the KOT (fig 4.30b), and the increased staining of cell membranes (desmosomes) in the ameloblastoma (fig 4.31). The close clustering of staining in all of the lesions, including the NPD Cyst, suggests that KLK8 is not significantly involved in odontogenic lesions or aggressiveness of these lesions. When all lesions are combined, KLKs 6 and 8 show statistically significant correlation by Spearman’s Rank Correlation Test (p=0.0017), confirming the close clustering demonstrated in Fig. 4.26.

KLK10 showed more varied expression (fig 4.26) among the lesions (KW p=0.0052) with increased expression in the neoplasms (ameloblastoma, KOT) and decreased expression in the controls (NPD Cyst and odontoma) (post-KW correlation tests: ameloblastoma vs NPDC: p<0.05; ameloblastoma vs odontoma: p<0.05), suggesting a greater role in neoplasia and aggressive behavior. Surprisingly, the LP cyst also showed higher levels of KLK10 expression (although not statistically significant). The significance of this finding is unknown.

KLK13 showed the greatest variability in staining intensity among the lesions (fig 4.26) (KW p<0.0001, considered highly statistically significant) and continued the trend seen with KLK10, of higher expression in the neoplasms and lower in the odontogenic cysts (post-KW correlation tests ameloblastoma vs odontoma p<0.001; ameloblastoma vs dentigerous cysts p<0.05; ameloblastoma vs LP cyst p<0.001; KOT vs LP cyst p<0.05; KOT vs dentigerous cyst p<0.05). This strongly suggests that
KLK13 is involved in the process of odontogenic neoplasia. In fact, there was no statistical difference between any of the odontogenic cysts and the control NPD Cyst indicating that the influence of KLK13 on cyst formation is not specific to odontogenic epithelium. The surprising finding that both KLK10 and KLK13 are more highly expressed in odontogenic neoplasms suggest that they may work synergistically as part of a KLK cascade. Although, this did not reach statistical significance (Spearman’s Rank Correlation Test p=0.1220).

KLK14 expression was again clustered and lower, and did not show sufficient variability to reach statistical significance. (KW p=0.1202) This suggests that KLK14 is not specifically involved in odontogenic neoplasia or cystogenesis.

5.2 Selected Observations on the Localization of Expression of KLKs

5.2.1 Reduced or Non-staining of Basal and Parabasal Layer Cells in Cysts.

A recurring finding was a partial or complete lack of staining (sparing) of basal and parabasal layer cells for some of the KLKs studied. The LP cyst and KOT showed basal sparing for KLKs 6,7,10 and 14, with increased expression in the middle epithelial layers (fig 4.29). The dentigerous cyst did not show this stratification difference. These observations suggest that KLKs 6,7,10 and 14 are not involved in cell cycling (proliferation) but are involved in differentiation, or at least maintenance, of specialized epithelium (LP cyst: odontogenic features of rests of Serres; KOT: parakeratinization; Dentigerous cysts: simple stratified squamous epithelium). This interpretation is supported by the findings for the control NPD
cyst, which also showed basal sparing for KLKs 6 and 7, but increased staining intensity in areas showing respiratory epithelium (KLKs 6, 8, and 13). However, the fact that both the control and the experimental cysts showed these changes indicates that the process is not specific to odontogenic cysts.

Klucky et al claimed that KLK6 is involved in cell proliferation, which is opposite to our view that KLK6 is not involved in cell cycling. However, their study was directed at e-cadherin degradation by KLK6 in cell separation and tissue invasion.

5.2.2 Variable Staining of Parakeratin in KOTs and Desmosomal Staining.

An interesting finding was the variability of staining between KLKs of the parakeratin layer of KOTs. There was consistently increased staining by KLKs 7 and 10 (fig 4.30a) but, paradoxically, decreased staining by KLKs 8 (fig 4.30b), 13 and 14. KLKs have been known to be present in skin and involved in the process of keratinization (KLK7 – stratum corneum formation) and especially desquamation (KLKs 5, 6, 7, 8, 10, 13, 14). KLK7 has been shown to degrade plakoglobins and corneodesmosin, destroying cell adhesion and therefore causing skin desquamation. KLK7 has been shown to be a true marker of terminal differentiation in keratinizing, cornifying squamous epithelium. KLK8 has also been shown to have role in the terminal differentiation of
The finding of increased KLK7 staining in an increasing gradient from parabasal to keratin layer is in agreement with previous findings of its involvement in the process of keratinization and desquamation. We found KLK10 to follow the same pattern (fig 4.30a). This suggests that KLK10 may work in a KLK cascade with KLK7 in the process of desquamation. Desquamation of the parakeratin occurs in KOTs. Therefore, the increased staining of KLKs 7 and 10 in the parakeratin layer of KOTs further demonstrates their role in desquamation. Since this is occurring in skin as well as the KOT, the involvement of the KLKs seems process driven, rather than tissue specific. The lack or partial lack of staining by KLKs 8, 13, and 14 is harder to explain, since these KLKs also have been shown to cause degradation of adhesion molecules (E cadherin, plakoglobin and desmoglein 1) in keratin desquamation in skin. One possible answer is that skin is orthokeratinized while KOTs are parakeratinized and the desquamation process may differ. In fact, our findings of desmosomal staining by KLKs 8 and/or 10 in LP cysts, dentigerous cysts, odontomas (fig 4.31), and ameloblastomas suggests that these molecules are involved in adhesion in odontogenic epithelium, rather than separation of cells. This interpretation would also explain the lack of KLKs 8, 13 and 14 in desquamating parakeratin.
5.2.3 Staining of Enamel Matrix in the Process of Odontogenesis

KLK4 has been studied extensively in the process of odontogenesis. (Clements et al., 2004; Hu et al., 2002; Hu et al., 2000; Simmer & Hu, 2002; Wright et al., 2006) (See Introduction: 1.4.8)

Enamel matrix, a combination of a number of known proteins including amelogenin (about 90% of matrix protein), enamelin, and ameloblastin, is deposited initially to form the tooth shape, and is then replaced during calcification in the maturation phase, by hydroxyapatite. For calcification to occur, the matrix must be broken down and removed. Two proteins are currently known to be involved in this degradation: MMP-20 (enamelysin) and KLK4. (Neville, Damm, Allen, & Bouquot, 2009) However, it is known that KLKs often work in cascades with other KLKs. In this study using enamel matrix produced in the odontomas, we found that KLKs 8 and 13 were found in high concentration in enamel matrix, with less intense staining by KLKs 6, 7, and 10. Normal appearing dentin did not stain and negative controls were negative for matrix staining. These findings indicate that the ameloblasts likely secrete KLKs 6, 7, 8, 10 and 13 into the enamel matrix where they may act synergistically with KLK4 in enamel matrix degradation during the maturation phase. It is possible that other KLKs not yet studied are also involved in this process. Future studies are needed to investigate the roles of various KLKs in enamel matrix degradation.

5.3 Semi-quantitative Estimation of KLK Expression

The intensity and percentage of staining of each specimen was estimated simultaneously by three individuals (see Methods). This, however, was somewhat subjective. An attempt was made to provide a score for each specimen objectively
with a computer program that can analyze specimens for staining intensity and percentage of staining of regions outlined by the researcher. However, for technical reasons, such as tissue overlap, inability to standardize staining against controls, vagueness of background staining, subjectivity in outlining, difficulty in outlining on a computer screen, and interpretation of colour variations, the computerized program was rendered less reliable than the initial human analysis. Therefore, after comparing results, subjective analysis was interpreted to be more reliable. (Axiovision, Carl Zeiss Microimaging GmbH, Goettingen, Germany).

5.4 The Expression of KLKs 6,7,8,10,13, and 14 in Odontogenic Cystic Lesions (Lateral Periodontal Cyst, Dentigerous Cyst, KOT) vs the Non-Odontogenic Control (Nasopalatine Duct Cyst).

All KLKs studied were found in the epithelium of all of the cysts studied, including the control, the NPD cyst, indicating a non-specific role for these KLKs in cystic epithelial tissues of the jaws. Relative to the control, the NPD cyst, only the KOT showed a significant increase in staining with KLK7 (post-KW correlation tests p<0.05), which can be adequately explained by the involvement of KLK7 in the process of desquamating keratin. The lack of a significant difference in staining of the NPD cyst and the odontogenic cysts with any of the other KLKs argues against a specific role of these KLKs in odontogenic cystogenesis. However, there were significant differences among the odontogenic cysts of different destructive potential, at least with respect to KLK13, where the staining in the KOT was found to be significantly greater than that seen in both of the other odontogenic cysts, the LP
cyst (p<0.05) and the dentigerous cyst (p<0.05). Since KLK13 was found to be underexpressed in the parakeratin layer of KOTs, the process of keratinization/keratin desquamation cannot explain these differences, suggesting that KLK13 may be involved in more aggressive behavior and increased potential for recurrence.

KLK10 appears to have a similar involvement as KLK13 (although not statistically significant), and perhaps they work together in a common pathway. Therefore, KLKs 10 and 13 may potentially be useful as biomarkers, if found in human serum, for the diagnosis and management of more aggressive odontogenic cystic lesions, such as the KOT.

5.5 The Expression of KLKs 6, 7, 8, 10, 13, and 14 in the Odontoma (Hamartoma Control) vs the Ameloblastoma (Aggressive Odontogenic Neoplasm)

To determine if any of these KLKs could impact the process of neoplasia in odontogenic epithelium, the staining of the hamartoma – the odontoma – was compared to the staining of the most aggressive neoplasm of all benign odontogenic tumors – the ameloblastoma. Importantly, the staining intensity of the ameloblastoma was significantly more intense than the odontoma for KLK6 (post-KW comparison tests p<0.05), KLK10 (post-KW comparison tests p<0.05) and KLK13 (post-KW comparison tests p<0.001) suggesting a role of these KLKs in more aggressive lesions. The upregulation of KLK13 in the KOT, relative to the other jaw cysts (see 5.4) supports this interpretation. Exactly how these KLKs are involved in aggressive behavior is unknown. It is unlikely that KLKs 6, 10 and 13 are involved in increased proliferation (see 5.2.1 basal layer sparing), so the increased expression
may simply be related to increased demands on differentiation as a secondary response to the increased neoplastic activity. Hashem et al have previously noted that KLK6 and KLK13 seem to be involved in a common pathway in a salivary gland tumor (pleomorphic adenoma). (N.N. Hashem, 2008) Our findings seem to further support this relationship between KLK6 and KLK13, and perhaps furthermore involving KLK10 in a common pathway or cascade in the development and progression of odontogenic neoplasms. However, this relationship was not shown to be statistically significant by Spearman’s Rank Correlation Test.

Given these findings, KLK13 may be a useful surrogate in situ marker for aggressive odontogenic epithelial lesions (KOT, ameloblastoma) similar to p16 for HPV detection in some mucosal squamous cell carcinomas.

Further research is needed to delineate the roles of KLKs 6, 10 and 13 in the development and progression of odontogenic neoplasms.

5.6 Diagnostic Markers

From our study, it appears that KLKs 6, 10, and 13 are potential candidates to be used as diagnostic markers for the more aggressive odontogenic lesions (See 5.4 and 5.5). Our studies show their usefulness as potential in-situ immunohistochemical markers for relative aggressiveness of jaw lesions, but they cannot determine odontogenic lineage. For these KLKs to be used as biomarkers for the studied odontogenic cysts and tumors, further investigation to determine if serum concentrations or salivary concentrations of KLKs, especially KLK13 for ameloblastoma, vary with the presence of this tumor, and if serum or salivary
concentration of KLK13 could predict recurrence after surgery. Salivary samples would be less invasive than obtaining serum samples for isolating kallikrein concentrations.

5.7 Correlations

KLK14 did not demonstrate any statistically significant differences using the Kruskal-Wallis test. Consequently, it showed the most correlation by Spearman’s Rank Correlation Test with KLKs 8, 10, and 13, when all lesions are considered (p=0.0045, p=0.0096, p=0.0369 respectively). Interestingly, there was a correlation of KLKs 8 and 14 for both NPD cyst and dentigerous cyst, again suggesting that KLK expression is not specific for odontogenic epithelium.
Chapter 6

CONCLUSIONS

1) For the first time, KLKs 6, 7, 8, 10, 13, and 14 have been documented and localized in the epithelium of human odontogenic cysts and tumors.

2) The localization of various KLKs in odontogenic epithelium and tissues of the studied cysts and tumors varied: a) There was sparing of the basal and parabasal layers in LP cyst and/or KOT; b) There was increased expression in the keratin layer of KOTs by KLKs 7 and 10; c) Cell membrane (desmosomal) staining was present in most odontogenic lesions with KLKs 8, 10, and sometimes 14; d) Enamel matrix in odontomas exhibited staining for KLKs 6, 7, 8, 10, and 13.

3) The role of the studied KLKs in cystogenesis of jaw cysts is not considered to be specific for odontogenic epithelium.

4) KLKs 6, 10, and 13 have shown evidence of involvement in the process of neoplasia in the ameloblastoma, and KLKs 10 and 13 in the KOT.

5) KLK10, and especially KLK13, showed greater expression in neoplastic tissues relative to cysts and hamartomas, suggesting a potential role as a tissue section in-situ marker for odontogenic neoplasia.

6) Elevated co-expression of KLKs 10 and 13, and possibly KLK6, in odontogenic neoplasms suggests that these may act in a KLK cascade. Similarly, co-expression of KLKs 6, 7, 8, 10, and 13 in enamel matrix suggests a cascade mechanism in conjunction with KLK4 in the resorption of enamel matrix during the maturation phase of amelogenesis.
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