IMMUNOHISTOCHEMICAL PROFILE OF ODONTOGENIC EPITHELIUM OF DEVELOPING DOG TEETH *(CANIS FAMILIARIS)*

By

SULETTE NEL

Submitted in partial fulfilment of the requirements for the degree of Master of Science (Odontology) in the School of Dentistry, Faculty of Health Sciences, University of Pretoria.

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DECLARATION OF OWN WORK

I declare that the dissertation, which I hereby submit for the degree of Master of Science (Odontology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Sulette Nel

SYNOPSIS OF THE DISSERTATION

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MSc (Odontology) in Oral Biology

ABSTRACT

Similarities between the acanthomatous epulis and ameloblastomas resulted in debate regarding the nature and origin of the acanthomatous epulis found in dogs. In an attempt to elucidate the origin and character of the acanthomatous epulides, this study aimed to find suitable cell markers to identify odontogenic epithelium versus oral epithelium in developing dog teeth in order to use in future research on the pathogenesis and pathology of odontogenic neoplasms in dogs. As specific markers for odontogenic epithelium have not been described in dog tissue, proposed markers of odontogenic epithelium of human and rat tissues were tested on developing dog teeth. Keratin 14, keratin 19, amelogenin, p75 neurotrophin receptor and calretinin have been proposed as markers for inner enamel epithelium and/or ameloblasts in human and rat tissue and was therefore included in this study.

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Keratin 14 and keratin 19 can not be regarded as specific markers of odontogenic epithelium as various other types of epithelium also stained positive with these markers. Amelogenin could be a promising marker to distinguish between odontogenic tumours and non-odontogenic tumours as it was only detected in odontogenic tissues in this study. However, amelogenin has also been observed in other tissues in dogs and rats, and therefore further studies on this protein will be needed to elucidate the expression profile of amelogenin in odontogenic versus non-odontogenic tissues in dogs. p75 Neurotrophin receptor expression was restricted to certain regions of the inner enamel epithelium and no staining was observed in other epithelial cells. It therefore seems to be a promising marker to differentiate between odontogenic and non-odontogenic epithelium, but the widespread staining observed in the mesenchymal tissue makes differentiation between odontogenic and non-odontogenic stromal elements impossible. Calretinin staining was observed in the alveolar epithelial cells directly overlying the developing tooth germ, proposed as the oral epithelium where the dental lamina takes origin from, as well as the dental laminae and Serres rests. No staining was observed in the rest of the oral epithelium and it can therefore be proposed that calretinin could be a useful marker to distinguish between odontogenic and non-odontogenic epithelial cells.

In light of the results found in this study on foetal tissue, the expression profile may be different in adult tissue. Odontogenic tumours in adult dogs may originate from remnants of odontogenic tissue like Serres rests and Malassez rests. It is therefore proposed that this study be repeated on adult dog tissue with specific reference to Serres rests, Malassez rests and the associated gingiva.

Key terms: odontogenic epithelium, odontogenesis, dog, canine, calretinin, keratin 14, keratin 19, amelogenin, p75 neurotrophin receptor

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LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
Alv. Epith.	Alveolar Epithelium
AMB	Ameloblasts
Bmp	Bone morphogenetic protein
CBP	Calcium binding protein
D	Diffuse
E	Day of embryonic development
F	Focal
Fgf	Fibroblast growth factor
Fig.	Figure
H&E	Heamatoxylin and eosin
HERS	Hertwig's epithelial root sheath
HIER	Heat induced epitope retrieval
IEE	Inner Enamel Epithelium
IF	Intermediary filament
K	Keratin
kD	Kilodalton
min	Minute
ml	Millilitre
NA	Not present in section evaluated
NGF	Nerve growth factor
nm	Nanometre
ODB	Odontoblasts
OEE	Outer Enamel Epithelium
p75NTR	p75 Neurotrophin receptor
PCR	Polymerase chain reaction
pI	Isoelectric point
S	Single
Shh	Sonic hedgehog
Stel. Ret.	Stellate Reticulum
Strat. Interm.	Stratum Intermedium
TNFR	Tumour necrosis factor receptor
Trk	Tyrosine kinase

CHAPTER 1

GENERAL INTRODUCTION

1.1   Odontogenesis

Developmental biology is a field of rapid progress and in recent years advanced gene technology have led to an explosion in the information and understanding of the molecular mechanisms regulating embryonic development [1, 2]. For the purpose of this study, dental development will be briefly reviewed.

During the embryonic period a group of cells separate from the forming neural tube, migrate and differentiate extensively and undergo epithelial-mesenchymal transformation. These cells, called the neural crest cells, form most of the connective tissue in the head and as it originates from neuroectoderm, the connective tissue in the head is known as ectomesenchyme [3-5]. The migration of the neural crest cells is critical for tooth formation as all the tissues of the tooth (except for the enamel and probably some cementum) are derived from these cells [3-5]. The maxillary teeth develop from the maxillary arches, which originate from the first branchial arch, as well as the frontonasal process. The mandibular teeth develop from the mandibular processes, also originating from the first branchial arch [5]. Odontogenesis initiates with the formation of a band of thickened oral epithelium in the future dental arches of the upper and lower jaws [5, 6]. This results from the epithelial cells dividing in a different plane, directing the cells to grow into the ectomesenchyme to form a series of dental and vestibular laminae [4]. The latter gives rise to the vestibule between the alveolar ridge and the cheek as the cells of the lamina enlarge and then degenerate to form a cleft [4, 5]. The dental laminae form the enamel organs of the developing teeth that differentiate through the bud, cap and bell stages [5, 6].

In the process of tooth initiation and development, over 300 genes and various transcription factors have been identified, resulting in a very complex field of study [2]. The expression of transcription factors are often regulated by signalling molecules, which in turn is again regulated by the same transcription factors and

therefore it should be kept in mind that various signalling cascades are still not completely understood. Fibroblast growth factor (Fgf), bone morphogenetic protein (Bmp), Sonic hedgehog (Shh) and wingless (Wnt) families are some of the most important and best studied signalling factors in odontogenesis [2, 7-12], but for the purpose of this dissertation only certain aspects of this intricate signalling system will be mentioned.

Most of the current knowledge regarding tooth development, has been gathered from laboratory mouse models, as it is not always possible to obtain human or dog embryos at specific known dates of gestation. It is important to remember that although mice have a genome that allows comparison with humans, significant differences do exist between the two dentitions as mice have a single dentition, lack premolars and canines, have a prominent diastema and the incisor teeth erupt continuously (elodont teeth) throughout life [5].

A series of sequential and reciprocal interactions between epithelial and ectomesenchymal cells are fundamental in tooth development [13]. The initiation of tooth development can be observed morphologically as an epithelial thickening around the tenth day of embryonic development (E10) in mice [11, 12] and at this stage Bmp-4 expression is evident in the thickened epithelial band [11, 13]. Bmp-4 has been proposed to spread to underlying ectomesenchyme with bud formation and then induce the expression of Msx-1 and Msx-2 genes in the dental ectomesenchyme [13, 14]. In mouse embryo sections, Bmp-4 expression is no longer detectable in the epithelium at E11.5 (bud and cap stages) [14], and it was therefore proposed that the transfer of inductive potential from the epithelium to the ectomesenchyme results from the shift of Bmp-4 expression and from the epithelium to the ectomesenchyme [15]. It has also been established that Msx-1 is required for Bmp-4 expression to be transferred from dental epithelium to ectomesenchyme and therefore it was suggested that Msx genes have a major function in development as it permits reciprocal inductive signalling to occur between tissue layers [15]. Bmp-4 and Msx-1 are co-expressed in the ectomesenchyme surrounding the invaginating tooth germs [14]. The expression pattern of the above mentioned signalling molecules is in agreement with the fact that the initiation of odontogenesis is at first determined by factors located in the first arch epithelium, influencing the ectomesenchyme,

and that this potential is then taken over by the ectomesenchyme [3, 4]. In murine teeth, for example, the ectomesenchyme has the full potential to elicit tooth formation after the twelfth day of gestation [4].

It was thought that Bmp-4 cannot substitute for all the inductive functions of the dental epithelium and therefore further studies revealed more factors in the initiation process [7]. Fgf-8 (and Fgf-9) is expressed in murine oral ectoderm as early as the tenth day of embryonic development (E10) [7, 10] and Fgf-8 is also able to induce Msx-1 expression in the dental ectomesenchyme [10]. Furthermore it was described that Fgf-8 can induce Fgf-3 expression in the dental ectomesenchyme in a manner that Msx-1 is required [7]. This induction of Fgf-3 expression by Fgf-8 occurs at the lamina-bud transition stage when odontogenic potential shifts from the epithelium to the ectomesenchyme and therefore Fgfs may be important factors in the epithelium-ectomesenchymal interactive signalling process [7]. The exact role of ectomesenchymal Fgfs like Fgf-3 has not been elucidated but what can be said is that the initiation step in odontogenesis can be regarded as at least two separate pathways that are Msx-1 dependant and can be induced by either Bmp-4 or Fgf-8 [7]. It has also been proposed that the Msx-1,Msx-2, Dlx1 and Dlx2 genes are subjected to differential induction by Bmp-4 and Fgf-8 [7].

The bud stage

Epithelial cells of the dental lamina proliferate into the neural crest-derived ectomesenchyme representing the bud stage (Fig. 1.1) [6]. Concurrently the ectomesenchymal cells adjacent to it do not produce extracellular substance and is therefore not separated from one another, leading to a condensation of ectomesenchyme [4]. At this stage of tooth development there is still a series of reciprocal epithelial-ectomesenchymal interaction taking place [16]. Sonic hedgehog (Shh) acts as a proliferative factor in the epithelium during invagination of the tooth bud [17] and its expression in the epithelium (together with that of Bmp-2) requires Bmp-4 expression in the ectomesenchyme [4, 13]. In the bud stage there is down regulation of Fgf-8 expression and from then on it is not detected in the developing tooth [10]. Shh also plays a role in maintaining survival of epithelial cells at the tip of the bud at the late-bud stage [17]. The

cells at the tip of the bud are potential precursors of enamel knot, and are therefore important in later morphogenesis of the tooth [17].
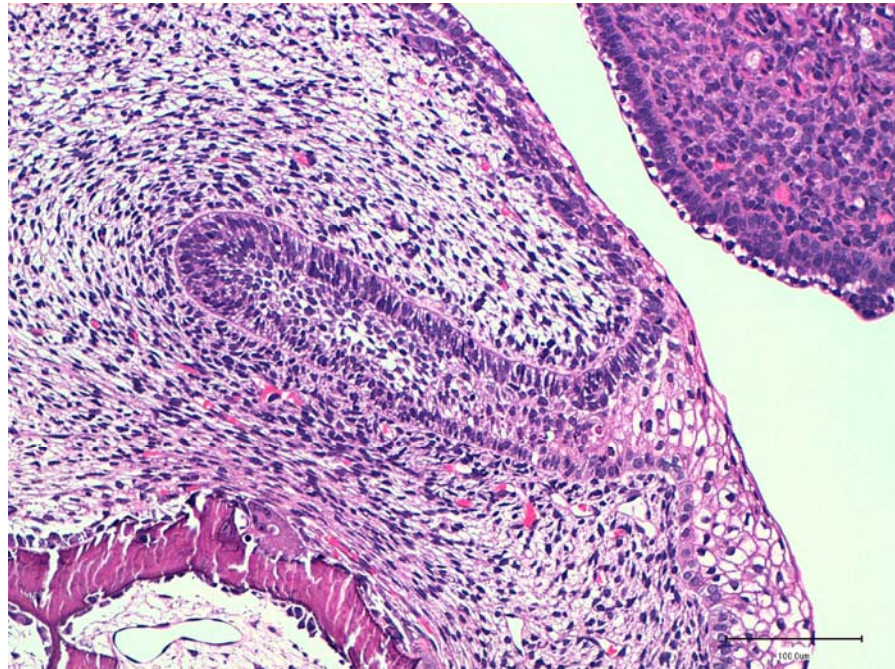
Fig. 1.1: *A tooth germ in the bud stage of development – epithelial cells of the dental lamina proliferate into the ectomesenchyme (scale bar 100µm, original magnification 200x).*

The cap stage

The cap stage resembles a "cap" of epithelial cells on a "ball" of condensed ectomesenchymal cells, representing the dental papilla (Fig. 1.2). The ectomesenchyme limiting the dental organ and papilla is called the dental follicle [4, 5]. Collectively all these developing dental tissues are known as the tooth germ [5, 6] or dental organ [4, 6]. During the cap stage, the epithelial cells in the centre of the enamel organ synthesize and secrete hydrophilic glycosaminoglycans, which pull water into the enamel organ. This leads to the epithelial cells being forced apart from one another, only retaining their desmosomal contacts. At this stage these cells resemble stars which led to the term stellate reticulum [4].
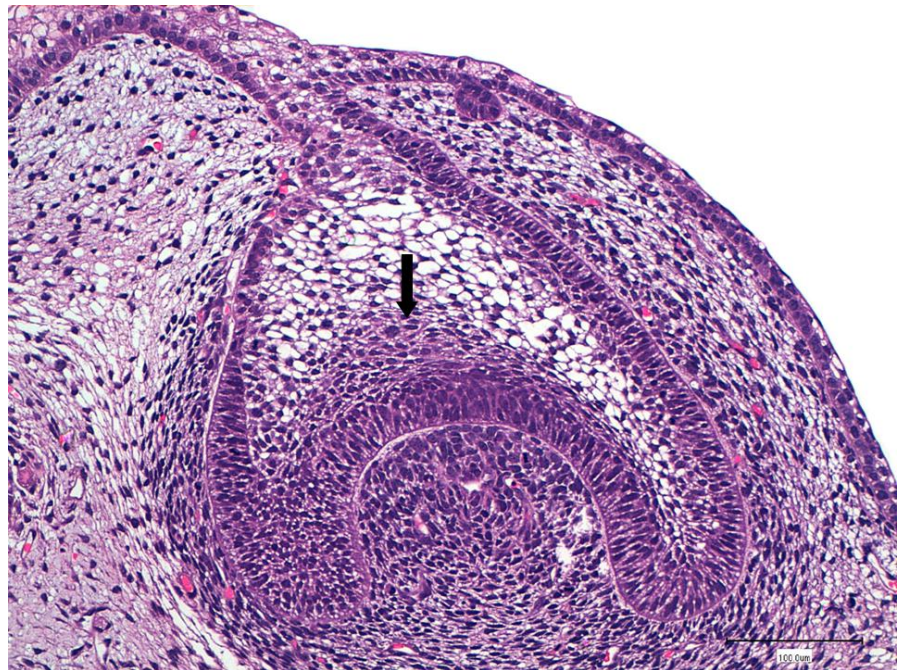
Fig. 1.2: *A tooth germ in the cap stage of development – the enamel organ (epithelial cells) represents a "cap" adjacent to condensed ectomesenchyme. The stellate reticulum is clearly visible in the centre of the enamel organ (asterisk) (scale bar 100μm, original magnification 200x).*

Enamel knots

Enamel knots are non-proliferating epithelial cells formed at the tip of the tooth bud, fully differentiated by the cap stage (Fig 1.3). Its formation is initiated by Bmp-4 expression in the ectomesenchyme and although these cells do not divide themselves, they stimulate the proliferation of nearby epithelial and ectomesenchymal cells [18-20]. Primary enamel knots are present in each tooth germ at the cap stage and as they disappear, secondary knots form at the future cusp tips in molars [4, 20]. It could also extend from the inner enamel epithelium to the outer enamel epithelium to form an enamel cord [4]. Transient expression of Fgf-4 in the enamel knots has been described because it disappears with the secondary enamel knots. Therefore Fgf-4 seems not to be associated with ameloblast cell differentiation as the expression thereof is absent in the other

areas of inner enamel epithelium [19]. It has been suggested that Fgf-4 diffuse from the enamel knot, stimulate ectomesenchymal cell division and also enhance proliferation of adjacent epithelial cells [19]. This poses a mechanism whereby the enamel knot controls the formation of the tooth cusps as it may stimulate cusp growth and guide folding of the inner enamel epithelial-mesenchymal interface as it does not proliferate itself [19]. Fgf-9 is also expressed in the enamel knots and it is has been proposed that Fgf-4 and/or Fgf-9 is involved in prevention of untimely apoptosis of enamel knot cells and may also regulate Msx-1 gene expression in the dental ectomesenchyme [10].

Fig. 1.3: *Enamel knot cells (arrow) visible in a developing tooth germ in the late cap stage (scale bar 100µm, original magnification 200x).*

The bell stage

As the cells of the tooth germ continue to proliferate, the enamel organ resembles a bell (Fig. 1.4). During the bell stage histodifferentiation and morphodifferentiation take place. At this stage the enamel organ (apart from other cells) consists of an outer layer of dental epithelium with a low cuboidal

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shape known as the outer enamel epithelium (OEE) and an inner layer of epithelial cells with high glycogen content, a short columnar shape and centrally placed nucleus known as the inner enamel epithelium (IEE) [4, 21]. The inner and outer dental epithelia are confluent at the cervical loop, where root formation will proceed after the crown attained its full size. These cells grow in an apical direction and form Hertwig's epithelial root sheath (HERS) that is responsible for root formation and induces the differentiation of odontoblasts in the roots [5, 6]. At this stage, epithelial cells between the IEE and stellate reticulum differentiate into a layer of cells with a high activity of the enzyme alkaline phosphatase, called the stratum intermedium [4].
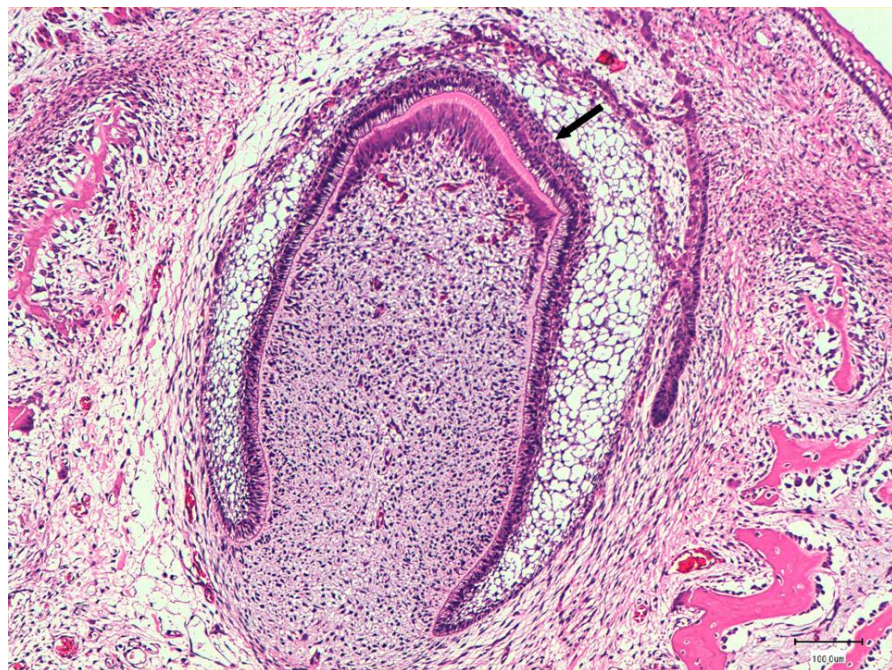
Fig. 1.4: *A tooth germ in the bell stage of development – the stratum intermedium (arrow) is visible between the stellate reticulum and differentiating ameloblasts (scale bar 100µm, original magnification 100x).*

The late bell stage

At the late bell stage, the future crown pattern is recognizable as the IEE completes its folding [4]. Terminal differentiation of the IEE will take place to form

enamel producing cells known as ameloblasts and the ectomesenchymal peripheral cell layer in the dental papilla will differentiate to form dentin-producing odontoblasts [12]. Differentiation starts at the tip of the developing cusp and proceeds apically. Signalling from the pre-ameloblasts to the pre-odontoblasts across the basement membrane initiate reciprocal signalling for final cytodifferentiation of the different cell layers [12]. Prior to ameloblast differentiation Bmp-4 gene expression shifts back from the ectomesenchyme to the epithelial cells transiently [11]. The transient expression of Bmp-2 and Bmp-4 in the ameloblasts and odontoblasts during terminal differentiation led to the suggestion that Bmp-2 and Bmp-4 gene expression is regulated by epithelial-mesenchymal interactions [11]. The first layer of predentin acts as a signal for the overlying IEE cells to differentiate into ameloblasts [5, 6, 12]. The short columnar cells of the IEE gradually elongate, the cell nucleus moves from the centre of the cell to a basal position near the stratum intermedium, and the cells also develop a prominent endoplasmic reticulum and Golgi apparatus. This process is also referred to as reversed polarity [12, 21]. When the first calcified matrix appears at the cuspal tip of the bell stage, the dental papilla is referred to as the dental pulp. During the bell stage the developing tooth is separated from the oral epithelium as the dental lamina breaks up into discrete islands of epithelial cells called Serres rests [4].

During the late bell stage, the processes of dentinogenesis and amelogenesis take place for dentin and enamel formation respectively. Ameloblasts undergo several differentiation processes and therefore three morphologically different stages can be detected namely the presecretory (consisting of ameloblast cytodifferentiation), secretory (the bulk of the enamel matrix formation and secretion), and maturation stages (associated with increased matrix mineralization) [22-24]. During the secretory process the ameloblast develops a distal extension called Tomes' process and the enamel matrix is secreted from the distal aspect of the cell [4]. As the maturation stage is reached water and organic material are removed from the enamel and additional inorganic material is incorporated into the maturing enamel [4].

1.2   Aim of study

Dogs frequently present with odontogenic neoplasms [25-27] and numerous cases of epulides have been diagnosed in the Department of Oral Pathology and Oral Biology. Various types of epulides have been described in dogs which includes the acanthomatous epulis [28]. It has been proposed that many of the epulides in dogs originate from the periodontal ligament as it resemble the stromal features thereof [28], but the stroma of an acanthomatous epulis does not necessarily resemble the periodontal ligament and it has been proposed to arise from the gingival epithelium or have an intraosseous odontogenic origin [29]. The acanthomatous epulis usually infiltrates bone extensively [28], and similarities between the acanthomatous epulis and ameloblastomas resulted in debate regarding the nature and origin of the acanthomatous epulis found in dogs [30]. Therefore it has been proposed that alternative nomenclature be used for acanthomatous epulis like peripheral ameloblastoma [31], or canine acanthomatous epulis [29].

In an attempt to elucidate the origin and character of the acanthomatous epulides, the aim of this study was to find suitable cell markers to identify odontogenic epithelium versus oral epithelium in developing dog teeth in order to use in future research on the pathogenesis and pathology of odontogenic neoplasms in dogs. As specific markers for odontogenic epithelium has not been described in dog tissue, proposed markers of odontogenic epithelium of human and rat tissues were tested on dog odontogenic tissue. Keratin 14, keratin 19, amelogenin, p75 neurotrophin receptor and calretinin have been proposed as markers for IEE and/or ameloblasts in human and rat tissue [32-36] and was therefore included in our study. Only one study on amelogenin expression in dog (canine) oral tissues and lesions have been published with the polymerase chain reaction (PCR) technique [30]. To the best of the author's knowledge, none of the other markers mentioned above has been tested in dogs.

CHAPTER 2

CALRETININ

2.1   Introduction

In the 1950s it became clear that calcium had a huge role to play in the regulation of cellular activities. Calcium has the ability to bind tightly with proteins, making it a highly suitable intracellular messenger [37]. In the search for intracellular calcium "sensors", intracellular calcium mediator proteins were identified and characterized during the 1970s and 80s [37]. The terms "Calcium mediator protein" and "Calcium-binding protein" are used interchangeably in the literature but it will be referred to as Calcium-binding proteins (CBPs) in this study. Two families of the CBPs were described, being the EF-hand family and the annexin family [37].

The EF-hand family is characterized by a protein structure known as the "EF-hand motif" or "calmodulin fold" [37]. Calmodulin has been described as the prototype of the CBPs [38], possibly leading to its name being used to describe such characteristic features of the typical protein. Kretsinger described the characteristic conformation of the CBPs as an α-helix, calcium-binding loop and a second α-helix, being referred to as the "EF hand" [39]. The structure of the helices E and F resembles a right hand with the forefinger symbolizing helix E and the thumb corresponding to helix F [40].

The EF-hand family consists of more than 160 different CBPs [37]. Some examples of this family of CBPs are: calmodulin, troponin C, myosin light chains and parvalbumin. Another important member of the EF-hand family of CBPs is the 28-kilodalton (kD) vitamin D-dependent intestinal CBP, also known as cholecalcin 28k, spot 35, or visinin (47a) and later renamed as calbindin. In 1987 another member of this family was isolated from chicken retinas with a molecular weight of 29 kD and named calretinin [38].

There is a great similarity between the amino acid sequence of calretinin and calbindin (on average 58%) as stretches of 14-15 amino acids have been identified that are virtually identical between the two proteins, and therefore cross

reactivity of antisera has been described between calbindin and calretinin [38, 41]. As the existence of calretinin was previously unknown, it is possible that some cell populations previously described as containing calbindin, in fact contained calretinin. It was hypothesized that calretinin and calbindin have other functions in addition to their calcium-binding properties as it has a highly conserved structure and exhibits a low evolutionary rate similar to essential proteins. Proposed additional functions of calretinin could involve a role in intracellular regulatory pathways as in the case of troponin C and calmodulin [41].

Calretinin expression in normal tissue

The expression of calretinin has been described in central and peripheral neural tissues [38, 42, 43], interstitial cells of the ovary [44], interstitial Leydig cells of the testes [45], medullary epithelial cells of avian thymus [46], pineal glial cells and a subpopulation of pinealocytes [47], pancreatic islet cells [48], periodontal Ruffini endings [49] and in various epithelial cells of developing odontogenic tissues of rats [50].

Calretinin expression in odontogenic tissue

A study on calretinin expression in the rat dental organ revealed focal expression of calretinin in the dental lamina, outer enamel epithelium, stellate reticulum and stratum intermedium. Diffuse and intense expression was however encountered in the IEE and presecretory ameloblasts with less intense staining observed in the cytoplasm of secretory ameloblasts [50]. The authors even suggested calretinin to play some role in enamel formation as calretinin expression remained negative in rat molar teeth over the cusp tips, where enamel is never formed. At the late cap and bell stages staining in the papilla was interpreted as representing neural elements with no staining of odontoblasts and other ectomesenchymal cells [50]. In accordance with a previous postulation regarding calbindin, the authors postulated that calretinin may act as a "calcium ferry" as its presence was only detected in cells that reside directly in the path of calcium transition towards the enamel matrix, resulting in the dynamic temporal and spatial distribution thereof [50, 51].

Calretinin expression in odontogenic tumours

Concurrent with the above mentioned study, calretinin expression was revealed in the epithelium of unicystic, solid and multicystic ameloblastomas [52, 53]. Positive staining for calretinin was detected in cells with ameloblastic features as well as in nondescript epithelial linings of unicystic ameloblastomas [52]. In contrast to the above mentioned finding calretinin stained mainly in the stellate reticulum-like cells in the ameloblastomas and not in the ameloblast-like cells [53]. Although the expression of calretinin in ameloblastomas was diffuse and intense, there were also areas completely devoid of staining and in unicystic ameloblastomas the cells with typical ameloblastic features often showed no or little positivity for calretinin while cells not resembling ameloblasts stained positive [53]. As a result it was stated that no obvious correlation between the staining pattern of calretinin in the normal odontogenic tissues and their related neoplasms could be observed [50].

In some cases loss of calretinin expression seemed to be associated with an inflammatory infiltrate in the ameloblastic epithelium [53]. Mast cells in odontogenic tumours and cysts has also been shown to stain positive for calretinin [53].

To the best of the author's knowledge, the study done by Mistry *et al*, in 2001 is the only one describing calretinin expression in odontogenic tissue. As diffuse and intense expression of calretinin was observed in the IEE and presecretory ameloblasts of rat tissue [50], it was decided to include calretinin in our study to evaluate its expression in developing dog odontogenic tissue.

2.2   Materials and Methods

Foetuses of large breed dogs were obtained, under ethical clearance of the Animal Use and Care Committee of the Faculty of Veterinary Sciences, University of Pretoria, South Africa, from female dogs scheduled for elective termination of pregnancy. Mixed breed dog foetuses were used in the study (*Canis familiaris*). Twenty four dog foetuses were fixed in 10% buffered formalin and then the heads were carefully cut into coronal sections (rostral to caudal), dehydrated and embedded in paraffin wax blocks. Those tissue samples that

contained calcified bone or dental hard tissues were decalcified in routine decalcifying solution consisting of 880ml distilled water, 70 ml nitric acid (65%) and 50 ml hydrochloric acid (36%) for 60 minutes and rinsed in running tap water for 60 minutes in order to make sectioning possible without tearing and disruption of normal anatomy. The tissue blocks were then processed overnight.

Tissue specimens were sectioned at 3µm, stained with haematoxylin and eosin (H&E) and microscopically examined by two individuals in order to select slides with well-formed cap and/or bell stage enamel organs in which the respective odontogenic epithelial cells were morphologically clearly identifiable. Sections were de-paraffinized in two changes of fresh xylene for 10 minutes each, re-hydrated with 2 changes of absolute ethanol, followed by 90, 70 and 50% graded alcohol solutions respectively. The sections were then washed in 6 changes of fresh distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 6 min at 37ºC where after the sections were washed in 5 changes of fresh distilled water. Heat induced epitope retrieval in EDTA (Ethylene diamine tetra-acetic acid disodium salt) buffer, pH 8.00, was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 min. The sections were rinsed in 6 changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS buffer, Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for 10 min. The sections were incubated in Calretinin (1:100) anti serum (Novocastra NCL-Calretinin, Novocastra Laboratories Ltd., Balliol Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW United Kingdom) for 60 minutes at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The sections were then incubated in Dako Envision+R System Labelled Polymer, anti mouse, HRP (Dako K4001, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 30 min at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 4 min at 37ºC, washed in

distilled water and counterstained in Haematoxylin for 1 minute at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA).

According to Novocastra the Clone 5A5 detects the calretinin protein predominantly in the cytoplasm, but it may also be seen in the nuclei of cells. Reddish-brown granular cytoplasmic and nuclear staining were interpreted as positive for the presence of calretinin. The peripheral nerve bundles were used as positive internal controls in each specimen (Fig. 2.1).
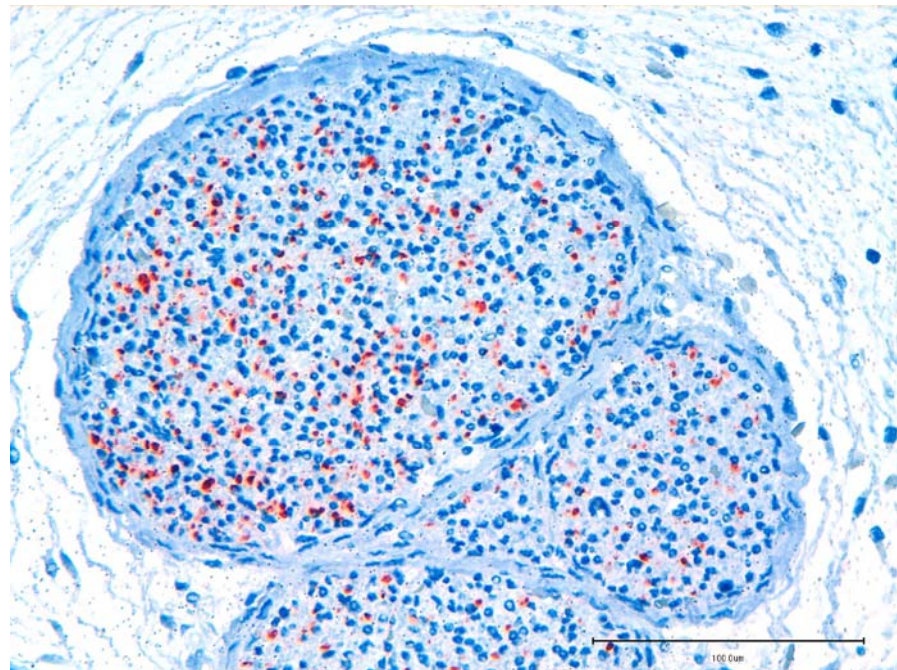

Fig. 2.1: *Peripheral nerve bundle staining positive for calretinin (scale bar 100µm, original magnification 400x).*

Staining in each case was noted as follows:

- S – Single
    - One or two cells in a specific field of cells stained positive
- F – Focal
    - Groups of cells that revealed positive staining, being interrupted by cells that did not stain
- D – Diffuse
    - General staining of the cells involved (90% or more of the cell type stained)
- + Weak staining
- ++ Intense staining

Because of the *in situ* nature of the study, different cell types of odontogenesis were distinguished from one another on the basis of their microscopic morphology and location in the developing tooth germ and adjacent structures. For the purpose of this study ameloblasts were defined as tall columnar cells, with prominent reversed polarity (nuclei aligned adjacent to the stratum intermedium) and located adjacent to formed dentin (Fig. 2.2). The inner enamel epithelial cells (IEE) were defined as short columnar cells on a basal membrane with centrally placed nuclei, opposite undifferentiated ectomesenchymal cells of the dental papilla without any signs of odontoblast differentiation or hard tissue formation (Fig. 2.2). The cells of the outer enamel epithelium were defined as low cuboidal cells with little cytoplasm at the periphery of the enamel organ. The oral epithelium on the alveolar ridge area from where the tooth germ originated, was referred to as the overlying alveolar epithelium. The term dental lamina was used when continuity of the lamina could be observed between the overlying alveolar epithelium and the developing tooth germ. When only discrete islands of epithelial cells were evident, it was termed Serres rests.
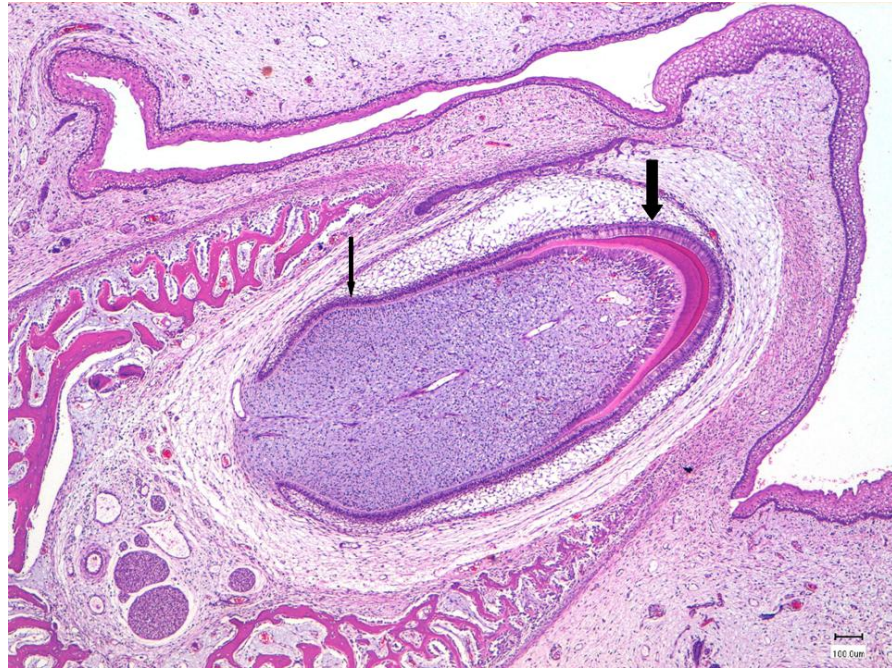
Fig. 2.2: *An example of an H&E stained section that was selected containing clearly identifiable IEE (thin arrow) and ameloblasts (thick arrow) (scale bar 100µm, original magnification 40x).*

2.3   Results

As the exact time of gestation of the developing dog foetuses were unknown, the foetuses were classified into 2 groups based on the odontogenic development stage. Foetuses 1-8, designated as group 1 only had tooth germs in the bud and cap stages of development with no ameloblast differentiation or dental hard tissue formation. The tooth germs of foetuses 9-24, designated as group 2, were already in the bell stage of development with visible cell differentiation and dental hard tissue formation.   Many presented with more than one section that conformed to the requirements of the inclusion criteria and in 15 cases a rostral (anterior) and a caudal (posterior) section of the same foetus were used for comparison.   In the remaining 9 cases, only one section was included in the study. A total of 39 sections were therefore harvested from the 24 foetuses for immunohistochemical investigation of calretinin expression. The number of tooth

germs on a single section varied from 1 to 4 and as the whole section was stained with the antibody there were more than one tooth germ that could be used in the evaluation process of some sections. Only one tooth germ, defined as the best representation of the cap and/of bell stage in the given section was chosen for analysis of the immunohistochemical stain. No discrepancies were however found between calretinin stains of the tooth germs on the same section.

Table 2.1 lists the results found for calretinin staining in dog odontogenic tissue. The group 1 foetuses showed calretinin staining of the OEE specifically on the mesial aspects of the tooth germ (Fig. 2.3), but no staining of OEE was detected in the group 2 foetuses.

No calretinin staining was detected in the ameloblasts, odontoblasts or epithelial cells of the stellate reticulum and stratum intermedium present at any stage of development (Fig. 2.4 and 2.5). The IEE showed no staining except in one section where weak, focal positivity was observed in enamel knot region (Fig. 2.6 and 2.7). The calretinin stain was repeated, but the result was the same.

Thirty of the 35 dental laminae evaluated, stained positive for calretinin (85,7%) (Fig. 2.8). Twenty four of the 28 cases where Serres rests were present, showed positive staining in the epithelial rests (85,7%) (Fig. 2.9). The overlying alveolar epithelium, in close approximation to the lamina and Serres rests, stained positive in 28 of the 39 cases evaluated (71,8%) (Fig. 2.3 and 2.8).

***Table 2.1*** *– Calretinin expression in dog odontogenic tissue.*

Foetus Nr.	AMB	IEE	OEE	ODB	Stel. Ret.	Strat. Interm.	Dental Lamina	Serres Rests	Alv. Epith.
					Calretinin				
1	NA	-	F++	-	-	-	F++	NA	D++
2	NA	-	F++	-	-	-	F++	NA	D++
3	NA	-	F++	-	-	-	F++	NA	D++
4	NA	-	F+	-	-	-	F++	NA	D++
5	NA	NA	NA	-	-	-	F++	NA	D++
6	NA	NA	NA	-	-	-	F++	NA	D++
7	NA	-	F++	-	-	-	F++	NA	D++
8	NA	-	F+	-	-	-	F++	NA	D++
9a	NA	-	-	-	-	-	S+	-	-
9b	-	-	-	-	-	-	S+	-	-
10a	-	-	-	-	-	-	S+	S++	S++
10b	-	F+	-	-	-	-	D++	D++	F++
11a	-	-	-	-	-	-	S+	F++	F++
11b	-	-	-	-	-	-	S+	S+	S+
12	-	-	-	-	-	-	D++	F++	F++
13a	-	-	-	-	-	-	-	-	-
13b	-	-	-	-	-	-	D++	D++	-
14a	-	-	-	-	-	-	-	NA	-
14b	-	-	-	-	-	-	D+	D++	S+
15a	-	-	-	-	-	-	-	-	-
15b	-	-	-	-	-	-	F+	F+	-
16a	-	-	-	-	-	-	-	S+	-
16b	NA	-	-	-	-	-	D++	D++	S++
17a	-	-	-	-	-	-	F+	S+	-
17b	-	-	-	-	-	-	D+	F++	F++
18a	-	-	-	-	-	-	-	F++	S+
18b	-	-	-	-	-	-	D+	D+	F++
19a	-	NA	-	-	-	-	NA	D+	S+
19b	-	-	-	-	-	-	D+	F+	F++
20a	-	-	-	-	-	-	NA	D+	S+
20b	NA	-	-	-	-	-	D+	D++	S+
21a	-	-	-	-	-	-	F+	D+	S+
21b	-	-	-	-	-	-	D++	D++	F++
22a	-	-	-	-	-	-	D+	F+	-
22b	NA	-	-	-	-	-	D++	D++	F++
23a	-	-	-	-	-	-	NA	D+	S+
23b	-	-	-	-	-	-	D++	NA	F++
24a	-	-	-	-	-	-	NA	NA	-
24b	-	-	-	-	-	-	D++	D++	F++

AMB – Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section.

Some interesting findings were positive calretinin staining seen in the epithelial cell rests in the ectomesenchyme where palatal fusion took place (referred to as "fusion epithelium") (Fig. 2.10). This was only noted in group 1 foetuses at a very early stage of development where fusion epithelium was still present and visible. Another interesting observation in this group was the strong positive calretinin staining in cells of unknown phenotype in the subepithelial mesenchyme of the dorsal tongue mucosa (Fig. 2.10). This disappeared in all cases where dental development had progressed to the late bell stage. Focal and sometimes diffuse staining of the respiratory mucosa was also observed in 28 of 30 sections that contained respiratory epithelium for evaluation (Fig. 2.11).
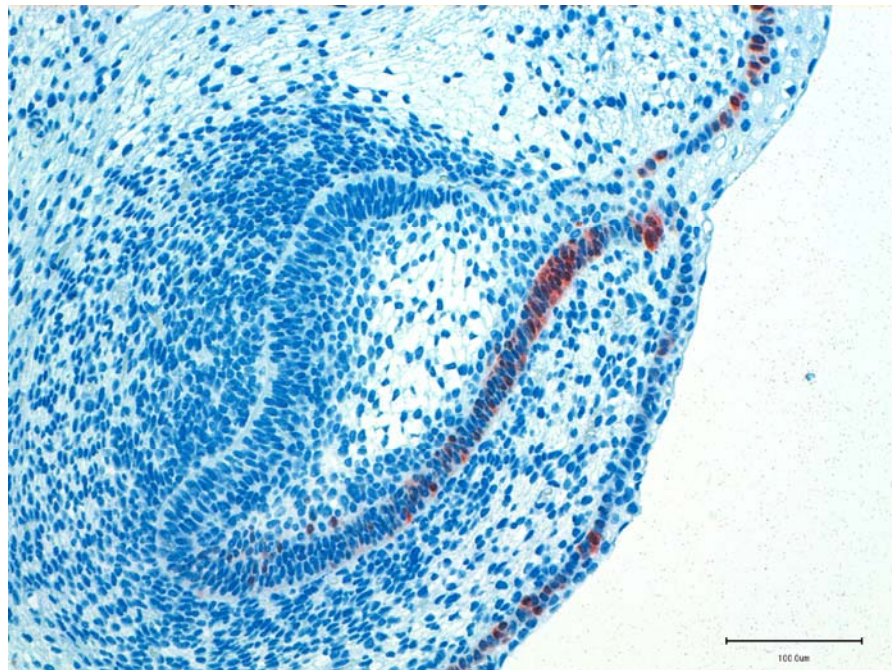
Fig. 2.3: *Positive calretinin staining of the OEE on the mesial aspect of the enamel organ as well as basal layer of overlying alveolar epithelium (scale bar 100µm, original magnification 200x).*
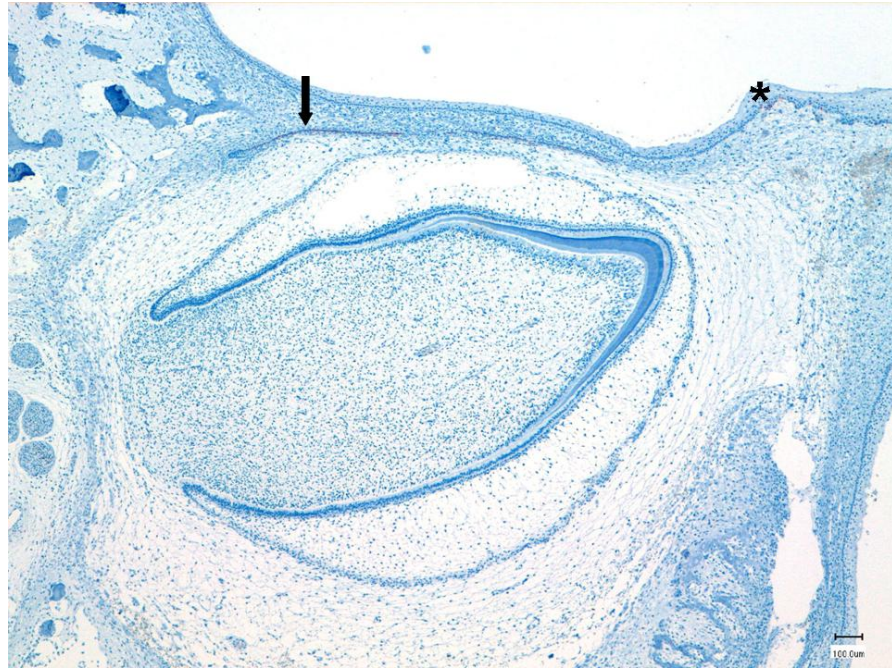
Fig. 2.4: *Calretinin staining restricted to the overlying alveolar epithelium (asterisk) and dental lamina (arrow) of a late bell stage tooth germ (scale bar 100µm, original magnification 40x).*
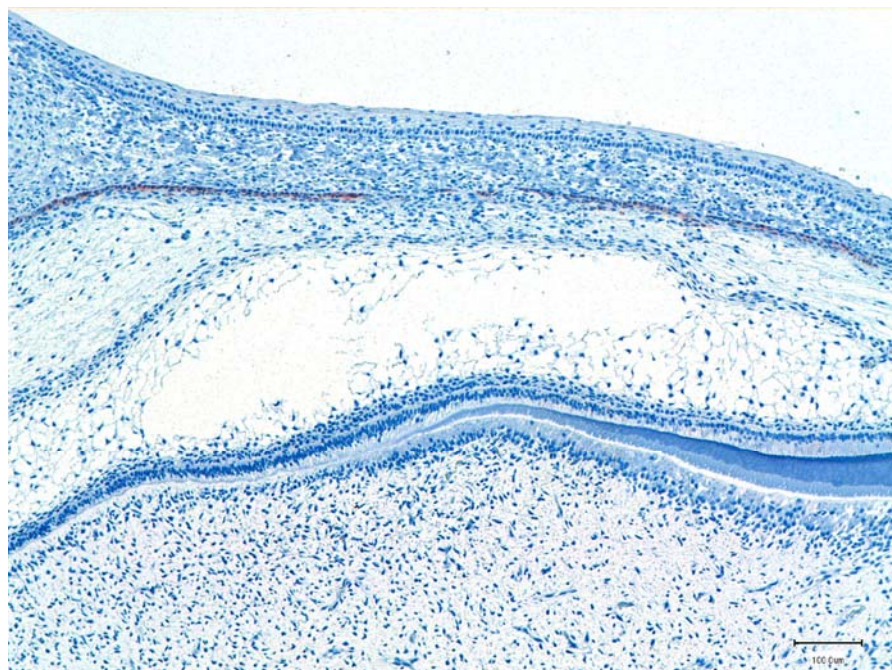
Fig. 2.5: *Higher magnification of calretinin staining in dental lamina as apposed to the other odontogenic tissues not staining (scale bar 100µm, original magnification 100x).*
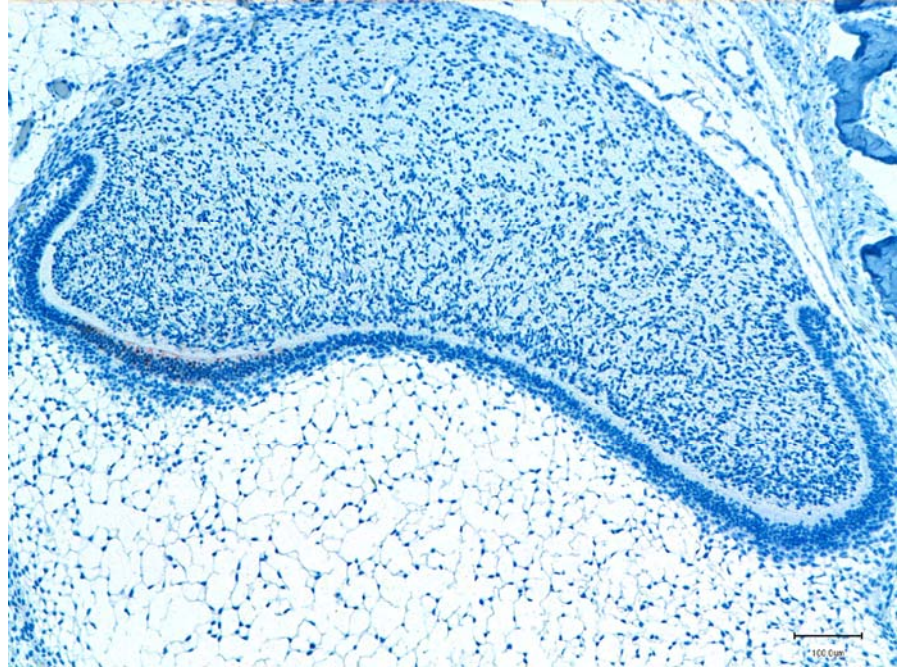
Fig. 2.6: *Focal calretinin expression in the IEE associated with the secondary enamel knot (scale bar 100µm, original magnification 100x).*
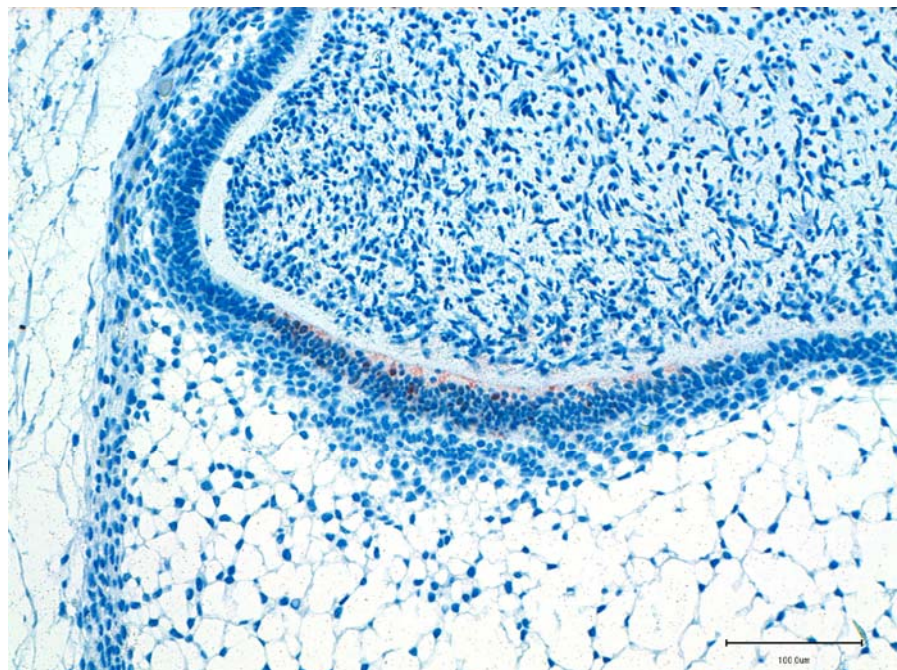
Fig. 2.7: *Higher magnification of focal calretinin expression in the IEE associated with the secondary enamel knot (scale bar 100µm, original magnification 200x).*
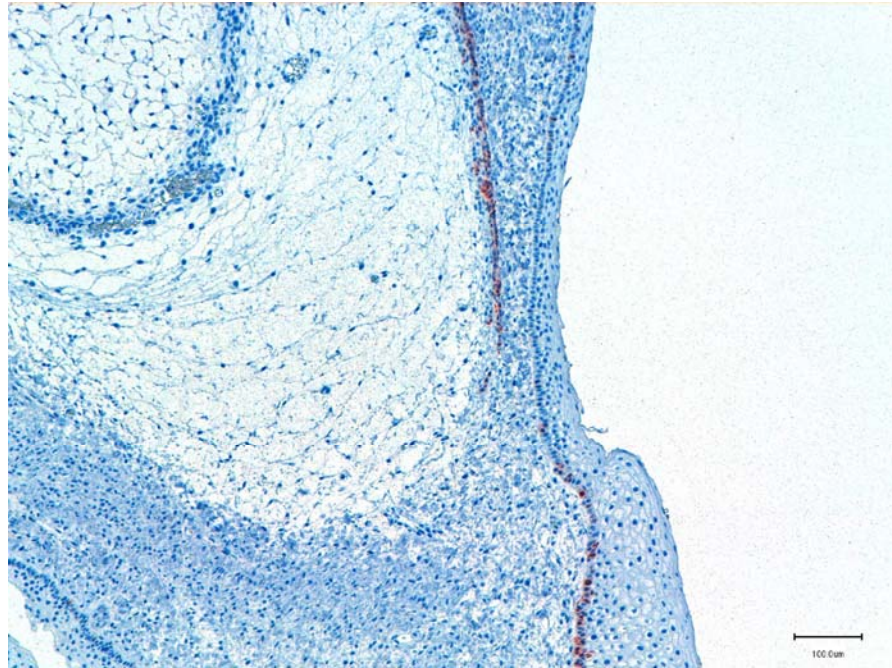
Fig. 2.8: *The dental lamina and basal layer of overlying alveolar epithelium revealing intense calretinin positivity (scale bar 100µm, original magnification 100x).*
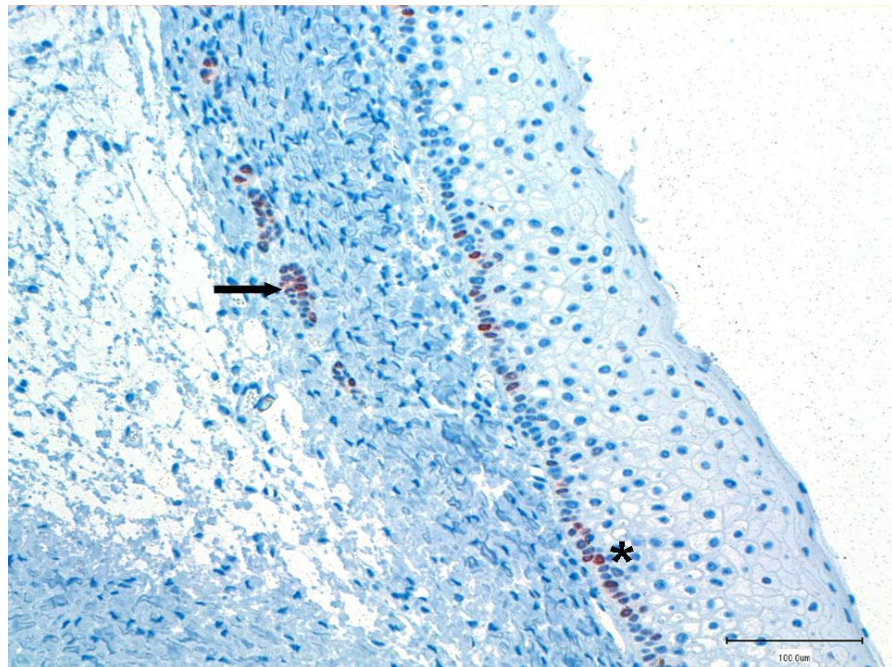
Fig. 2.9: *Calretinin expression in Serres rests (arrow) and basal layer of associated alveolar epithelium (asterisk) (scale bar 100µm, original magnification 200x).*

Fig. 2.10: *Calretinin expression in the palatal fusion epithelium, tongue mesenchyme and mesial aspect of the OEE in a group 1 foetus (original magnification 40x).*
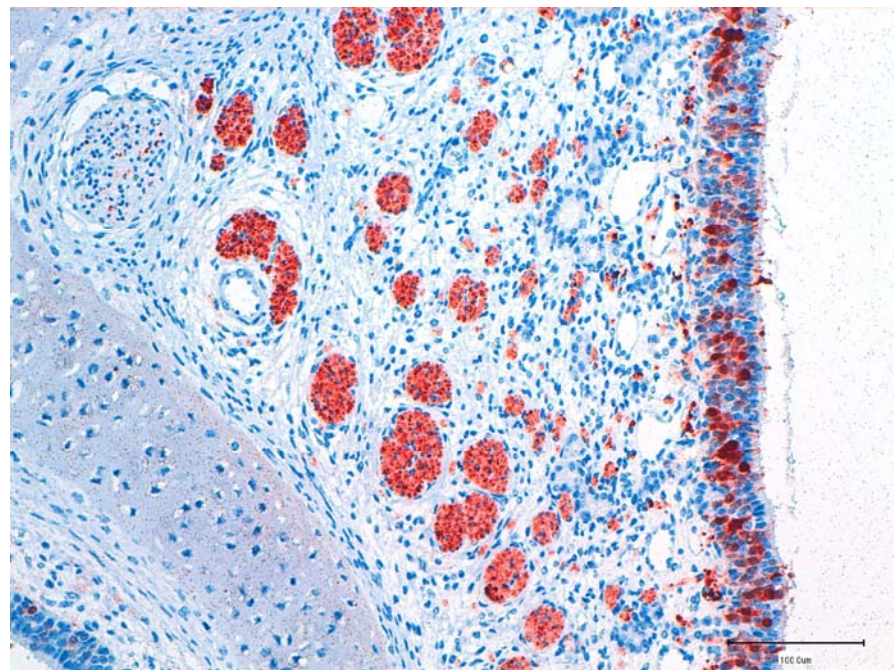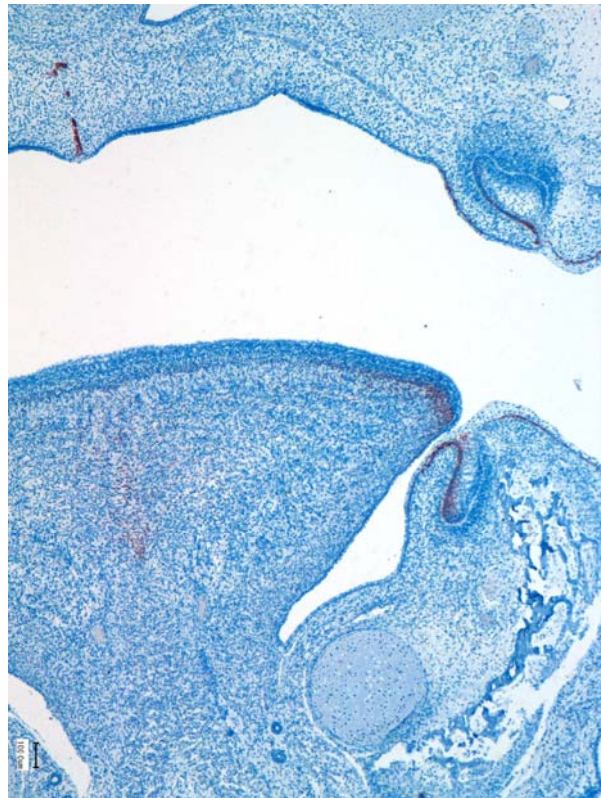
Fig. 2.11: *Intense expression of calretinin in the epithelium of the respiratory mucosa and associated developing salivary glands (scale bar 100μm, original magnification 200x).*

2.4   Discussion

In this study no calretinin expression was seen in ameloblasts, odontoblasts, stellate reticulum or stratum intermedium in any of the cases (Table 2.1). In contrast there was a definite staining pattern in the epithelium of the dental laminae, Serres rests and immediate overlying alveolar epithelium in 85,7%, 85,7% and 71,8% of the cases that contained these elements for evaluation, respectively. There were however discrepancies in the intensity of staining in these cells, as in some cases calretinin expression was diffuse and intense and in others weak and focal or expression was only seen in single cells.

One section revealed calretinin expression in the IEE of a tooth germ in the late cap stage. This was an interesting observation as it was the only positive result obtained in the IEE through 36 sections evaluated. A reason for this single detection of calretinin expression could be that sections presenting bell stage enamel organs were preferred over cap stage enamel organs when selected, as the former permitted evaluation of both IEE and ameloblasts. On this specific section another tooth germ was in fact chosen for evaluation as it complied better with the selection criteria but is was decided that these results should be included for the sake of completeness. In this specific section the evaluated dental organ was in the cap stage and it was the only tooth germ in the selected sections that was regarded as a molar tooth germ with secondary enamel knot formation. Positive staining of the cells of the IEE associated with the secondary enamel knot of the future buccal cusp was found, but the IEE of the future lingual cusp showed no staining. A possible explanation for this finding could be the transient nature of the enamel knot. The enamel knot on the buccal aspect of the tooth was clearly detectable, but the one on the lingual aspect was not so well defined. It is possible that the two areas were at different stages of development at the time and that the lingual enamel knot was already in the process of apoptosis but the inability to visualize a proper lingual enamel knot could also have been due to the plane of section. It is suggested that calretinin could be present in the IEE for a very rapid and transient period during secondary enamel knot formation, but further studies are needed to confirm this postulate.

It is clear that the results from this study differ drastically from those found in the study done in 2001 where intense expression of calretinin was found in the IEE and presecretory ameloblasts of rat molars [50]. Possible explanations are that different animal species and other antibodies were used, but still one would expect to see more similar expression patterns. In the study of 2001 polyclonal rabbit anticalretinin was used against rat tissue and in the current study lyophilized mouse monoclonal antibody directed against human calretinin was used on dog tissues. In the 2001 study, specimens were not demineralised as they described increased background and decreased staining intensity during a pilot study. In the current study 12 sections were not demineralised and 27 demineralised for 1 hour. All the cases evaluated in the current study had positive internal controls and no increased background staining was observed in the demineralised sections, most probably as a result of improved techniques used. Cross reactivity of the antiserum directed against calretinin with calbindin could also result in false positive results and such a possibility was therefore discussed with representatives of Novocastra and they gave the assurance that the antibodies are specific for calretinin binding.

The variation of calretinin expression could be due to species differences. In 1993 it was noted that in humans and monkeys the number of calretinin immunoreactive neurons in the cerebral cortex were larger than in rats [54]. Another example is that calretinin expression was found in a subpopulation of pinealocytes of hamsters but not in gerbil and guinea-pig pinealocytes and therefore interspecies variation could account for the different expression patterns observed [47].

It is also possible that the expression of calretinin may be age- or stage of development-related as it was already mentioned in 1995 that calretinin expression was mainly restricted to nerve cells in adult animals, but that during the developmental stages expression was more widespread [55]. The current study supports the above mentioned finding as the OEE revealed intense unilateral calretinin expression only in embryos that were in a very early stage of development (group 1) with no expression in the older embryos (group 2).

It has been suggested that calretinin expression may be linked with the metabolic activity of a cell as metabolic changes of the cell coincide with changes of calretinin expression [53]. Results from the current study support this finding as calretinin expression appeared intense and diffuse in certain areas of the respiratory mucosa whilst other areas on the same section showed no staining. The discrepancy could be due to the cells enrolling different metabolic activities at the given time.

The exact date of gestation of the dog embryos that were used in this study could not be determined, as the embryos were obtained from routine terminations of pregnancies. This is a shortcoming in the current study as the expression of calretinin seems to be related to certain developmental stages as mentioned above. Still it could be observed that some of the embryos were very early in the stages of development as they did not have nails, hair or hard tissue formation in the developing tooth germ (group 1), while others were further developed (group 2) and the differences were annotated. For this reason we could only describe different patterns of calretinin expression according to the stages of tooth development.

An interesting observation was that in four of the cases the anterior section revealed little or no positivity while the posterior sections expressed diffuse positive staining patterns. This phenomenon is not easily explained and it is speculated that the variable factor could be one or a combination of factors. Fixation could have played a role as it is not known exactly how soon the specimens were put into neutral buffered formalin after the foetuses were removed from the uterus as well as the exact duration of the fixation process. The size of the foetus is a variable factor as the formalin would penetrate more readily to the central aspects of a smaller foetus as opposed to larger foetuses. Tissue blocks taken from the periphery of the specimen would also have been exposed to formalin for a longer period of time than the blocks selected from a deeper level. Sections taken from the posterior aspect of the jaws were thicker and the dental organs covered with more bone and muscle compared to those located in the anterior aspects of the jaws. This would potentially influence the fixation, depending on the time the foetuses were in the formalin until cut. One could therefore anticipate that the fixation of the posterior aspects of the

specimen would be suboptimal, but the results of the posterior sections correlated with the rest of the studied specimens and therefore this proposal can not be validated. The effect of decalcification could also be influenced by the degree of fixation and if suboptimal, false negative or increased background staining could result. This would also have resulted in the inverse of what was detected as suboptimal fixation would be expected in the posterior sections and not in the anterior sections. The anterior sections that revealed no calretinin staining were repeated and the results were the same. Therefore the exact reason for the differing staining patterns can not be explained and remains elusive.

The recommended antibody dilution, incubation time as well as the incubation time of the labelled polymer of the manufacturer was used. These dilutions and times are provided as a guide and it is possible for reasons mentioned above that the dilution and incubation times of the negative sections could be adjusted.

Heat induced epitope retrieval (HIER) was performed for 16 seconds at 120ºC using the Pascal pressurized heating chamber. This setting is also used for our routine histopathology work and was standard throughout the project. The manufacturer has a recommended range for doing HIER according to the application. Dako recommends the setting of 30 seconds at 125ºC as optimal, but tissue damage is of such an extent at this setting that interpretation of the results is impeded. Irrespective of the complications of the technique, 12 sections were repeated with an altered HIER technique of 125ºC for 30 seconds. Comparative results are given in table 2.2, listing the results of the altered technique below that of the original technique applied. The sections that showed diffuse and intense staining with the original method did so again with the alternative method. An important finding was that the same cells that originally showed positive staining did so again and that no new cell types revealed positive calretinin staining. However the sections that revealed such poor positivity with the original method that it was annotated as "no positive staining" revealed improved staining intensity with the alternative method. As a result observed staining increased from 85,7%, 85,7% and 71,8% using the original technique to 96.9%, 93.1% and 79.5% respectively for the dental lamina, Serres rests and associated alveolar epithelium evaluated, using the altered technique.

The alternative method was not used initially as it results in clearly detectable tissue damage. In the current study we aspired towards obtaining a method with the most accurate results without tissue damage in order to retain the original microscopical anatomy of all the cells. Calretinin appears to be technique sensitive and we suggest that both techniques be carried out to eliminate false negative results.

Table 2.2 *– Results from the original and altered (in bold) techniques used for calretinin expression in dog odontogenic tissue.*

Calretinin									
Foetus nr	AMB	IEE	OEE	ODB	Stel. Ret.	Strat. Interm.	Dental lamina	Serres Rests	Alv. Epith.
1	NA	-	F++	-	-	-	F++	NA	D++
1	**NA**	**-**	**F++**	**-**	**-**	**-**	**F++**	**NA**	**D++**
9a	NA	-	-	-	-	-	S+	-	-
9a	**NA**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**S+**
9b	-	-	-	-	-	-	S+	-	-
9b	**-**	**-**	**-**	**-**	**-**	**-**	**S+**	**-**	**S+**
10b	-	F+	-	-	-	-	D++	D++	F++
10b	**-**	**F+**	**-**	**-**	**-**	**-**	**D++**	**D++**	**D++**
11b	-	-	-	-	-	-	S+	S+	S+
11b	**-**	**-**	**-**	**-**	**-**	**-**	**F++**	**F++**	**D++**
13a	-	-	-	-	-	-	NA	-	-
13a	**-**	**-**	**-**	**-**	**-**	**-**	**NA**	**S+**	**S+**
14a	-	-	-	-	-	-	-	NA	-
14a	**-**	**-**	**-**	**-**	**-**	**-**	**NA**	**S+**	**S+**
15a	-	-	-	-	-	-	-	-	-
15a	**-**	**-**	**-**	**-**	**-**	**-**	**NA**	**S+**	**-**
16a	-	-	-	-	-	-	-	S+	-
16a	**-**	**-**	**-**	**-**	**-**	**-**	**S+**	**S+**	**S+**
17b	-	-	-	-	-	-	D++	F++	F++
17b	**-**	**-**	**-**	**-**	**-**	**-**	**D++**	**NA**	**F++**
20b	NA	-	-	-	-	-	D+	D++	S+
20b	**-**	**-**	**-**	**-**	**-**	**-**	**NA**	**D++**	**S+**
24a	-	-	-	-	-	-	NA	NA	-
24a	**-**	**-**	**-**	**-**	**-**	**-**	**NA**	**NA**	**-**

AMB – Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section.

2.5 Conclusion

From the results of this study, it can be concluded that although calretinin is not a marker for ameloblasts and inner enamel epithelium in dog teeth, it can be regarded as a marker for odontogenic epithelium as the overlying alveolar epithelium, dental laminae and Serres rests revealed positive staining in the majority of cases. It should however be kept in mind that various factors alter the expression pattern of calretinin and one could therefore question the reliability of results found under various conditions.

CHAPTER 3

KERATIN 14 AND KERATIN 19

3.1   Introduction

Actin-containing microfilaments, intermediate filaments (IFs) and tubulin-
containing mictotubules are the three principal structural elements of the
cytoskeleton of an eukaryotic cell [4, 56, 57]. Intermediate filaments (IFs) are
composed of different proteins and are approximately 10nm in diameter [4, 58-
60]. IFs are said to be mechanical integrators of the cytoplasm as they are
central to the cytoarchitecture and structural integrity of the cell [59].

In general, 1% of the cell protein constitutes IFs, but in certain cell types like
epidermal keratinocytes and neurons, IFs are abundant and accounts for up to
85% of the total protein content of the cells [59]. An IF protein consists of a
central α-helical domain (the rod) with non-helical head (amino-end/N-terminal)
and tail (carboxy-end/C-terminal) domains [57]. Two α-helical polypeptide chains
intertwine with parallel orientation to one another to form a coiled-coil rod [57,
59]. The non-helical head and tail segments vary in length and amino-acid
composition [57].

There are six types of IFs and the most complex of them all is the keratin (K)
filaments (also known as cytokeratin (CK) filaments) that constitute type I and II
of the IFs [56]. Keratin filaments contain keratin-like proteins and have a high
specificity for epithelial cells [60, 61]. In 1982 Moll *et al.* compiled a catalogue of
human keratins and assigned a number (1-19) to each keratin as their molecular
weights, ranging from 40-68 kilodalton (kD), were previously used to distinguish
between them [60].

Based on molecular weight, the keratins can be divided into high and low
molecular forms and also into basic and acidic forms based on the isoelectric
point (pI) [56, 61]. Originally Moll and co-workers divided the keratins into two
groups being the acidic type I keratins (K9-K19) and the basic-to-neutral type II

keratins (K1-K8) [60]. Since then the typical characteristics of keratin proteins have been elucidated to such and extend that it is now known that type I keratins are regarded as light in weight (smaller), have an acidic isoelectric point (pI) and are encoded by genes located on the long arm of chromosome 17, while type II keratins are heavier (bigger), have a basic pI and are encoded by genes on the long arm of chromosome 12 [56, 60, 61]. K9 has a pI of 5.4 and molecular weight of 64 and therefore authors have grouped this keratin under both type I and II keratins [56, 60]. According to the latest literature however, it should be regarded as a type I keratin [62]. Another exception in the above mentioned classification system, is the discovery that the gene encoding K18 is situated next to the gene for K8 on chromosome 12 and not on the proposed chromosome 17 [63].

With the discovery of more keratin genes, a new nomenclature for all keratins was proposed by Hesse *et al* in 2004 [64, 65]. A Keratin Nomenclature Committee was formed at the 2004 Gordon Conference on Intermediate Filaments in Oxford that included members of the Human Genome Nomenclature Committee and the Mouse Genome Nomenclature Committee as well as active investigators in the keratin field [62]. Genome analyses contributed to the demonstration of a total of 54 functional keratin genes in humans of which 28 belong to the keratin type I group and 26 to the type II group [62]. The committee proposed four categories of keratins: (A) human epithelial keratins, (B) human hair keratins, (C) non-human epithelial/hair keratins and (D) human keratin pseudogenes, which are numerically arranged in the following order: (Table 3.1)[62].

The Moll designation for the epithelial keratins as mentioned above has been retained for historical reasons and for the fact that numerous existing publications refer to the nomenclature established by Moll *et al* [62]. Therefore the numerical terms of K14 and K19 fortunately remained unchanged and major changes are mainly restricted to recently identified epithelial keratins. The terms "keratins" and "cytokeratins" have been used interchangeably in the past, and to facilitate communication and understanding, it has been advocated that the term "keratins" as well as the revised nomenclature of the Keratin Nomenclature Committee

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2006 rather be used [62]. For that reason the term keratin (K) we will be used for the purpose of this project.

Table 3.1 - *Numbering scheme of keratin categories [62].*

Category	Number range
• Human type I epithelial keratins	• 9-28
• Human type I hair keratins	• 31-40
• Nonhuman type I epithelial and hair keratins	• 41-70
• Human type II epithelial keratins	• 1-8 and 71-80
• Human type II hair keratins	• 81-86
• Nonhuman type II epithelial and hair keratins	• 87-120
• Type II keratin pseudogenes	• 121-220
• Type I keratin pseudogenes	• 221 →

General expression of keratins

In general there tends to be co-expression of keratin proteins as most basic high molecular weight keratins pair with acidic low molecular weight keratins [4, 56, 66]. The basic member is always larger than the acidic member by approximately 8kD [66]. Type I and II keratins associate as heterodimers and these intermolecular associations can be compared to the strongest non-covalent interactions that exists in nature [67]. The paired association between K5 and K14 is an example of such a heterodimeric complex and this association is probably responsible for the coiled-coil structure of epidermal IFs [67].

As keratins are expressed in different combinations of polypeptides in different epithelia, epithelial cells can be characterized by the specific pattern of its keratin expression [60, 68]. K7, K8 and K18 are referred to as the "simple" keratins as they are expressed in simple epithelia, like ductal luminal cells. The keratin pairs K5 and K14, K4 and K13 , K1 and K10 or K11 are associated with epithelial stratification [61]. Different subsets of keratins are expressed during the life cycle

of a cell [56] and the pattern of keratin expression has been described to vary with developmental stages, tissue differentiation [69, 70], and pathological changes [71]. The subepithelial mesenchymal tissue could also influence the keratin expression pattern of the epithelium [72]. On the other hand, the keratin profile of epithelial cells has been widely accepted to remain conserved during malignant transformation and therefore it is used in tumour typing of various carcinomas [56, 60]. More recent research reports that the keratin profile may change after malignant transformation, but that the profile would remain constant for that tumour [56, 73]. In the past IFs were considered as relatively stable cytoskeletal structures, but currently it is well recognized that they are highly dynamic proteins *in vivo* that allows rapid localized restructuring [74].

Functions of keratins

The keratins appear to integrate adjacent epithelial cells as it forms a complex network throughout the cytoplasm that abuts the desmosomal junction [56]. It has been proposed that the keratins loop through the matrix of desmosomal plaques rather than terminate within them [57], and it was later observed that keratin bundles could also attach to desmoplakin proteins on the intercellular surface of the desmosomal and hemidesmosomal plaques [56]. Other functions that were initially proposed for keratins are involvement in maintenance of the metabolic homeostasis of a cell [75] and regulation of apoptosis signalling [76]. Recently keratin functions were summarized as follows: keratins are involved in resistance against mechanical stress, regulation of ion transport, maintenance of cell junctions, plays a role in cell cycle regulation, attenuate apoptosis and maintains polarity of cells [68, 74]

3.1.1 Keratin 14

K14 is a type I IF with an isoelectric pH of 5.3, molecular weight of 50 kD, is encoded by genes located on chromosome 17q [56, 60, 77] and is said to confer physical resilience to basal cells [77]. Together with K5, K14 is a primary keratin of stratified squamous epithelia [61, 68].

K14 expression in normal tissue

In normal tissue samples the expression of K14 has been detected in epithelial cells of the tongue, epiglottis, oesophagus, anal canal, breast, basal cells of epidermis and squamous epithelium, outer root sheath of hair follicles, respiratory epithelium, acini of prostate and acini and intercalated ducts of salivary glands as well as transitional bladder cells [60, 78, 79]. K14 staining has been observed in myoepithelial cells of breast and salivary glands and ducts [79, 80]. No K14 immunoreactivity has been observed in adrenocortical, hepatic or gastric epithelial cells, neither in mesenchymal, lymphoid or neural cells [78].

K14 expression in oral epithelium

K14 mRNA and protein have been detected in the basal layer of non-keratinized epithelium of human oral mucosa, but strong staining patterns have been observed in the full thickness of keratinized epithelium (excluding the stratum corneum) [61, 81].

K14 expression in odontogenic tissue

K14 is the main intermediate filament of odontogenic epithelium and a marker often utilized in the detection of ameloblasts and IEE [32, 34, 35, 82]. Immunohistochemical studies have shown strong K14 positivity in IEE of human foetuses at the early bell stage [32] but at late bell stage, a weaker label for K14 was detected in fully differentiated ameloblasts [32, 82]. In contrast, the IEE cells of rat tooth germs in the proliferation stage show weak expression of K14, but intense staining in pre-ameloblasts and even more so in ameloblasts [83]. K14 has been observed throughout the dental epithelium of rat incisors and first molars, but not in the dental mesenchyme and is therefore a good marker for dental epithelium [35, 36].

K14 gene mutations

It is currently well known that point mutations in K14 (or K5) genes result in Epidermolysis Bullosa Simplex (EBS). This altered gene expression leads to

mechanical stress-induced intra-epithelial blistering [57]. It has been hypothesized that cells become fragile and prone to breakage upon mechanical stress without a proper IF network [57]. Most severe cases of EBS can be accounted for by K5 or K14 gene mutations, but isolated mild cases reveal no such mutations [59]. It has been found however that in the absence of K14 in basal cells, a wide variety of suprabasal networks can form, composed of different keratins [84].

3.1.2   Keratin 19

K19 is also a type I IF with an isoelectric pH of 5.2, molecular weight of 40 kD and encoded by genes located on chromosome 17q [56, 60, 77]. K19, unlike the other IF proteins (including other type I keratins), does not have a non-α-helical tail portion [85]. The authors who detected this feature of K19, did not propose any differences in assembly properties for this protein as compared to others [85].

K19 is referred to as part of the "simple keratins" like K8, K18, as well as K7 and K20 [68], mainly marking simple epithelia [66]. In a more recent study, K19 was classified under the same group as K14, namely keratins of stratified epithelia [74].

In 1985 K19 was proposed to pair with K7, K8 or K5 [86]. In 1989, K8 was regarded as the partner of K19 in filament formation [87], while others reported no known basic equivalent for K19 [61, 88]. As K8 and K18 are regarded as naturally occurring complexes [86], it has been postulated that if K8 production precedes or exceeds the synthesis of K18, this unbalanced state would induce K19 synthesis [88].

K19 expression in normal tissue

K19 expression has been described in luminal cells of salivary ducts at all levels and in acinar and ductal myoepithelial cells, but without staining of acinar cells themselves [80, 89]. Although K19 has been described in all the epithelial cells

of human foetal pancreas [90], only ductal cells stained intensely in adult tissue [91].

Homogenous positivity for K19 has been detected in human endometrium, urinary- and gall bladder epithelium [92]. A mosaic of negative and positive staining cells have been described in prostate epithelia and mammary gland luminal cells [92] and a complex and heterogeneous pattern has been described for hair follicles and non-keratinizing squamous epithelia of the oesophagus as the basal layer either staining exclusively or more intense than the rest of the epithelium [92]. Positive staining of special cell types include epithelial cells in the taste buds of the tongue and Merkel cells in some stratified squamous epithelia [92].

No staining of K19 has been found in human adult epidermal cells [92, 93], sebaceous glands and liver hepatocytes [92, 94].

K19 expression in oral epithelium

Although K19 has previously been detected in basal and parabasal cells of human alveolar mucosa (keratinized epithelium) [95], various studies reported different results. It has been described that K19 expression in human tissue is not found in keratinized stratified squamous epithelia, but it is present in low levels in the basal layer of nonkeratinized oral epithelia [92, 93]. This corresponds to later findings where K19 mRNA and protein was expressed in non-keratinized epithelium, but keratinized epithelium expressed K19 mRNA only [81]. K19 expression has however been observed throughout the basal cell layer and some parabasal cells of inflamed gingiva, while healthy human gingiva revealed positive staining for K19 in scattered cells located in or near the basal cell layer [96]. An upregulation of K19 in the sulcular and oral gingival epithelium has also been described in chronic periodontal diseases [97] and therefore it was proposed that a strong relationship between K19 expression and the severity of gingival inflammation exists [96, 98].

K19 expression in odontogenic tissue

K19 has been described in the stellate reticulum, stratum intermedium, secretory ameloblasts as well as ameloblasts in the maturation stage of monkeys (*Mamama mulatta* and *Macaca nemestrina*), with disappearance of expression as reduced enamel epithelium was transformed into junctional epithelium [99]. In human foetuses K19 (as well as K8) has been described in all cells of the dental lamina and enamel organ [32, 87] and it has been described to weakly label the IEE at the early bell stage, while intense positive staining in the fully differentiated ameloblasts was observed [32]. For this reason, K19 has been postulated to be a marker of ameloblast differentiation [32].

K19 gene mutation

Although no specific disease has been associated with K19 gene mutation, the possibility has been suggested that such genetic defects could lead to cell fragility as most IFs function to impart mechanical integrity [57]. This was proven in a study on K19 and K18 null mice, as it lead to cytolysis in trophoblast giant cells and early embryonic death [100].

3.2   Materials and Methods

Foetuses of large breed dogs were obtained, under ethical clearance of the Animal Use and Care Committee of the Faculty of Veterinary Sciences, University of Pretoria, South Africa, from female dogs scheduled for elective termination of pregnancy. Mixed breed dog foetuses were used in the study (*Canis familiaris*). Twenty four foetuses were fixed in 10% buffered formalin and then the heads were carefully cut into coronal sections (rostral to caudal), dehydrated and embedded in paraffin wax blocks. Those tissue samples that contained calcified bone or dental hard tissues were decalcified in routine decalcifying solution consisting of 880ml distilled water, 70 ml nitric acid (65%) and 50 ml hydrochloric acid (36%) for 60 minutes and rinsed in running tap water for 60 minutes in order to make sectioning possible without tearing and disruption of normal anatomy. The tissue blocks were then processed overnight.

Tissue specimens were sectioned at 3μm, stained with haematoxylin and eosin (H&E) and microscopically examined by two individuals in order to select sections with well-formed cap or bell stage enamel organs in which the respective odontogenic epithelial cells were morphologically clearly identifiable. Sections were de-paraffinized in two changes of fresh xylene for 10 minutes each, re-hydrated with 2 changes of absolute ethanol, followed by 90, 70 and 50% graded alcohol solutions respectively. The sections were then washed in 6 changes of fresh distilled water.

K14 and K19 were respectively treated as follows: Heat Induced Epitope Retrieval (HIER) in citric acid buffer; pH 6, was performed on the K14 sections using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 min. The sections were washed in 5 changes of fresh distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 6 min at 37ºC where after the sections were rinsed in 6 changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS buffer, Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for 10 min at room temperature.

Sections to be stained for K19 were first treated with 3% hydrogen peroxide for 6 min at 37ºC to quench endogenous peroxidase activity where after the sections were rinsed in 6 changes of fresh distilled water. Heat induced epitope retrieval in EDTA (Ethylene diamine tetra-acetic acid disodium salt) buffer; pH 8.00, was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 min. The sections were rinsed in 6 changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS buffer, Sigma P4417, Sigma-Aldrich

Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for 10 min at room temperature.

The sections were incubated in Cytokeratin 14 (1:60) and Cytokeratin 19 (1:100) anti serum respectively (Novocastra NCL-Cytokeratin 14 [NCL-LL002] and NCL-CK19, Novocastra Laboratories Ltd., Balliol Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW United Kingdom) for 60 minutes at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The sections were then incubated in Dako Envision+R System Labelled Polymer, anti mouse, HRP (Dako K4001, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 30 min at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 4 min at 37ºC, washed in distilled water and counterstained in Haematoxylin for 1 minute at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA).

Reddish-brown granular cytoplasmic staining was interpreted as positive for the presence of K14 and K19. As recommended by Novocastra skin and muscle tissue were used respectively for internal positive and negative controls for K14 and the same positive control is recommended for K19.

Staining in each case was noted as follows:
- S – Single
    - One or two cells in a specific field of cells stained positive
- F – Focal
    - Groups of cells that revealed positive staining, being interrupted by cells that did not stain
- D – Diffuse
    - General staining of the cells involved (90% or more of the cell type stained)
- + Weak staining
- ++ Intense staining

Because of the *in situ* nature of the study, the different cell types of odontogenesis were distinguished from one another based on their microscopic morphology and location in the developing tooth germ and adjacent structures. For the purpose of this study ameloblasts were defined as tall columnar cells, with prominent reversed polarity (nuclei aligned adjacent to the stratum intermedium) and located adjacent to formed dentin. The IEE cells were defined as short columnar cells on a basal membrane with centrally placed nuclei, opposite undifferentiated ectomesenchymal cells of the dental papilla without any signs of odontoblast differentiation or hard tissue formation. The cells of the outer enamel epithelium (OEE) were defined as low cuboidal cells with little cytoplasm at the periphery of the enamel organ. The oral epithelium on the alveolar ridge area from where the tooth germ originated, was referred to as the overlying alveolar epithelium. The term dental lamina was used when continuity of the lamina could be observed between the overlying alveolar epithelium and the developing tooth germ. When only discrete islands of epithelial cells were evident it was termed Serres rests.

3.3   Results

As the exact time of gestation of the developing dog foetuses were unknown, the foetuses were classified into 2 groups based on the odontogenic development stage. Foetuses 1-8, designated as group 1, only had tooth germs in the bud and cap stages of development with no ameloblast differentiation or dental hard tissue formation. The tooth germs of foetuses 9-24, designated as group 2, were already in the bell stage of development with visible cell differentiation and hard tissue formation in the developing tooth germs. Many specimens presented with more than one section that conformed to the requirements of the inclusion criteria and in 15 cases a rostral (anterior) and a caudal (posterior) section of the same foetus were used for comparison. In the remaining 9 cases, only one section was included in the study. A total of 39 sections were therefore harvested from the 24 foetuses for immunohistochemical investigation of K14 and K19 expression. The number of tooth germs on a single section varied from 1 to 4 and as the whole section was stained with the antibody there were some sections that contained more than one tooth germ that could be used in the evaluation

process. Only one tooth germ defined as the best representation of the bell stage in the given section was chosen for analysis of the immunohistochemical stain. No discrepancies were however found between K14 and K19 stains of the different tooth germs on the same section.

3.3.1 Keratin 14

Table 3.2 lists the results found for K14 staining in dog odontogenic tissue. Skin was used as internal positive control as the basal layer of the epidermis stained positive in group 1 and 2 foetuses. Muscle tissue represented the internal negative controls as prescribed by Novocastra.

Intense and diffuse K14 staining was evident in the IEE of group 1 foetuses (Fig 3.1) while the group 2 foetuses revealed mainly focal staining in the same epithelium (Fig 3.2).

No ameloblasts were present in the group 1 foetuses. In the group 2 foetuses pre-secretory ameloblasts revealed no staining of K14 while the secretory ameloblasts stained intense and diffusely throughout the examined sections (Fig. 3.2 and 3.3). Three sections contained mainly pre-secretory ameloblasts and only a few secretory ameloblasts and therefore the annotation of focal staining for these cases in table 3.2. The intensity of the staining of the secretory ameloblasts seemed to decrease as enamel secretion continued. Post-secretory ameloblasts could not be evaluated as no maturation stage ameloblasts were present in the sections evaluated.

*Table 3.2* – *Keratin 14 expression in dog odontogenic tissue.*

Foetus nr.	AMB	IEE	OEE	ODB	Stel. Ret.	Strat. Interm.	Dental lamina	Serres Rsts	Alv. Epith.
1	NA	D++	D++	-	D++	D++	D++	NA	D++
2c	NA	D++	D++	-	D++	D++	D++	NA	D++
3	NA	D++	D++	-	D++	NA	D++	NA	D++
4	NA	D++	D++	-	D++	D++	D++	NA	D++
5	NA	NA	NA	NA	NA	NA	D++	NA	D++
6	NA	NA	NA	NA	NA	NA	D++	NA	D++
7	NA	D++	D++	-	D++	D++	D++	NA	D++
8	NA	NA	D++	-	D++	D++	D++	NA	D++
9a	NA	D++	D++	-	D++	F+	D++	D++	D++
9b	D++	D++	D++	-	D++	F+	D++	D++	D++
10a	D++	F+	D++	-	F+	F+	D++	D++	D++
10b	D++	F+	D++	-	F+	F+	D++	D++	D++
11a	D++	D++	D++	-	D+	F+	D++	D++	D++
11b	D++	F+	D+	-	S+	S+	D++	D++	D++
12	D++	F+	D++	-	S+	S+	D++	D++	D++
13a	D++	F+	F+	-	S+	S+	D++	D++	D++
13b	D++	F+	F+	-	S+	S+	D++	D++	D++
14a	D++	NA	F+	-	S+	-	D++	D++	D++
14b	D++	F+	F+	-	-	-	NA	D++	D++
15a	D++	F+	F+	-	-	-	D++	D++	D++
15b	D++	F+	F+	-	-	-	D++	D++	D++
16a	F+ #	F+	F+	-	-	-	D++	D++	D++
16b	NA	F+	F+	-	F+	F+	D++	D++	D++
17a	F+ #	F+	F+	-	-	-	D++	D++	D++
17b	D++	F+	F+	-	-	-	D++	D++	D++
18a	D+	NA	F+	-	F+	S+	D++	D++	D++
18b	D++	F+	F+	-	-	-	D++	D++	D++
19a	D++	NA	F+	-	F+	S+	NA	D++	D++
19b	D++	-	F+	-	-	-	D++	D++	D++
20a	D++	NA	D++	-	S+	S+	NA	D++	D++
20b	F+ #	NA	D+	-	-	-	NA	D++	D++
21a	D++	NA	F+	-	-	-	NA	NA	D++
21b	D++	-	F+	-	-	-	D++	D++	D++
22a	NA	NA	F+	NA	-	NA	NA	D+	D++
22b	NA	F+	F+	-	F+	F+	NA	D+	D++
23a	D++	NA	F+	-	-	-	D+	D+	D++
23b	D++	F+	F+	-	-	-	D+	D+	D++
24a	NA	NA	NA	NA	NA	NA	NA	NA	D++
24b	D++	F+	F+	-	-	-	D++	NA	D++

AMB – Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section, **#** Presecretory ameloblasts were mainly present on these sections and did not stain. The few secretory ameloblasts that were present stained positive, resulting in focal positivity of the evaluated cell layer.

Apart from diffuse and focal distribution and differing intensities, all of the sections revealed positive staining for K14 in the OEE (Fig 3.1 and 3.2). No positive staining was detected in any of the odontoblasts evaluated (Fig 3.2 and 3.3).

Intense and diffuse staining of the stellate reticulum and stratum intermedium was seen in group 1 foetuses (Fig 3.1) while this pattern could not be detected in the group 2 sections (Fig 3.2 and 3.3). Intense staining patterns were observed in all alveolar epithelial cells (full thickness), dental laminae and Serres rests (Fig 3.1, 3.2 and 3.4).

An interesting finding was that the group 1 foetuses revealed no K14 staining on the dorsal aspect of the tongue (Fig 3.5 and 3.6), while these cells stained intense and diffusely positive in the group 2 foetuses (Fig 3.7).
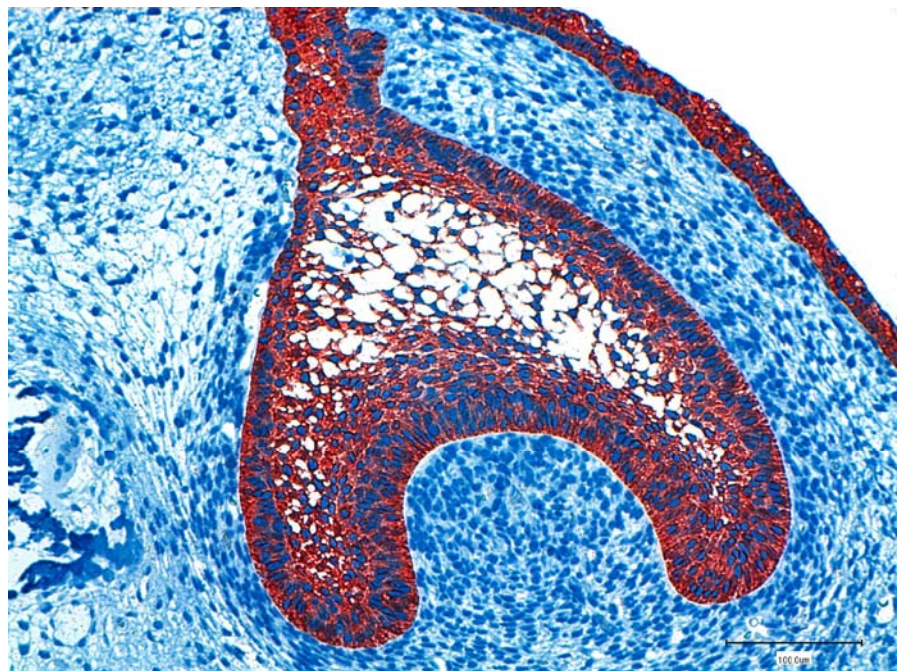
Fig. 3.1: *K14 expression in the epithelium of a late cap-early bell stage tooth germ of a group 1 foetus (scale bar 100µm, original magnification 200x).*
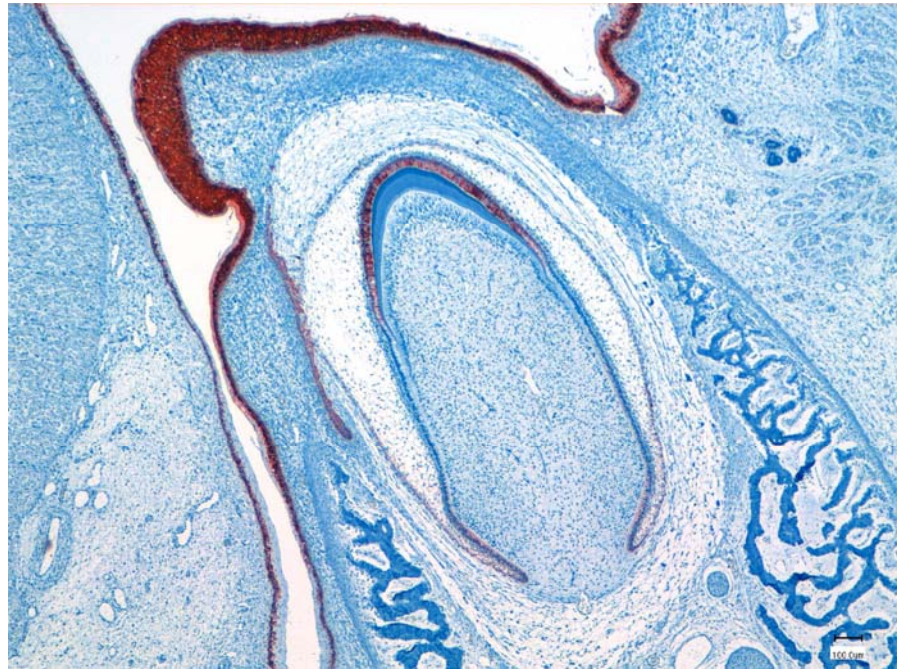
Fig. 3.2: *K14 expression observed in the secretory ameloblasts, IEE, OEE, dental lamina, Serres rests and overlying alveolar epithelium in the late bell stage tooth germ of a group 2 foetus (scale bar 100µm, original magnification 40x).*
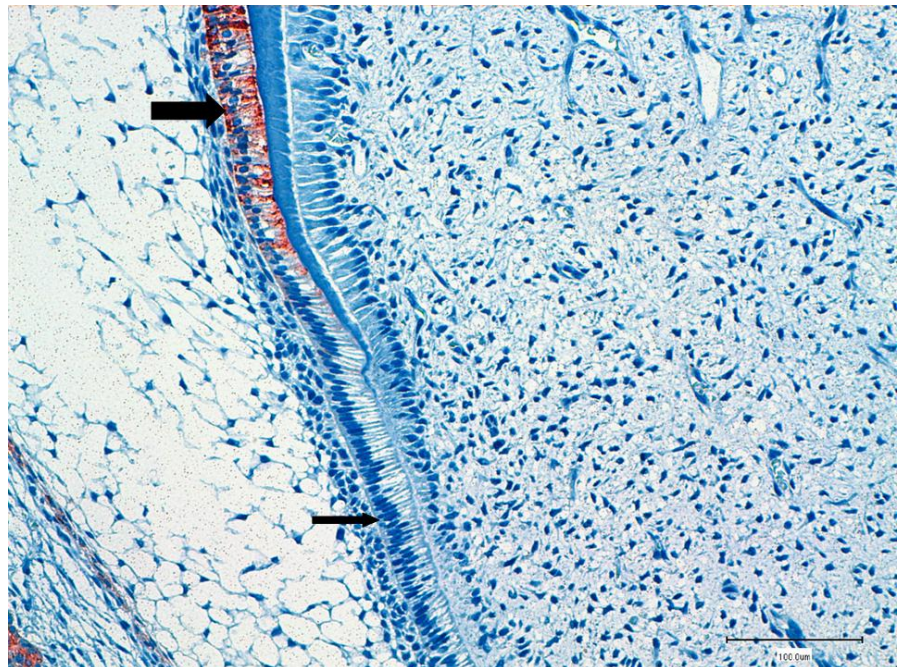
Fig. 3.3: *No K14 expression in pre-secretory ameloblasts (thin arrow) while intense and diffuse staining of the secretory ameloblasts (thick arrow) can be observed (scale bar 100µm, original magnification 200x).*

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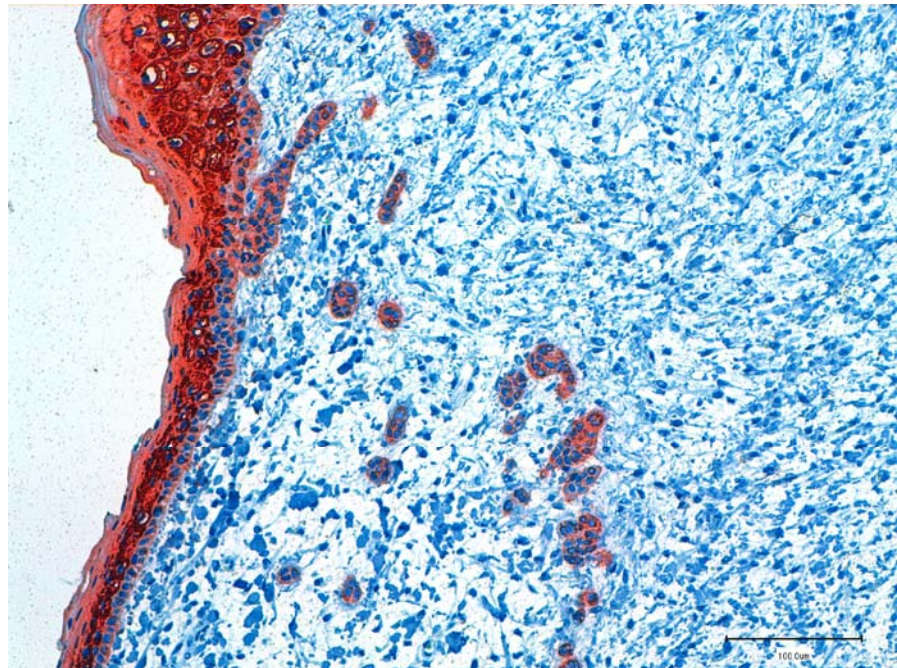
Fig. 3.4: *Positive staining for K14 in the overlying alveolar epithelium and Serres rests (scale bar 100µm, original magnification 200x).*
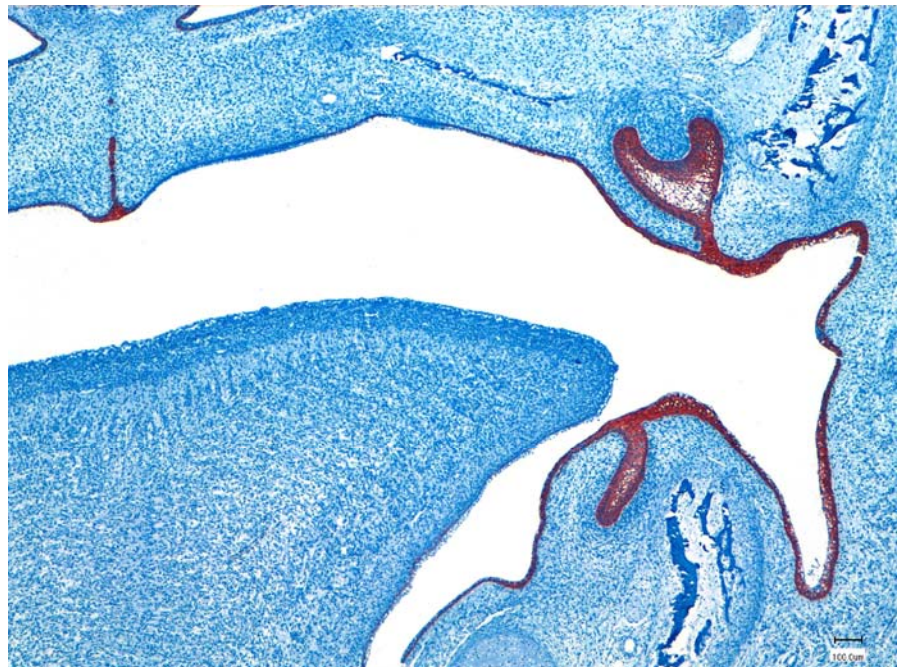
Fig. 3.5: *General view of K14 expression in a group 1 foetus (scale bar 100µm, original magnification 40x).*

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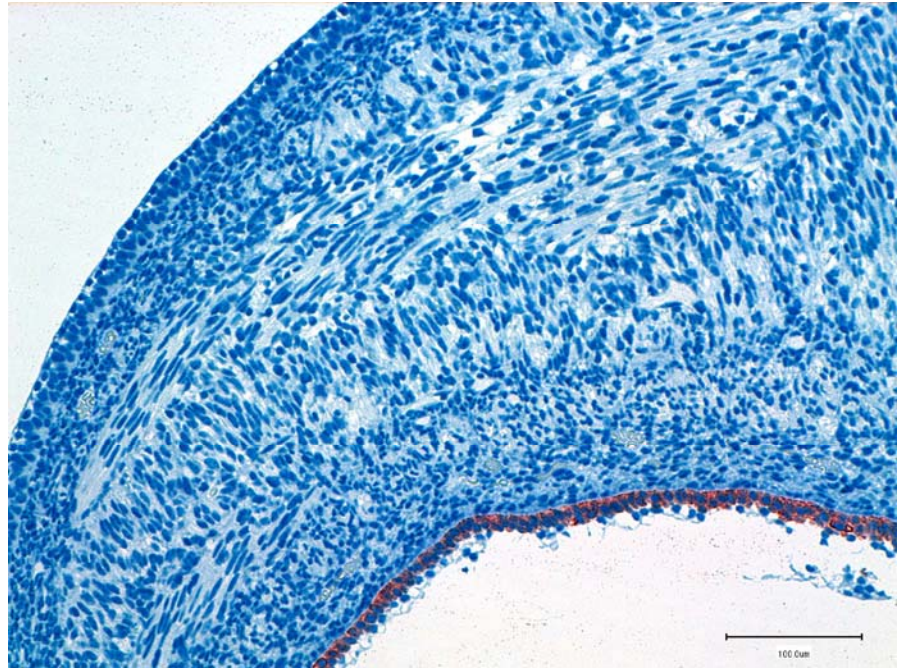
Fig. 3.6: *K14 staining in the epithelium of the ventral aspect of the tongue with absent staining in the epithelium of the dorsal aspect in a group 1 foetus (scale bar 100µm, original magnification 200x).*
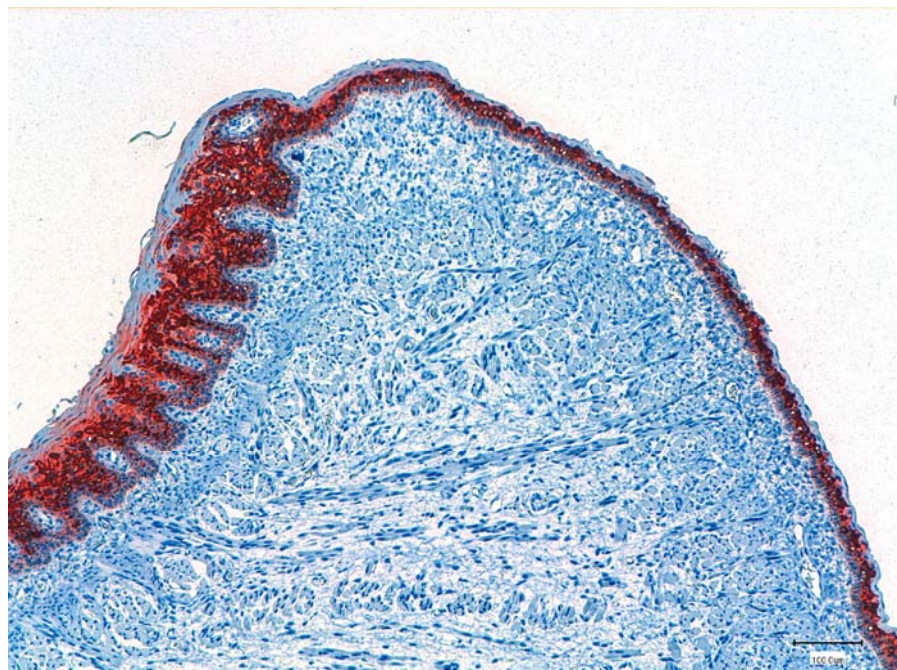
Fig. 3.7: *K14 expression in the epithelium of the dorsal and ventral aspects of the tongue in a group 2 foetus (scale bar 100µm, original magnification 100x).*

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3.3.2　Keratin 19

Table 3.3 lists the results found for K19 staining in dog odontogenic tissue. The positive control prescribed by Novocastra could not be utilised as skin stained positive in group 1 foetuses but not in group 2 foetuses. Both salivary gland duct epithelium as well as respiratory mucosa stained positive for K19 throughout the sections (Fig 3.8 and 3.9).

In group 1 and 2 foetuses the cells of the dental organ stained positive with variable intensities and distribution patterns (see table 3.3). The IEE cells of the group 1 foetuses revealed a heterogeneous pattern of staining for K19 as differing staining intensities were observed (Fig 3.10) and in group 2 foetuses diffuse expression of these cells were observed except in the cervical loop region where IEE did not stain (Fig 3.11). Therefore, the annotation of focal staining was given to various sections (table 3.3).

No ameloblasts were present in the group 1 foetuses. The pre-secretory and secretory ameloblasts in group 2 foetuses stained diffusely positive for K19 although intensities varied (see table 3.3 and Fig 3.11).

No staining of K19 was observed in the odontoblasts (Fig 3.11). Positive staining was observed in the full thickness of the overlying alveolar epithelial cells in group 1 foetuses (Fig 3.13), but in the group 2 sections it was mainly restricted to the superficial epithelial cell layers (Fig 3.11 and 3.12). Diffuse staining was observed in the dental lamina and Serres rests (Fig 3.11 and 3.12).

In group 1 foetuses diffuse staining of the epidermal epithelium was observed as well as respiratory mucosa epithelium and keratinized and non-keratinized epithelium lining the oral cavity (Fig 3.13), including the dorsal aspect of the tongue (Fig 3.14). In group 2 foetuses, the staining pattern of the tongue diminished to only suprabasal cells staining (Fig 3.15) and K19 expression was lost in epidermal epithelium. Most sections evaluated revealed diffuse staining of suprabasal epithelial cells only (keratinized and non-keratinized areas), while single cell positivity was observed in the basal and suprabasal layers of keratinized and non-keratinized oral epithelium of some sections. Constant
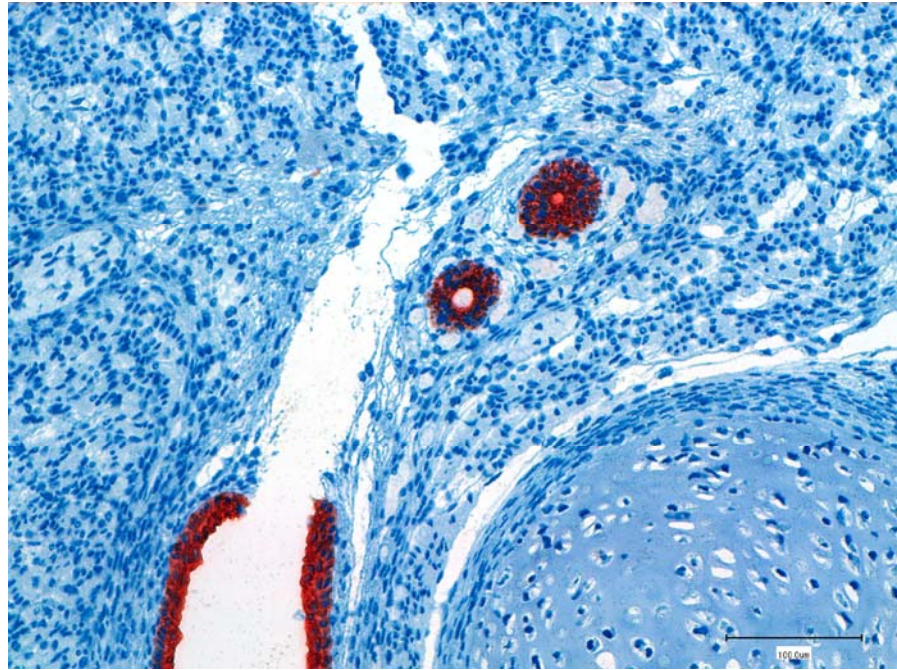
staining of the respiratory mucosa (Fig 3.9) as well as acinary and ductal epithelial cells of the salivary glands (Fig 3.8) were observed in group 1 and 2 foetuses.

Table 3.3 – *Keratin 19 expression in dog odontogenic tissue.*

Foetus nr.	AMB	IEE	OEE	ODB	Stel. Ret.	Strat. Interm.	Dental lamina	Serres Rests	Alv. Epith.
					Keratin 19				
1	NA	F+	D+	NA	D+	D++	D+	NA	D++
2	NA	F+	D+	NA	D++	D++	D+	NA	D++
3	NA	S+	D+	NA	D++	NA	D+	NA	D++
4	NA	F+	D+	NA	D++	D++	D+	NA	D++
5	NA	NA	NA	NA	NA	NA	D++	NA	D++
6	NA	NA	NA	NA	NA	NA	D++	NA	D++
7	NA	F+	D++	NA	D++	D++	D++	NA	D++
8	NA	NA	D+	NA	D++	D++	D++	NA	D++
9a	D++	D++	D++	-	D++	D++	D++	D++	F+ *
9b	D++	D++	D++	-	D++	D++	D++	D++	D+ *
10a	D++	D++	D++	-	D++	D++	D++	D++	S+
10b	D++	D++	D++	-	D++	D++	D++	D++	S+
11a	D++	F+	D++	-	D++	D++	D++	D++	S+
11b	D+	D+	D++	-	D+	D+	D+	D+	S+ *
12	D+	D++	D++	-	D++	D++	D++	D++	F+
13a	D++	D+	D++	-	D++	D++	D++	D++	S+
13b	D++	D++	D++	-	D++	D++	D+	D+	S+
14a	D+	D+	D+	-	D+	D++	D+	D+	D+ *
14b	D+	F+	D+	-	D+	D+	D+	D+	D+ *
15a	D+	F+	F+	-	F+	F+	NA	D++	S+
15b	D++	F+	F+	-	F+	F+	D++	D++	S+ *
16a	D++	F+	F+	-	F+	F+	D+	D+	F+ *
16b	NA	F+	D+	-	D++	D++	D++	D++	D++ *
17a	D+	F+	F+	-	D+	D+	D+	D+	F+
17b	D++	D++	D+	-	D+	D+	D+	D+	D+ *
18a	D++	D++	D++	-	D++	D++	D+	D+	D+ *
18b	D++	D++	D++	-	D++	D++	D++	D++	D++ *
19a	D+	NA	D+	-	D++	D++	NA	D++	F+
19b	D+	D+	D+	-	D+	D+	D+	NA	F+ *
20a	D+	D+	D+	-	D+	D+	NA	D+	D+ *
20b	D++	NA	D+	-	D+	D+	D+	D++	D+ *
21a	D++	F+	F+	-	F+	F+	D+	D+	D+ *
21b	D+	F+	F+	-	D+	D+	D+	D+	D++ *
22a	D+	F+	F+	-	F+	F+	D+	D+	D+ *
22b	NA	D+	D+	-	D+	D+	NA	D++	D+ *
23a	D++	D++	D++	-	D++	D++	D++	D++	D+ *
23b	D++	D+	F+	-	F+	F+	D+	NA	D+ *
24a	D+	D+	F+	-	F+	D+	NA	NA	D+ *
24b	NA	NA	NA	-	NA	NA	D+	D++	D+ *

AMB – Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section, ***** Only superficial layers stained.

Fig. 3.8: *Positive staining for K19 in the salivary duct epithelium (scale bar 100μm, original magnification 200x).*
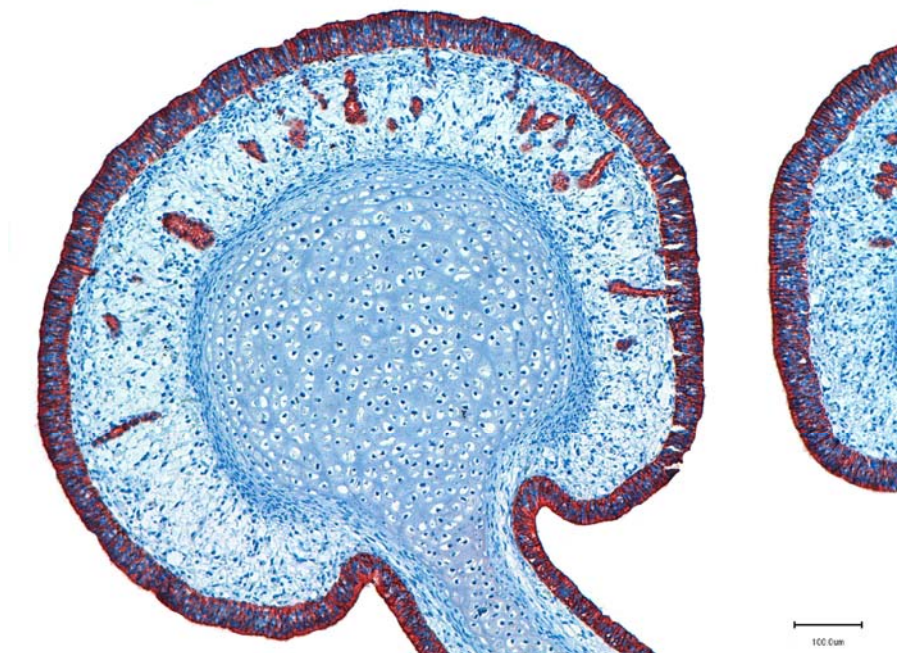
Fig. 3.9: *K19 staining in the epithelium and developing minor salivary glands of the respiratory mucosa (scale bar 100μm, original magnification 100x).*
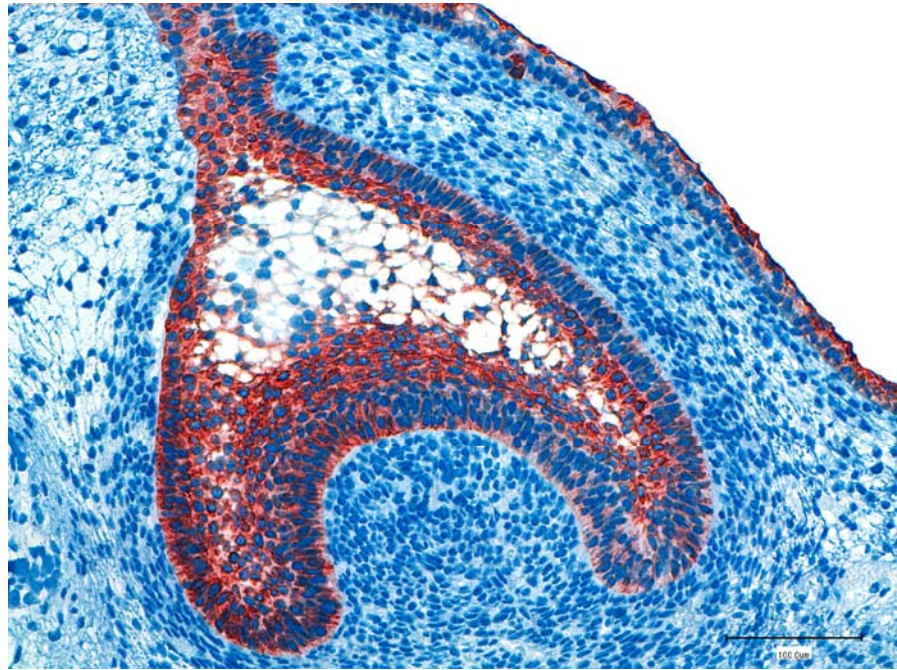
Fig. 3.10: *K19 expression in the epithelium of a late cap- early bell stage tooth germ (scale bar 100µm, original magnification 200x).*
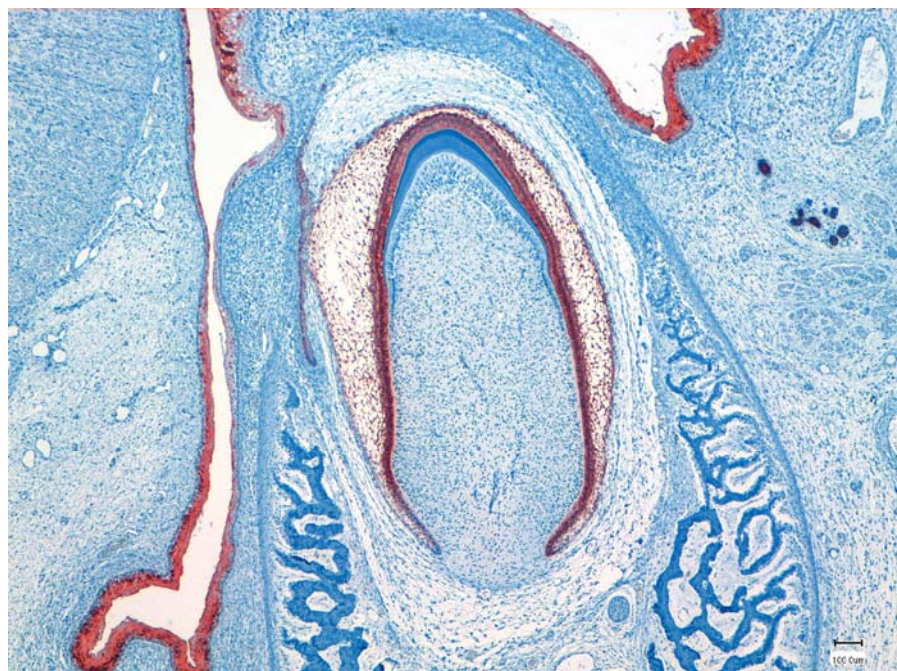
Fig. 3.11: *K19 expression observed in the odontogenic epithelium of a group 2 foetus with the tooth germ in the late bell stage of development (scale bar 100µm, original magnification 40x).*
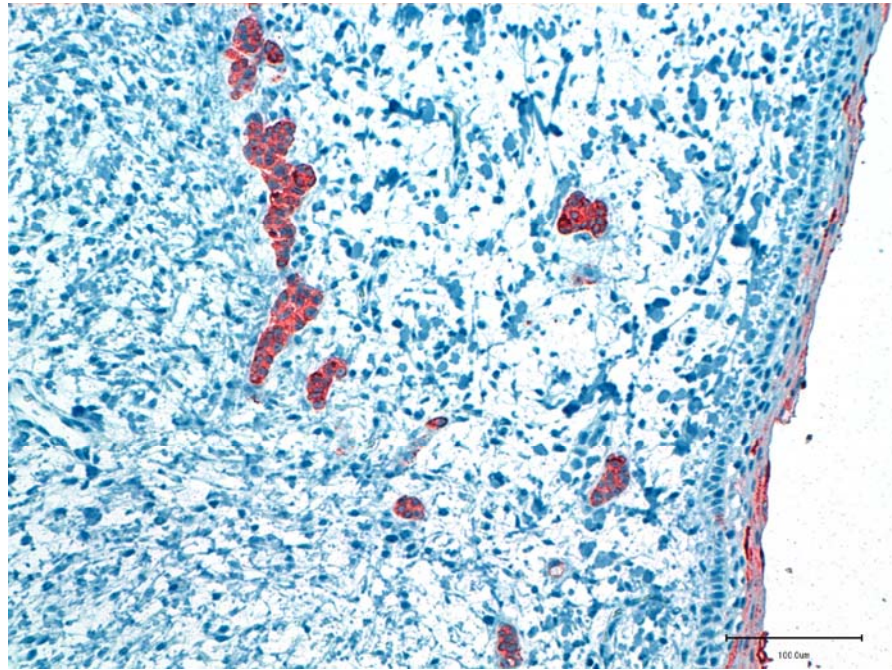
Fig. 3.12: *Positive staining for K19 in the Serres rests and superficial cell layers of the overlying alveolar epithelium (scale bar 100μm, original magnification 100x).*

Fig. 3.13: *General overview of K19 expression in a group 1 foetus (original magnification 40x).*
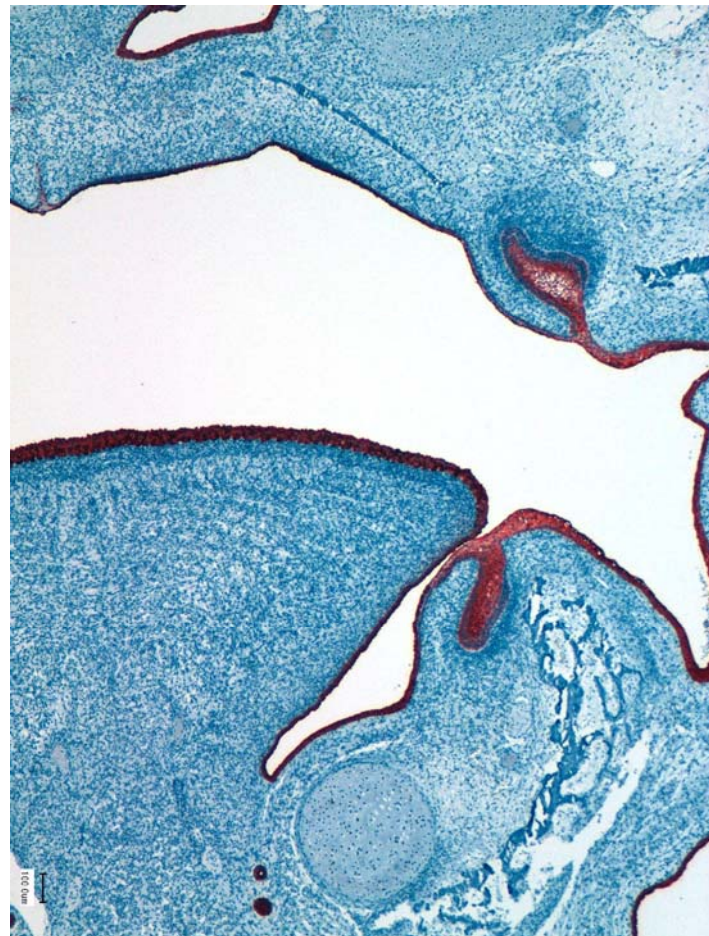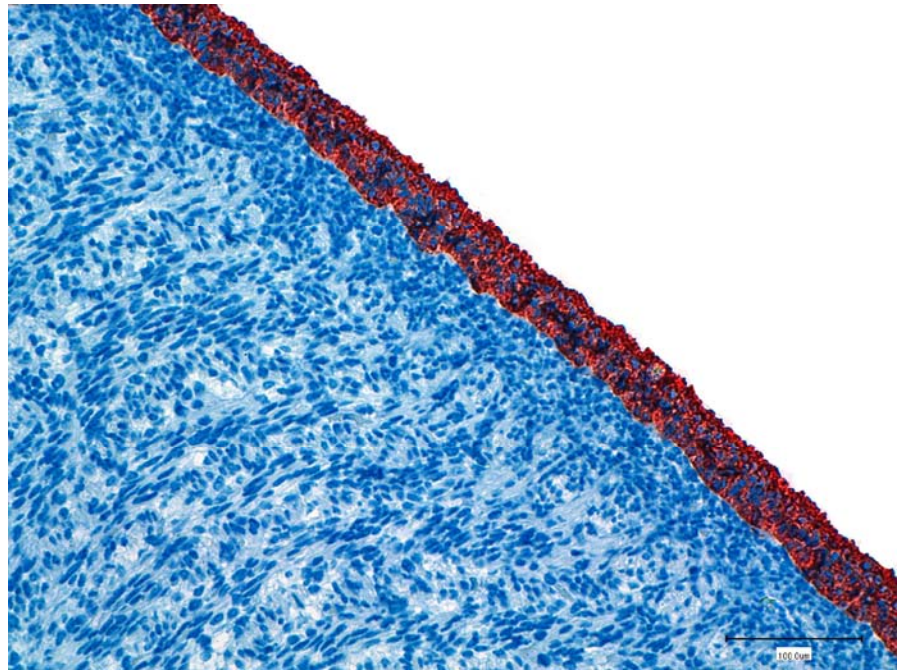
Fig. 3.14: *K19 expression in the epithelial cells of the dorsal aspect of the tongue of a group 1 foetus (scale bar 100µm, original magnification 200x).*
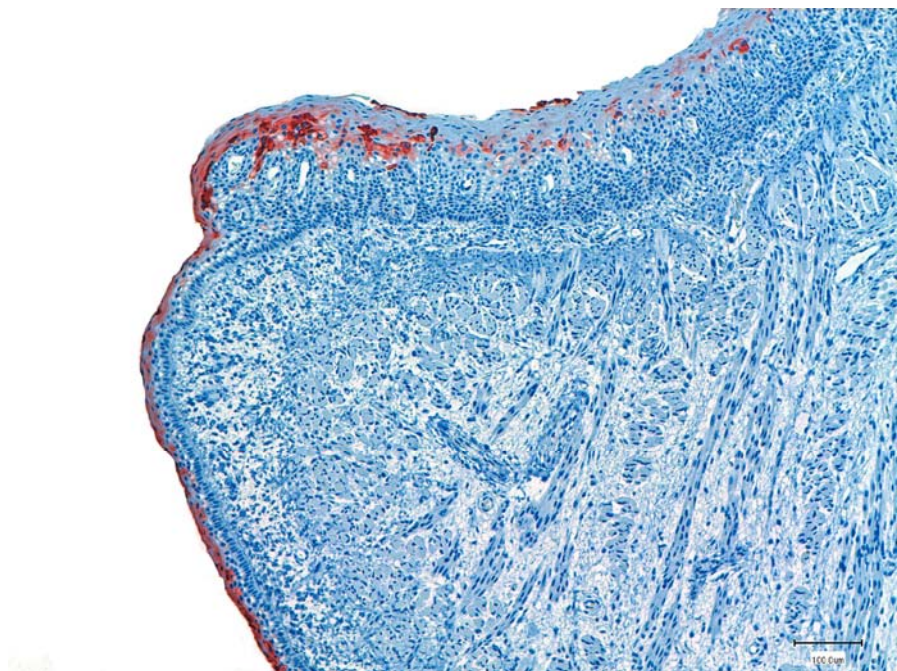
Fig. 3.15: *K19 expression in the superficial layers of the epithelium on the dorsal and lateral aspects of the tongue in a group 2 foetus (scale bar 100µm, original magnification 100x).*

3.4 Discussion

3.4.1 Keratin 14

In accordance to the study of Yan *et al*. on human foetal odontogenic tissue, K14 revealed a cytoplasmic staining pattern in the current study [82]. Intense positive staining was observed in all epithelial cells in the group 1 foetuses examined. During the process of differentiation the IEE stained intensely positive, the presecretory ameloblasts revealed no staining for K14, but secretory ameloblasts regained intense and diffuse K14 staining. Towards the end of the secretory phase the staining of ameloblasts became less intense. Conflicting results were however obtained by Tabata and colleagues as they described weak staining of K14 in the IEE cells, intense staining in presecretory ameloblasts and much more intense in ameloblasts of rats [83]. They even proposed K14 to be a useful cell- and differentiation stage-specific marker for ameloblast-lineage cells [83]. In 2003 K14 expression was described in all the cells of the enamel organ except in the secretory ameloblasts in areas of advanced amelogenesis [34]. The authors proposed that loss of K14 expression in regions of advanced amelogenesis could be the consequence of the cellular secretory processes taking place in these cells [34]. K14 has been described as an anchorage protein that might have a role in supporting and preserving the epithelial-mesenchymal interactions [32, 59] and therefore it has been speculated that ameloblasts need to lose K14 to disengage its anchorage as these cells migrate to deposit enamel matrix [32]. Although it cannot be sure whether the various author's definition of pre-ameloblasts, presecretory ameloblasts, and secretory ameloblasts conform to one another, there seems to be remarkable differences in the respective results found.

It is difficult to explain the loss and gain of K14 expression that were found in differentiating ameloblasts in the current study. Although it has been generally presumed that IFs are the most stable component of the mammalian cytoskeleton, several lines of evidence suggest that IFs are dynamic structures, not only with respect to their ability to reorganize within a cell, but also with regard to the assembly of their networks [57]. This could be a possible explanation for the observed dynamic expression profiles in the process of

ameloblast differentiation in the current study. Various other factors could have an influence on the expression pattern of keratin protein. Keratins have an estimated half life between 84 and 104 hours [101], are able to assemble in vitro in the absence of auxiliary proteins or factors and the information necessary for the formation of a filament is suggested to be intrinsic, within the primary sequence of the keratin filament polypeptide [57]. It has also been speculated that post-transcriptional controls could play a part in the interdependent keratin expression patterns [81, 92] and therefore the protein might not be detected although the mRNA is present. Several modalities have been proposed for keratin protein regulation including phosphorylation, caspase cleavage, ubiquitination, glucosylation, transglutamination and interaction with associated proteins [57, 58]. The exact mechanism of the *in vivo* regulation of keratins still needs to be elucidated as numerous aspects thereof remain to be determined [56, 58, 100].

Another possible explanation for the intriguing expression pattern of K14 observed in the current study could be the phenomenon of selective masking. It has been observed that antibody reaction with keratin filaments of mitotic cultured rat kangaroo cells fail to react in interphase cells as the result of cell cycle-dependant selective masking [102]. According to Franke and co-workers masking could be due to an intrinsic change in the arrangement of the keratin polypeptides or specific association with a non-keratin protein [102]. Co-assembly of K14 and amelogenin has been demonstrated and it has been suggested that K14 functions as a chaperon for nascent amelogenin polypeptides during amelogenesis [103]. It has been hypothesized that the N-terminal region of K14 is bound to the ATMP motif of amelogenin, and that it may only pair with K5 after disassociation with amelogenin [103]. Antibodies used in the current study were directed against the C-terminal ends of the keratin proteins (Novocastra) and is postulated that if the K14 terminal region (to which antibodies are directed) were bound to another molecule, detection of the K14 protein would be hampered.

The occurrence of selective and complete masking of a determinant in the IF system should emphasize the need to interpret negative results with caution [102]. For this reason it has been proposed that immunoblotting tests on proteins

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from cells under question as well as treatments with unmasking reagents be used as controls for possible masking of keratin filaments [66, 102].

Formaldehyde fixation and paraffin embedding can cause weak or even false-negative keratin staining [66]. It has also been reported that over-fixed tissue results in difficult and even impossible retrieval of keratin immunoreactivity [91] Therefore it has been proposed that deparaffinized sections should be treated with proteases like pepsin, trypsin or pronase [66], but although Proteinase K has been used to increase accessibility and probe penetration, it has been shown that a reduced signal may actually be observed after proteinase K digestion [81]. In the current study heat induced epitope retrieval (HIER) was tested in different buffers and as the best results were obtained with EDTA buffer, ph 8, it was used in this study.

In agreement with previous studies on human and rat tissue, no K14 staining was observed in odontoblasts or other mesenchymal cells in the current study, while positive staining was observed throughout the dental laminae [34, 83]. The positive K14 staining that was observed in the Serres rests of the current study have also been reported previously in human foetuses [32].

K14 has been described as a marker for cells of the stratum intermedium in rats [36], but the current study resulted in differing expression profiles of K14 in early and late developmental stages. Intense and diffuse staining of the stellate reticulum and stratum intermedium was seen in early developmental stages (group 1) with little or no staining observed in later stages (group 2). This is supported by results from Domingues and colleagues who also described K14 staining of the stellate reticulum to be stronger at the early bell stage of human tissue and Crivelini *et al.* who reported no staining of the stellate reticulum and stratum intermedium in areas of advanced amelogenesis in human foetuses [32, 34].

An interesting observation was the staining pattern of K14 in keratinized epithelium as the staining pattern spread diffusely through the whole thickness of the keratinized epithelium (excluding the stratum corneum), while restricted to the basal layer of non-keratinized epithelia. This supports previous studies on

human tissues [61, 81]. It has been suggested that the need for large amounts of K14 (and 5) filaments may be reduced in the non-keratinized epithelium as it endures less masticatory forces compared to the masticatory mucosa [81].

Numerous conflicting results have been proposed regarding keratin expression patterns and their proposed associated functions. K14 has been proposed to be down regulated in epidermal cells that commit to differentiation [57] and on the other hand described as a marker for differentiation of the ameloblasts cell lineage with increased intensity in the process of differentiation [83]. The variable keratin staining patterns described in the literature may be as a result of different antibodies used and also different tissue preparation methods influencing the tissue antigenicity, for example demineralization [61]. Even the presence of EBV that has been documented to influence K14 expression patterns [104].

3.4.2 Keratin 19

The prescribed positive control for K19 could not be used throughout the sections as the group 2 foetuses did not reveal K19 positivity in epidermal cells. It should be kept in mind that the antibody was developed against human K19 and that it has been shown that the keratin expression patterns between the two species differ. One example is the constant expression of K6 in the epidermal epithelium of dogs as opposed to no K6 expression in human skin [105]. Constant staining of the ductal epithelial cells of the salivary glands was observed and it is therefore recommend that these cells should rather be used as positive control for K19 in dog tissue.

In the current study the K19 staining pattern in the IEE varied from weak single cell staining, to intense and diffuse staining. The pre-secretory and secretory ameloblasts however stained intense and diffusely positive for K19. Therefore this study agrees with the study of Domingues and co-workers to some extend as they found weak K19 staining in the IEE with intense positive staining of the fully differentiated ameloblasts of human tissue [32]. Crivelini *et al.* hypothesized that K19 staining characterizes only ameloblasts and preameloblasts with complete differentiation in human foetuses [34]. These differences could be due to individual interpretation of what should be regarded as weak positive as opposed

to recording a negative result. Still, the observed staining pattern of the IEE in this study implies that less staining is evident in the undifferentiated cells.

The constant positivity obtained in OEE, stellate reticulum, stratum intermedium, ameloblasts, dental lamina and Serres rests is in agreement with the results from previous studies on human foetuses and monkeys [87, 99].

In the current study diffuse K19 expression was detected in the basal and suprabasal cells throughout the oral epithelium of the very young foetuses (group 1). This expression pattern changed in the group 2 foetuses to single cell positivity in basal layers which then further diminished to expression of only suprabasal cells of keratinized and non-keratinized epithelium. The results of this study propose that this altered expression pattern could be the result of the developmental changes of the foetal epithelium. The effect of aging or the progress in development seen in the current study is in keeping with a previous study reporting K19 expression in pancreatic epithelial cells and hepatoblasts during early foetal development with loss of this expression in adult tissue [90, 94, 106].

The observed pattern of suprabasal epithelium staining in keratinized and non-keratinized oral epithelial cells in group 2 foetuses has also been described in human foetuses [34]. These results differ drastically from those obtained in 1986 on human child and adult tissue as they suggested K19 expression to be related to cells with potential proliferative capacity as it was often detected in basal epithelial cells and not in suprabasal layers [92]. As oral epithelial expression of K19 seemingly diminished with development in our study, we support the proposal that K19 expression in the basal layer and even parabasal cells could occur as a result of inflammation in postnatal tissue [96, 98]. In 1990 Ouhayoun *et al*. concluded that K19 expression has a strong relationship to the severity of gingival inflammation. In healthy gingival epithelium, a total absence of K19 staining was observed, while in moderately inflamed tissue a more frequent presence of K19 could be detected and strong staining was evident in all strata of markedly inflamed pocket epithelium [96].

When each article on K19 staining is evaluated, uniform staining patterns were described in each animal case evaluated by a specific author. When the results are however compared with those of other studies, some minor and other grave differences exist. Possible reasons were sought to explain these discrepancies. Bartek *et al.* reported considerable variability due to different fixation techniques of tissue and found mainly basal cells layers staining for K19 on frozen sections and additional suprabasal staining patterns in methacarn-fixed paraffin-embedded tissues [92]. That could explain the differing results obtained in the current study as compared to that of Bartek and colleagues [92] In the same study three different monospecific monoclonal antibodies were directed against K19 (BA16, BA17 and A53-B/A2) in human tissue and fewer cells stained with BA 16 than the other two [92]. Therefore the differing antibodies used could also result in different results obtained.

Interspecies differences is another factor that can also be held responsible for differing results as the K19 expression pattern in human oral tissue could not be observed in pig oral mucosa. The molecular weight of K19 is only 40 kD and the smallest protein detected in porcine tissue is 48 kD [95]. As K19 has been proposed to associate with K5, K7 and K8, different complexes are formed [86]. This could possibly result in conformational changes of the targeted epitope and influence K19 expression profile.   Therefore, there seems to be numerous variable factors that could influence the staining pattern of K19.

3.4.3   Keratin 14 and Keratin 19

Domingues *et al.* reported opposite expression patterns for K14 and K19 in human tissue as K14 was down regulated with ameloblast differentiation and K19 up regulated [32]. In the current study down regulation of K14 was found in cells starting to differentiate (polarizing pre-secretory ameloblasts), but this phenomenon was only transient as secretory ameloblasts regained expression of K14. K19 expression however was constant in the pre-secretory and secretory ameloblasts.   In general there seemed to be a tendency towards opposite expression profiles as the basal and parabasal oral epithelial cells stained positive for K14 and the stratum corneum stained positive for K19. Acidic and basic keratins are stabilized when expressed together and when expressed alone

it is rapidly degraded [107]. Therefore, it has been suggested that K14 may compete with K19 for a common pool of type II keratin like K5 and in this way the abundant expression of K14 protein in keratinized epithelium may repress K19 protein expression at the post-transcriptional level [81]. This is supported by the finding that K19 mRNA is expressed in keratinized epithelium but the protein is not [81]. However, form the results of this study it can not be postulated that expression of the one needs to be down regulated to gain expression of the other as K14 and K19 expression do occur concurrently in secretory ameloblasts.

In a study on keratin expression in the oral epithelium of different species it was shown that antihuman keratin antibodies stain animal tissues, but the pattern of staining can differ from the known profile of human keratin expression [108]. This can result from different keratinisation patterns between species, variations in differentiation pathways, variable cross-reactivity between human antibodies and animal keratins, the nature of the tissue analyzed and the influence of local factors or functional requirements on translational mechanisms [108]. It was postulated in 1994 that keratins are not needed in certain species, cell types, or stages of development, but are critical in others [57]. One example is the constant expression of K6 in the epidermis of dogs as opposed to no K6 expression in human skin [105]. Therefore, interspecies variability needs to be kept in mind when different studies of keratin profiles are compared.

Different keratin expression patterns between foetal and adult tissues have been proposed in 1990 as the pattern of keratin expression differed between tissue taken at the floor of the mouth of human foetuses and adult specimens [93]. The current study clearly supports this finding and the differing ages of specimens used is surely another factor influencing the comparability of different studies.

3.5 Conclusion

Recently proteomic analysis was done on keratins. As a result of the sequence homology, extractability and posttranslational modifications of keratins, the analysis is complex and challenging but this technique poses great possibilities for future research as numerous questions regarding keratins still prevail [58, 74, 100].

In agreement with Brouillard who suggested that a standardized method needs to be put in place for keratin proteomic research [109], the current study also propose that standardized guidelines regarding terminology as proposed by Schweizer and colleagues be used in future research projects [62]. A lot of confusion arises from the fact that some authors refer to cytokeratins and others to keratins and the nomenclatures for keratin or cytokeratin antibodies also require re-evaluation to preclude future misnomers. Standardized guidelines regarding monoclonal and polyclonal keratin antibodies could also aid future research methodology and interpretation of results.

The results of keratin expression is contradictory when it comes to odontogenic epithelia [61] as keratins are usually divided into keratins of simple epithelia and keratins of stratified epithelia and the enamel organ consists of unusual stratified epithelium undergoing many functional changes during development. The keratin profile of the enamel organ and specifically IEE and ameloblasts seems to be complex as these cells even obtain functions associated with simple and glandular epithelia [99]. Keratin expression in odontogenic tissue therefore remains complex.

From the results of this study it is concluded that K14 and K19 are non-specific markers for odontogenic epithelium as other oral and respiratory epithelial cells also stained for K14 and K19 and the odontogenic epithelial cells did not stain exclusively.

CHAPTER 4

AMELOGENIN

4.1   Introduction

Amelogenins are enamel matrix proteins secreted by the ameloblasts and constitute over 90 % of the developing enamel extracellular matrix during odontogenesis [22, 110, 111]. Amelogenins are mostly 20 to 25 kD proteins, primarily hydrophobic and contain proline, glutamine, leucine and histidine amino acid residues [112-114]. This protein molecule consists of three distinct regions: a hydrophobic core sequence of 100-130 residues enriched in prolines and glutamines, a N-terminal region (45 residues) and an acidic hydrophilic C-(carboxy-) terminal region (13-15 residues) [112, 115]. The N-terminal domain is rich in tyrosine and therefore it is referred to as the TRAP segment (tyrosine-rich amelogenin peptide) [112]. The N- and C- terminal regions have been proposed to be highly conserved across mammalian species, as opposed to the variable hydrophobic central region of the molecule [112, 116, 117].

Amelogenins have been proposed to self-assemble and form nanospheres that constitute the basic building blocks of the enamel extracellular matrix structural framework [115, 118]. In this model the N-terminal domain has been implicated in the formation of nanospheres, whereas the C-terminal region may contribute to homogeneity and stability of the nanospheres, preventing their fusion to form larger assemblies [115].   It was recently proposed that all regions of the amelogenin molecule aggregate *via* hydrophobic bonds, except the hydrophilic C-terminal region, to form a micelle core [119]. This proposal suggests that amelogenin may then form super-assemblies through the C-termini [119] and the assembly and apparent solubility has been proposed to be affected by temperature and pH changes [110, 112].

A complex mixture of amelogenin peptides is found in the enamel layer of developing teeth as amelogenins undergo alternative RNA splicing and proteolytic processing of formed proteins [120]. Various isoforms of amelogenin

have been characterized by alternative splicing at the mRNA level [121, 122]. Enamel proteins are believed to be cleaved by specific enzymes, undergo a series of proteolytic events and are then degraded and removed from the enamel matrix as enamel matures [123-125]. The metalloprotease enamelysin (MMP-20) [126] has been described to cleave the surface-accessible amelogenin C-terminal of amelogenin in the initial developmental stages [112, 119, 127], while the serine protease EMSP1 [128] has been implicated in the degradation process of the exposed middle portion during the early maturation phase [119, 129]. This controlled proteolysis results in disassembly of the micelles to liberate space for crystal growth [119]. It has previously been proposed that dendritic cells are involved in the elimination of amelogenin from the enamel organ [130], but more recently it was suggested that endocytosis and post-endocytotic processing of amelogenin or amelogenin peptides occurs at the ameloblast Tomes' process and could therefore be a possible mechanism for protein removal from the extracellular matrix [131].

The coding region of the amelogenin gene is conserved among different species and is found on the X and Y chromosomes in human and bovine genes, but only on the X chromosome in rat, murine and porcine genes [132-134]. The X and Y forms are designated AMGX-Human and AMGY-Human [132, 135], but older literature refer to AMELX and AMELY genes [132]. The conserved coding region for amelogenin led to the suggestion that amelogenin could be a new phylogenetic marker for ordinal mammal relationships and therefore it could be useful to determine the evolutionary relatedness among various species [136].

Expression pattern of amelogenin

In the past amelogenin has been regarded as a protein exclusive to enamel and its formative cells, the ameloblasts [137-140]. It has been proposed and used as a stage specific marker for ameloblast differentiation as amelogenin expression was not detected in IEE while expression was found in pre-ameloblasts and ameloblasts [33, 82, 83]. Small amounts of amelogenin mRNA has been described in pre-secretory ameloblasts before the onset of biomineralization [138, 139] while intense expression was found in differentiated ameloblasts in the secretory stage with sustained expression during the maturation or early

maturation stage [138, 139, 141]. It was previously widely accepted that amelogenin mRNA is not present in odontoblasts, dental pulp, bone or along the developing tooth root and the conclusion was made that amelogenin is an enamel-specific protein in developing rat and mouse molars [138-141].

However, amelogenin has been detected in hamster mantle dentin [113]. It was proposed that amelogenin diffuse or translocate into the predentin and pre-odontoblast layer and that the odontoblasts endocytose and digest the amelogenin protein [113, 142]. It was therefore concluded that transcription of the amelogenin gene does not occur in odontoblasts [113]. It has also been suggested that the presence of trace amounts of amelogenin splice products in dentin could be the result of contamination [125].

In 2002 amelogenin mRNA was detected in odontoblasts of porcine teeth using reverse transcription-polymerase chain reaction (RT-PCR) [120]. The level of amelogenin mRNA expressed by secretory ameloblasts was however shown to be more than 1000x the level found in odontoblasts [120]. In a similar study relatively small amounts of amelogenin mRNA and protein was detected in rat odontoblasts [143]. Amelogenin expression was described to occur within a limited developmental period as it was observed in the young odontoblasts forming early mantle dentin [143]. Weak staining of odontoblasts was also detected with immunohistochemistry studies in rat incisors [36].

In 2006 it was suggested that odontoblasts synthesize and secrete amelogenins after *in situ* hybridization and immunohistochemistry studies were done on human odontogenic tissue [144]. Amelogenin mRNA was localized in the dentin layer containing the odontoblastic processes and was not detected in the odontoblast cell bodies which contained type I collagen mRNA [144]. It is an interesting observation as one would anticipate amelogenin mRNA expression in close approximation to the cell nucleus if the protein were transcribed in the cell. These results were in direct contrast to that obtained in mouse models as amelogenin transcript was not observed in mouse odontoblasts. It was therefore suggested that the differing expression patterns obtained for human and mouse tissues could be an interspecies variation [141, 144].

The mRNA of splice forms of amelogenin have been detected in periodontal ligament cells (cementoblasts) of wild-type mice [145] and recently amelogenin mRNA and protein was described in dog and rat osteoblasts, osteoclasts, in some osteocytes and also in articular cartilage chondrocytes [146]. It was therefore proposed that amelogenin is also expressed in mesenchymal cells and is not only an ectodermal ameloblasts-specific protein as previously believed [145, 146].

Interspecies conformity of amelogenin

The amino-acid sequences of amelogenin found in dog tissue have shown 88% homology with the human amelogenin derived from the X chromosome and 85,1% homology to the human Y chromosome derived amelogenin [30]. It has also been suggested that the splicing pattern in the dog is more closely related to that of man or pig than that of mice [30]. However, the full translated sequence of dog amelogenin was published for the first time in 2007 [146].

Functions of amelogenin

A variety of functions have been proposed for amelogenin, but its primary role is to provide the necessary milieu for the development of the mineralized enamel [132]. Amelogenin has been proposed to act as a proton buffer that absorbs the large amounts of hydrogen ions generated during hydroxyapatite formation and to inhibit crystal growth, without changing the crystal morphology [147]. As enamel crystals first grow in their length during the secretory phase of amelogenesis and then in thickness during the maturation stage, it was proposed that full length amelogenin molecules bind to developing enamel crystals to prevent premature crystal fusion during early stages of amelogenesis [148]. The stepwise processing of amelogenins was shown to affect its affinity to bind to apatite crystals [148], but the persistence of large quantities of amelogenin cleavage products as well as the conserved C-terminus have been suggestive of the intact protein as well as cleavage products functioning in enamel formation [149]. It was previously proposed that amelogenin is not required for the initiation of crystal formation but regulates the enamel thickness and plays a part in the organization of the crystal pattern [150]. However, it was recently suggested that

the nanosphere supramolecular structure of amelogenin could promote the heterogeneous nucleation of calcium phosphate by acting as a nucleation template and concentrating charge at the nanosphere surface [151]. With regard to the above mentioned functions, amelogenin has been implicated in the pathogenesis of Amelogenesis imperfecta as well in dental flourosis [150, 152, 153].

The role of amelogenin in epithelial-mesenchymal signalling during tooth development has been proposed by various authors [125, 133, 138]. Through this signalling interaction amelogenin expression in pre-odontoblasts has been implicated to inhibit the secretion of ameloblast matrix and the maturation thereof until after the initial layer of dentin has been mineralized [132]. It has also been suggested that vitamin D regulates amelogenin expression in odontoblasts and is therefore part of this interactive process [143].

Amelogenin expression has been observed on the dentin surface at the apical end of developing human tooth roots [154]. When porcine enamel matrix was placed in experimental cavities created in extracted monkey incisors, a tissue identical to acellular extrinsic fibre cementum was formed and therefore it was proposed that amelogenin could have an inductive effect on some cells in the dental follicle [154]. These findings led to the development of a porcine enamel matrix derivative called EMDOGAIN, essentially a mixture of amelogenins [154-157]. EMDOGAIN was developed as a treatment for periodontal repair and it has shown to result in increased levels of bone formation as well as increased periodontal attachment [156, 158, 159]. The therapeutic effects in periodontal regeneration has been proposed to be due to the cell-adhesive activity of amelogenin as well as its proposed function as a growth factor [114, 160]. EMDOGAIN has opposite effects on mesenchymal and epithelial cells as it promotes regeneration of periodontal and cementum attachment to exposed tooth surfaces but inhibits epithelial cell attachment [132]. In a study on amelogenin-null mice it was also indicated that amelogenins may prevent abnormal resorption of cementum [145].

Low-molecular-weight amelogenins have been suggested to have a positive effect on cell-cycle progression of dental pulp cells. This effect has been

proposed to have potential use to promote proliferation of dental pulp tissue in the presence of injury, resulting in reparative dentin formation [144].

Amelogenin polypeptides have been shown to be the components in dentin extracellular matrix capable of inducing osteogenesis and chondrogenesis at ectopic sites [125]. Amelogenin may have roles in both promoting osteogenesis as well as inhibiting osteoclastogenesis. It has been implicated in the promotion of osteoblast proliferation and differentiation as well as stimulation of osteoprotegerin expression [161]. Amelogenin has also been demonstrated to inhibit the expression of RANKL and M-CSF dramatically by its presence in osteoblasts and therefore inhibiting RANKL-mediated osteoclastogenesis [145, 162]. The expression of amelogenin in osteoblasts and osteoclasts at the mineralization front led to the speculation that amelogenin has a role in the cross-talk between these two cell types to control the process of osteoclastogenesis [146]. It has even been postulated that the cementum, osteoblasts and PDL cells maintain the periodontal ligament and regulate root resorption via amelogenin [162]. Amelogenin may also have a role in inducing mesenchymal stem cell recruitment during the process of long bone and periodontal repair [146].

As the amelogenin gene is present on the X (AMEL X / AMGX-Human) and Y (AMEL Y / AMGY-Human) chromosomes of humans [132, 134, 135, 163] and these genes differ in size, it has been used to differentiate males from females [164, 165]. However, deletion of the amelogenin gene on the Y chromosome has been detected in five Indian males in a study of 270 male samples and therefore it has been suggested that investigators should not rely solely on amelogenin for gender determination [166]. An interesting finding is that the individuals with the AMELY/AMGY-Human gene deletion have reportedly no visibly abnormal male phenotype and therefore it has been proposed that additional Y-chromosome markers should be included in gender determination tests performed for detecting the presence of male DNA [167].

4.2 Materials and Methods

Foetuses of large breed dogs were obtained, under ethical clearance of the Animal Use and Care Committee of the Faculty of Veterinary Sciences,

University of Pretoria, South Africa, from female dogs scheduled for elective termination of pregnancy. Mixed breed dog foetuses were used in the study (*Canis familiaris*). Twenty four foetuses were fixed in 10% buffered formalin and then the heads were carefully cut into coronal sections (rostral to caudal), dehydrated and embedded in paraffin wax blocks. Those tissue samples that contained calcified bone or dental hard tissues were decalcified in routine decalcifying solution (HNO$_3$ 70ml, HCL 50ml, Distilled water 880ml) for 60 minutes and rinsed in running tap water for 60 minutes in order to make sectioning possible without tearing and disruption of normal anatomy. The tissue blocks were then processed overnight.

Tissue specimens were sectioned at 3μm, stained with haematoxylin and eosin (H&E) and microscopically examined by two induviduals to select slides with well-formed cap or bell stage enamel organs in which the respective odontogenic epithelial cells were morphologically clearly identifiable.

Sections were de-paraffinized in two changes of fresh xylene for 10 minutes each, re-hydrated with two changes of absolute ethanol, followed by 90, 70 and 50% graded alcohol solutions respectively. The sections were then washed in 6 changes of fresh distilled water. Heat induced epitope retrieval in citric acid buffer; pH 6 was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 min. The sections were rinsed in 6 changes of fresh distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 6 min at 37ºC where after the sections were washed in 5 changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS buffer, Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for 10 min at room temperature. The sections were incubated in Amelogenin (FL-191) anti serum (1:100) (Santa Cruz Biotechnology sc-32892, Santa Cruz Biotechnology, Inc, 2145 Delaware Ave, Santa Cruz, California 95060) for 60 minutes at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The sections were then incubated in

Dako Envision+R System Labelled Polymer, anti rabbit, HRP (Dako K4009, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 30 min at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 4 min at 37ºC, washed in distilled water and counterstained in Haematoxylin for 1 minute at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA).

Reddish-brown granular cytoplasmic staining was interpreted as positive for the presence of amelogenin. A section containing secretory ameloblasts was used throughout the different batches as a positive control for amelogenin.

Staining in each case was noted as follows:
- S – Single
  - One or two cells in a specific field of cells stained positive
- F – Focal
  - Groups of cells that revealed positive staining, being interrupted by cells that did not stain
- D – Diffuse
  - General staining of the cells involved (90% or more of the cell type stained)
- + Weak staining
- ++ Intense staining

Because of the *in situ* nature of the study, we distinguished between the different cell types of odontogenesis on the basis of their microscopic morphology and location in the developing tooth germ and adjacent structures. For the purpose of this study the IEE cells were defined as short columnar cells on a basal membrane with centrally placed nuclei, opposite undifferentiated ectomesenchymal cells of the dental papilla without any signs of odontoblast differentiation or hard tissue formation. Secretory ameloblasts were defined as

tall columnar cells with prominent reversed polarity (nuclei aligned adjacent to the stratum intermedium) and located adjacent to formed enamel and dentin. Presecretory ameloblasts were defined as tall columnar cells with reversed polarity, but without the presence of enamel product adjacent to these cells. The cells of the outer enamel epithelium were defined as low cuboidal cells with little cytoplasm at the periphery of the enamel organ. The oral epithelium on the alveolar ridge area from where the tooth germ originated, was referred to as the overlying alveolar epithelium. The term dental lamina was used when continuity of the lamina could be observed between the overlying oral epithelium and the developing tooth germ. When only discrete islands of epithelial cells were evident it was termed Serres rests.

4.3   Results

As the exact time of gestation of the developing dog foetuses were unknown, the foetuses were classified into 2 groups based on the odontogenic development stage. Foetuses 1-8, designated as group 1, only had tooth germs in the bud and cap stages of development with no ameloblast differentiation or dental hard tissue formation. The tooth germs of foetuses 9-24, designated as group 2, were already in the bell stage of development with visible cell differentiation and hard tissue formation. Many presented with more than one section that conformed to the requirements of the inclusion criteria and in 15 cases a rostral (anterior) and a caudal (posterior) section of the same foetus were used for comparison. In the remaining 9 cases, only one section was included in the study. A total of 39 sections were therefore harvested from the 24 foetuses for immunohistochemical investigation of amelogenin expression. The number of tooth germs on a single section varied from 1 to 4 and as the whole section was stained with the antibody there were sometimes more than one tooth germ that could be used in the evaluation process of some sections. Only one tooth germ defined as the best representation of the bell stage in the given section was chosen for analysis of the immunohistochemical stain. No discrepancies were however found between amelogenin stains of the tooth germs on the same section.

Table 4.1 lists the results found for amelogenin staining in dog odontogenic tissue. No staining for amelogenin could be detected in the group 1 foetuses. In

these foetuses no dental hard tissue formation was observed as differentiated ameloblasts and odontoblasts were not present. Diffuse granular staining for the amelogenin protein was observed in all secretory ameloblasts (Fig 4.1 and 4.4). Small amounts of amelogenin were observed in presecretory ameloblasts (Fig 4.2 and 4.3) and the pattern of staining was in close approximation to the cell nucleus.

No staining was detected in the IEE, OEE, stellate reticulum, stratum intermedium, dental laminae, Serres rests or alveolar epithelium (Fig 4.1).

Weak staining was observed in some odontoblasts in 6 of the 39 sections evaluated (Fig 4.5 and 4.6). The staining pattern seemed to be localized to the intercellular spaces between the odontoblasts and other pulp cells, resembling background staining. Staining of these sections was repeated and the same pattern of staining was observed. No amelogenin staining was observed in or around odontoblasts at advanced stages of odontogenesis.

***Table 4.1*** *– Amelogenin expression in dog odontogenic tissue.*

Amelogenin									
Foetus nr.	**Secr. AMB**	**IEE**	**OEE**	**ODB**	**Stel. Ret.**	**Strat. Interm.**	**Dental lamina**	**Serres rests**	**Alv. Epith.**
1	NA	-	-	NA	-	-	-	NA	-
2	NA	-	-	NA	-	-	-	NA	-
3	NA	-	-	NA	-	-	-	NA	-
4	NA	-	-	NA	-	-	-	NA	-
5	NA	NA	NA	NA	NA	NA	-	NA	-
6	NA	NA	NA	NA	NA	NA	-	NA	-
7	NA	-	-	NA	-	-	-	NA	-
8	NA	-	-	NA	-	NA	-	NA	-
9a	D+	-	-	-	-	-	-	-	-
9b	D+	-	-	U	-	-	-	-	-
10a	D+	-	-	-	-	-	-	-	-
10b	D+	-	-	-	-	-	-	-	-
11a	D++	-	-	-	-	-	-	-	-
11b	D+	-	-	-	-	-	-	-	-
12	D+	-	-	-	-	-	-	-	-
13a	D+	-	-	-	-	-	-	-	-
13b	D+	-	-	-	-	-	-	-	-
14a	D+	-	-	-	-	-	-	-	-
14b	NA	-	-	-	-	-	-	-	-
15a	D+	-	-	-	-	-	-	-	-
15b	D+	-	-	U	-	-	-	-	-
16a	NA	-	-	-	-	-	-	-	-
16b	NA	-	-	-	-	-	-	-	-
17a	D+	-	-	-	-	-	-	-	-
17b	D+	-	-	-	-	-	-	-	-
18a	D++	-	-	U	-	-	-	-	-
18b	D+	-	-	U	-	-	-	-	-
19a	D++	-	-	-	-	-	-	-	-
19b	D+	-	-	-	-	-	-	-	-
20a	D+	-	-	U	-	-	-	-	-
20b	D+	-	-	U	-	-	-	-	-
21a	D+	-	-	-	-	-	-	-	-
21b	D+	-	-	-	-	-	-	-	-
22a	D+	-	-	-	-	-	-	-	-
22b	NA	-	-	-	-	-	-	-	-
23a	D+	-	-	-	-	-	-	-	-
23b	D++	-	-	-	-	-	-	-	-
24a	NA	NA	NA	NA	NA	NA	NA	NA	-
24b	D++	-	-	-	-	-	-	-	-

Secr. AMB – Secretory Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section, **U** – Uncertain.
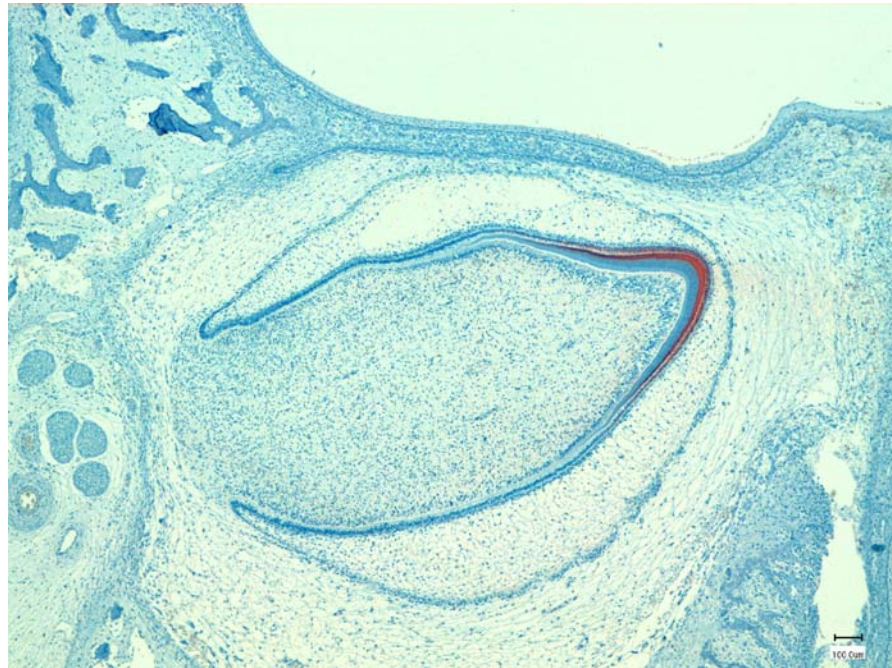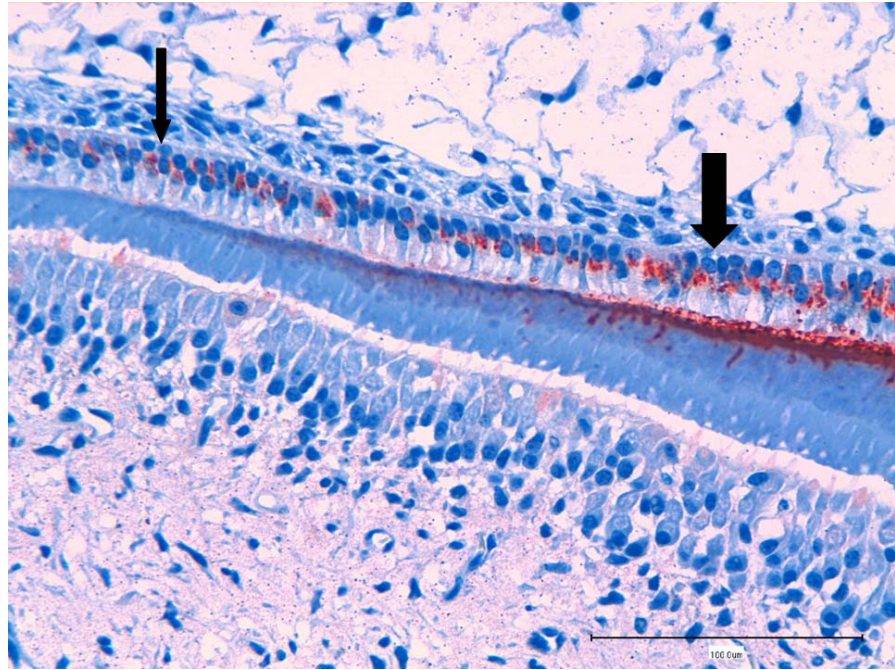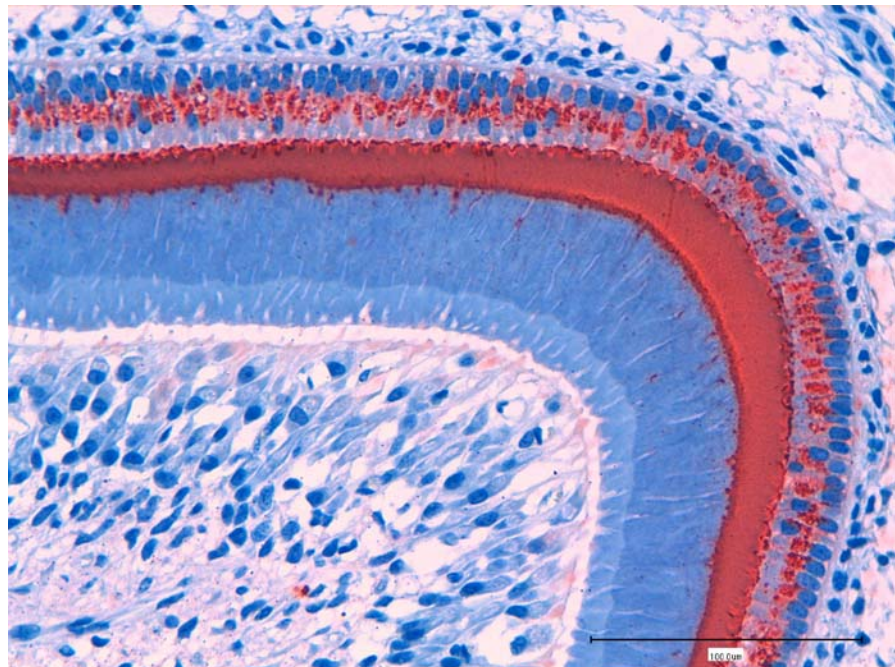
Fig. 4.1: *Diffuse amelogenin staining observed in the secretory ameloblasts of a bell stage tooth germ (group 2 foetus) (scale bar 100μm, original magnification 40x).*
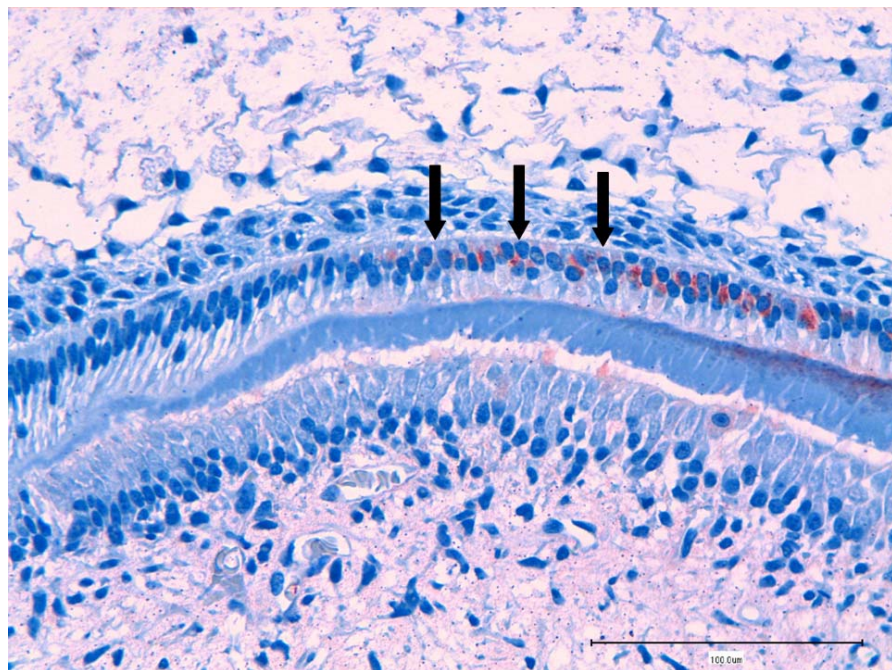
Fig. 4.2: *Focal positivity for amelogenin observed in the presecretory ameloblasts (arrows) (scale bar 100μm, original magnification 400x).*

Fig. 4.3: *Amelogenin staining in the presecretory (thin arrow) and secretory ameloblasts (thick arrow) (scale bar 100µm, original magnification 400x).*

Fig. 4.4: *Intense and diffuse amelogenin staining observed in the secretory ameloblasts (scale bar 100µm, original magnification 400x).*
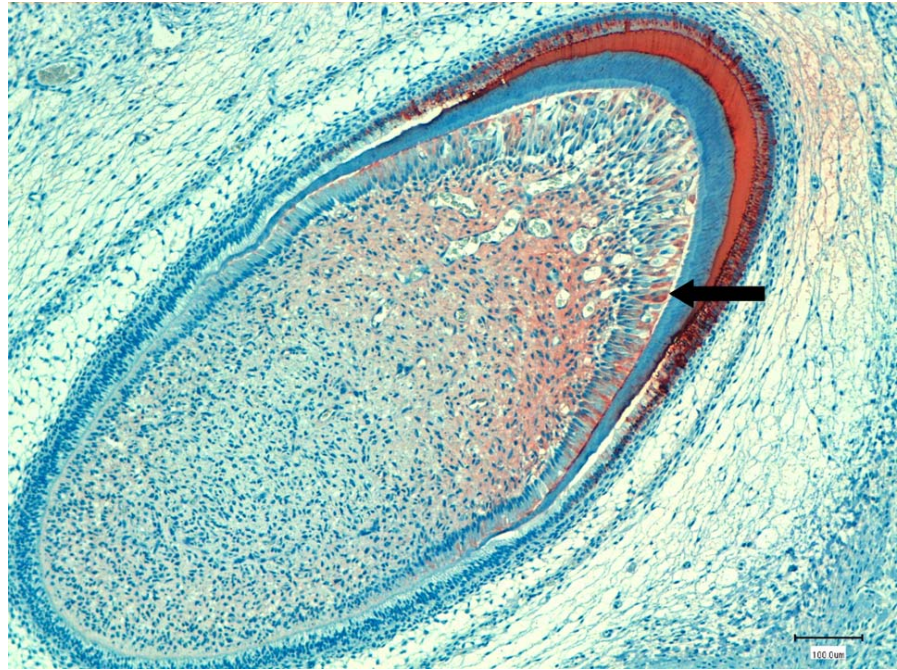
Fig. 4.5: *Focal staining of amelogenin in some odontoblasts (arrow) (scale bar 100μm, original magnification 100x).*
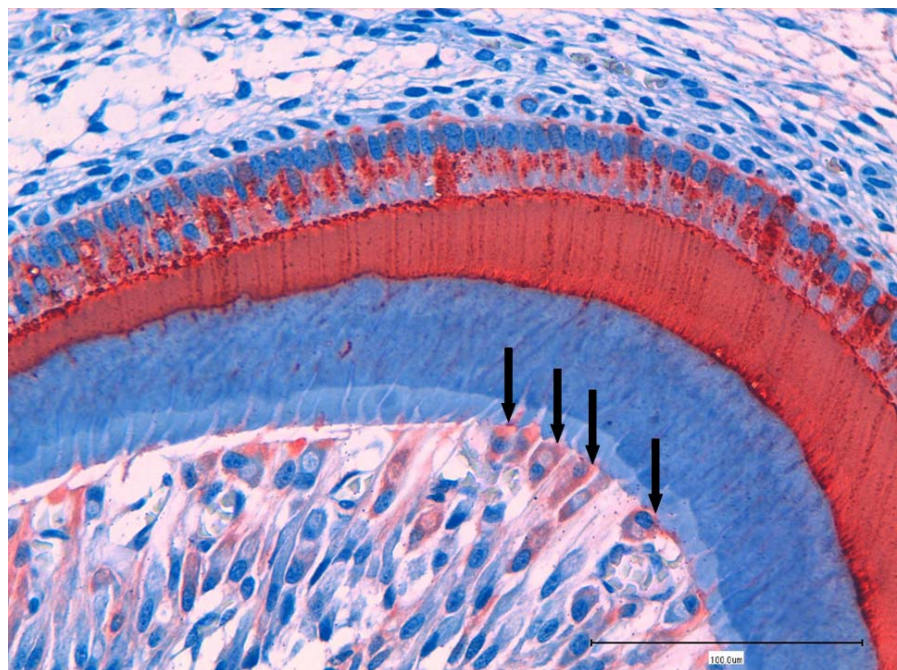
Fig. 4.6: *Higher magnification of some odontoblasts that stained positive for amelogenin (arrows) (scale bar 100μm, original magnification 400x).*

4.4 Discussion

The staining pattern observed in the current study supports previous studies as expression of the amelogenin protein was observed throughout differentiated ameloblasts in the secretory stage [138, 139, 141]. As only small amounts of amelogenin protein was detected in most presecretory ameloblasts evaluated in the current study, it supports the possibility that amelogenin mRNA is mainly found in presecretory ameloblasts before the onset of biomineralization [138, 139].

The odontoblasts revealed an intriguing staining pattern in the current study as only single cell positivity was observed in 6 sections. The reddish-brown stain appeared to be localized to the intercellular spaces and it therefore supports the hypothesis that amelogenin diffuse or translocate into the predentin and odontoblast layer and that the odontoblasts endocytose and digest the amelogenin protein [113, 142]. It is also possible that the single cell positivity observed in odontoblasts of this study was artifactual. The amelogenin protein that is in fact located outside the cell could have been misinterpreted as intracellular staining of amelogenin due to superimposition. As it was previously reported that expression of amelogenin is limited to young odontoblasts associated with early mantle dentin formation, it was proposed that a development-dependant pattern of expression exists for amelogenin [143]. The current study supports this proposal as no staining was observed in odontoblasts at advanced stages of odontogenesis and the single cells that did seem to stain were associated with the mantle dentin.

During epithelial-ectomesenchymal interaction, transient expression of several dental hard tissue matrix proteins by the opposing cell type occurs to mediate terminal cytodifferentiation of odontogenic cells [4, 143]. Previously dentin sialophosphoprotein, and ameloblastin were recognized in this phenomenon, but amelogenin has been proposed to be part of this group of matrix proteins [132, 143]. It has been suggested that the highly restricted temporal expression pattern of amelogenin in odontoblasts and ameloblasts may dictate matrix deposition, mineralization and maturation [143]. This proposal seems to be plausible, but if amelogenin was expressed in odontoblasts due to the process of

epithelium-ectomesenchymal interaction one would expect to find more diffuse expression of amelogenin in the participating cells and not positivity of single odontoblasts in only 20% of the evaluated sections.

There is also the slight possibility that the amelogenin proteins in odontoblasts were bound to some other molecule and were therefore not detectable. It has, for example, previously been proposed that amelogenin have a binding affinity for keratin [168]. It was therefore proposed that intracellular amelogenin-keratin binding may be functionally involved in inhibiting amelogenin assembly and that the reason for such interaction could be to facilitate amelogenin secretion [168].

4.5   Conclusion

The study on amelogenins is complex as the amelogenin gene is located on the X and Y chromosomes of some species and only on the X chromosome of others [132-135]. This complexity is exacerbated by the generation of various isoforms through alternative mRNA splicing and post-translational protein modifications [121, 149, 169, 170]. It has also been found in mouse models that the splicing pattern could change during odontogenesis [121]. Therefore, various factors should be kept in mind when studying this protein.

Amelogenin always stained secretory ameloblasts diffusely but due to the positive amelogenin staining properties previously described in odontoblasts [36, 144], cementoblasts, periodontal ligament cells [145], osteoblasts, osteoclasts, osteocytes and chondrocytes [146] the marker is not specific for ameloblasts only. Amelogenin can however be regarded a marker for odontogenic epithelium as no staining was observed in any other epithelial cells.

CHAPTER 5

p75 NEUROTROPHIN RECEPTOR (p75NTR)

5.1   Introduction

A complex interplay between diffusible molecules and their cell surface receptors are responsible for the development and maintenance of the nervous system [171]. The neurotrophins are the best characterized mammalian neuronal differentiation factors [171] and are comprised of four proteins: nerve growth factor (NGF), the prototype of the neurotrophins, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) [172-174]. The diverse biological functions of the neurotrophins are mediated by signal transduction systems. This is initiated through interactions with cell surface receptors [171, 175]. In 1985 it was observed that two classes of nerve growth factor receptors (NGFRs) exists, originally described as high and low affinity receptors [175]. It includes the tropomyosin-related kinase (Trk) tyrosine kinase receptors (high affinity receptor) and the p75 neurotrophin receptor (p75NTR) (low affinity) [171].

p75NTR was cloned and referred to as the low affinity NGF receptor (LNGFR) [176, 177], but as the neurotrophin family expanded it was determined that p75NTR bound all of the neurotrophins with approximately equal affinity in most cells [178, 179] and therefore it was proposed that this receptor be designated low-affinity neurotrophin receptor (LANR) [178]. In embryonic chick sympathetic neurons NT-3 bound to p75NTR with high affinity [180] and it was shown that the binding affinity of this "low affinity" neurotrophin receptor was actually similar to that of the Trk receptors [181]. Therefore this receptor cannot be described as a low affinity receptor in general. Various names have been designated to this receptor including Tumour Necrosis Factor Receptor – II (TNF-R II) [182], but for the purpose of this study it will be referred to as p75 neurotrophin receptor (p75NTR).

Structure of p75NTR

p75NTR is a member of the Tumour Necrosis Factor Receptor (TNFR) superfamily. All members of the TNFR superfamily contain structurally related cysteine-rich modules in their extra-cellular domains [171, 182] and p75NTR conforms to this criteria as its extra-cellular domain contains four repeated modules of six cysteines [183, 184]. Another feature in keeping with the TNFR family is the intra cellular death domain module, being the most prominent intracellular feature of p75NTR [171, 185].

Although p75NTR was the first identified member of the TNFR family, it is an unusual or unique member due to several factors [171, 182, 185]. p75NTR binds the homodimer NGF, while other death receptor family members bind trimeric ligands [185]. p75NTR has the ability to act as a tyrosine kinase co-receptor and it has the propensity to dimerize rather than trimerize [171, 185]. It has also been proposed that binding of dimeric NGF to p75NTR inhibits dimerization of the receptor [185].

The p75NTR receptor has been described to consist of three domains: an extracellular domain that binds to neurotrophins, a transmembrane domain and an intracellular domain that contains the "death domain" [185]. It has been described that p75NTR isoforms are produced by alternative splicing and proteolysis [171]. Alternative splicing of the p75NTR gene has been proposed to generate a p75NTR isoform that is incapable of neurotrophin binding [186], while a mettaloproteinase-like activity has been proposed to cleave the full length receptor to generate a soluble extracellular domain capable of neurotrophin binding [171, 187-189]. The functions of the post-synthetic truncations and modifications of p75NTR has not been elucidated and it therefore remains a subject of research and controversy [171, 181, 185].

Precursors of neurotrophins are initially synthesized as pro-neurotrophins, and are cleaved to produce the mature proteins [184, 190, 191]. The proform of NGF (pro-NGF) is a high affinity ligand for p75NTR, whereas the proteolytically cleaved mature NGF is the preferred ligand for TrkA [192, 193]. This has led to the speculation that pro-NGF could be the important ligand for p75NTR and not

NGF [185]. p75NTR may therefore have a mechanism for ligand discrimination as already proposed in 1995 and may therefore selectively modulate the biological actions of specific members of the neurotrophin families [172].

There is evidence that complexes form between the p75NTR and Trk receptors, but they do not bind directly to each other [194, 195]. It could be as a result of these interactions that p75NTR influences the conformations of Trk receptors and that it therefore modifies the specificity and affinity of the receptor for ligand-binding [196, 197]. It has been proposed that for neurotrophin signalling to take place through p75NTR, disassembly of p75NTR dimers may take place and assembly of asymmetric 2:1 neurotrhophin/p75NTR complexes [198]. These complexes could potentially engage Trk receptors to form trimolecular signalling complexes [198]

It has been proposed that p75NTR can be influenced by co-receptors like NogoR (NgR) and Lingo-1, which cooperate to prevent p75NTR activiation, or sortilin, which shares the pro-neurotrophin ligands with p75NTR and directs its activity towards apoptosis [199-201]. These co-receptors have been implicated as fundamental in the functionality of p75NTR, but more recently it was proposed that they should be considered as auxiliary factors of p75NTR, enhancing or inhibiting its activity [202]

<u>Functions</u>

Growth factor receptors can be seen as intermediate factors between intracellular responses and adaptations to extracellular events [185]. It has been postulated that p75NTR could serve as a neuronal receptor for the rabies virus [203], but although the *in vitro* evidence is convincing, there is still a lack of *in vivo* results to confirm the postulate [204].

One of the most prominent biological functions of p75NTR is that it can induce cell death as it contains a death domain sequence [205, 206]. The first direct evidence of p75NTR–mediated apoptosis was reported in 1993 as it was shown that over expression of p75NTR facilitated apoptosis when unbound, but binding with NGF inhibited cell death induced by p75NTR [207]. It was however later on indicated that the apoptotic actions of p75NTR were also ligand-mediated and

therefore suggested that ligand binding does not abolish its ability to mediate apoptosis [208]. It has also been proposed that Trk activation silences p75NTR apoptotic signalling as p75NTR only mediated apoptosis when Trk was suboptimally activated or inactive [208]. It was subsequently realized that p75NTR can act as a signal transducer to either induce or prevent apoptosis [209]. For example it has been shown that Schwann cells can exhibit p75NTR-dependant apoptosis [210], but that under other circumstances it can be influenced by p75NTR-mediated survival signals [211]. Pro-NGF has been shown to be the pathophysiological ligand responsible for cell-death program activation through p75NTR signaling after brain injury [212]. It has therefore been proposed that interference in the binding of pro-NGF with p75NTR may provide a therapeutic approach for the treatment of disorders involving neuronal loss [212]. The p75NTR-dependant signalling pathways for apoptotic response are however not completely understood and a question that still needs to be answered is how p75NTR choose when to induce or inhibit apoptosis [213]. It has been proposed that p75NTR can promote life in collaboration with Trks and mature neurotrophins but that it can induce cell death when it engages pro-NGF and Sortilin (the authentic pro-NGF receptor) [213]. There is spontaneous association between Sortilin and p75NTR as pro-NGF simultaneously binds p75NTR and Sortilin [200].

p75NTR expression has been observed in tissue that correlates with sites of dense neural innervation [214]. However, a consistent correlation between the p75NTR expression and the degree of innervation could not always be detected and it was therefore proposed that the expression of p75NTR in non-neuronal tissue only modulates the innervation patterns thereof [215, 216]. In neural tissue p75NTR has been proposed to be a key player in the regulation of neuronal growth, but the precise signalling mechanisms has not been elucidated [181, 217, 218]. Ceramide could play a role in this process as it has been implicated to act as a second messenger to induce apoptotic or trophic effects of p75NTR [202].

The variable functional aspects of p75NTR could derive from the finding that these transmembrane proteins are able to exert functions other than those initiated by their cognate ligands [185]. In this respect it is becoming clear that

some of the functions of p75NTR do not require neurotrophin binding or the presence of the entire receptor [185]. One example is that p75NTR has been shown to play a role in the mediation of cellular response to oxidative stress [219] and it was suggested that this role may not require the extracellular domain of p75NTR or binding of neurotrophins [219].

p75NTR expression in odontogenic tissue

In 1990 it was reported that in bud stage rat molars, cells of the dental lamina, epithelial cells at the tip of the growing tooth bud, as well as associated mesenchymal cells stained positive for p75NTR [220]. In contrast, it was later described that p75NTR does not stain the dental epithelium of bud stage rat molars, but faint staining was observed in the condensed mesenchyme [221].

In the cap stage intense staining has been observed in the IEE of rat molars and less intense staining of the OEE and stellate reticulum cells [220]. Later on, cap stage rat molars also revealed p75NTR staining in cells of the IEE with added staining of the stratum intermedium, dental papilla and dental follicle [214, 221].

In the bell stage staining has been observed in proliferating cells of the IEE [214, 220, 222], some cells of the stratum intermedium and in pre-odontoblasts/polarizing odontoblasts [214, 220, 221]. Absent staining has been observed in pre-ameloblasts before terminal differentiation into ameloblasts [220, 221], outer enamel epithelium, cervical loop cells, Hertwig's epithelial root sheath (HERS) cells, cementoblasts [220] and functional odontoblasts [214]. The expression of p75NTR has been described to be lost progressively as the cells of the IEE became post-mitotic, polarized and differentiated into ameloblasts [214].

More recent studies revealed immunoreactivity against p75NTR to be restricted to the IEE, the dental papilla and the dental follicle of rat incisors [35, 36] and therefore p75NTR has been used as a marker for IEE cells [35, 36].

In adult rat tissue the ectomesenchymal cells of the pulp revealed strong p75NTR immunoreactivity that became concentrated in the sub-odontoblastic regions of

the crown [220]. These findings were supported by others as the cells underlying the functional odontoblasts (sub-odontoblastic layer cells) stained positively for p75NTR [214, 221] and the nerve fibers of the sub-odontic plexus also revealed positive staining for p75NTR [214]

One study has also reported the presence of p75NTR in the basal epithelial cells of the junctional epithelium and in adjacent sulcular epithelium as it has been proposed to be a marker for intraepithelial nerve fibres and their associated epithelial cells in adult rats [223, 224].

General expression of p75NTR

p75NTR is expressed in cells derived from all three germinal layers in very early stages of embryogenesis [225], but it becomes progressively restricted to specific cell populations as development proceeds [213]. Neural crest cells express p75NTR as they migrate and levels increase as these cells undergo neuronal differentiation [171]. p75NTR has been found in the central as well as peripheral nervous systems [171]. Postnatally, low levels of p75NTR has been described in motor neurons [226], while others proposed that motor neurons do not normally express p75NTR [172]. In adult tissue p75NTR expression has been observed in sensory and sympathetic neurons as well as in cell bodies of some parasympathetic and enteric neurons [227].

Marked increase in p75NTR has however been detected in certain conditions including stroke, epileptic seizures, focal ischemia, axotomy, Alzheimer's disease and mechanical injury [228-232]. As p75NTR expression is lost in various post natal tissues the re-expression thereof has been proposed to enhance the action of trophic factors, including NGF [229], assist in neuronal outgrowth [230] and resist neuronal cell death [228]. Others however suggested that this up-regulated expression pattern mediates cell death (apoptosis) [228, 233, 234]. It therefore seems that the p75NTR expression is secondary to the above mentioned insults and that a complex mechanism could be responsible for the differing results that the up-regulated receptor could induce.

Interestingly, enriched p75NTR expression has been found at a number of epithelial/mesenchymal boundaries. p75NTR is abundant in mesenchyme associated with developing organs and structures (teeth, hair follicles, salivary glands, kidneys, testis, muscles, maxillary pad and lungs) [215, 216, 220, 222, 235-238].

Gene mutation

It has been shown that gene mutation of the p75NTR in mice leads to a loss of sensory neurons and a lack of sympathetic innervation of the pineal gland and in a subset of sweat glands in the footpad [239, 240]. The lack of a major effect as the result of p75NTR knockout has been attributed to the possible compensation of other members of the receptor family or the action of other neurotrophic receptor molecules like TrkA [221].

5.2   Materials and Methods

Foetuses of large breed dogs were obtained, under ethical clearance of the Animal Use and Care Committee of the Faculty of Veterinary Sciences, University of Pretoria, South Africa, from female dogs scheduled for elective termination of pregnancy. Mixed breed dog foetuses were used in the study (*Canis familiaris*). Twenty four foetuses were fixed in 10% buffered formalin and then the heads were carefully cut into coronal sections (rostral to caudal), dehydrated and embedded in paraffin wax blocks. Those tissue samples that contained calcified bone or dental hard tissues were decalcified in routine decalcifying solution (HNO_3 70ml, HCL 50ml, Distilled water 880ml) for 60 minutes and rinsed in running tap water for 60 minutes in order to make sectioning possible without tearing and disruption of normal anatomy. The tissue blocks were then processed overnight.

Tissue specimens were sectioned at 3µm, stained with haematoxylin and eosin (H&E) and microscopically examined by two individuals to select slides with well-formed cap or preferably bell stage enamel organs in which the respective odontogenic epithelial cells were morphologically clearly identifiable.

Sections were de-paraffinized in two changes of fresh xylene for 10 minutes each, re-hydrated with 2 changes of absolute ethanol, followed by 90, 70 and 50% graded alcohol solutions respectively. The sections were then washed in 6 changes of fresh distilled water. Heat induced epitope retrieval in citric acid buffer; pH 6.00, was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 min. The sections were washed in 5 changes of fresh distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 6 min at 37ºC where after the sections were rinsed in 6 changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS buffer, Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for 10 min at room temperature. The sections were incubated in gp75 Nerve Growth Factor Receptor (1:50) anti-serum (Novocastra NCL-NGFR Novocastra Laboratories Ltd., Balliol Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW United Kingdom) for 60 minutes at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The sections were then incubated in Dako Envision+R System Labelled Polymer, anti mouse, HRP (Dako K4001, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 30 min at room temperature and rinsed in 3 changes of fresh PBS for 5 minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA) for 4 min at 37ºC, washed in distilled water and counterstained in Haematoxylin for 1 minute at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025, Dako Cytomation Inc. 6392 Via Real, Carpinteria, California 93013 USA).

Reddish-brown granular cytoplasmic staining was interpreted as positive for the presence of p75NTR. p75NTR expression has been described in peripheral neural bundles and it was therefore used as positive internal control throughout the evaluated sections of our study (Fig. 5.1) [171, 222].
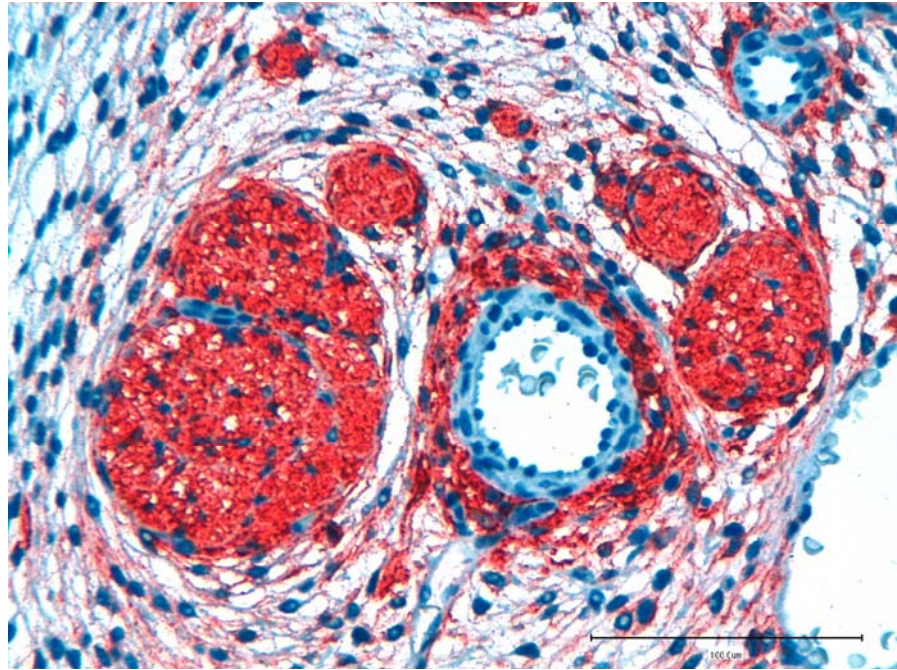
Fig. 5.1: *Intense positive staining for p75NTR in peripheral nerve bundles and blood vessel adventitia, most probably representing nerve fibres innervating the blood vessel walls (scale bar 100µm, original magnification 400x).*

Staining in each case was noted as follows:

- S – Single
  - One or two cells in a specific field of cells stained positive
- F – Focal
  - Groups of cells that revealed positive staining, being interrupted by cells that did not stain
- D – Diffuse
  - General staining of the cells involved (90% or more of the cell type stained)
- + Weak staining
- ++ Intense staining

Because of the *in situ* nature of the study, we distinguished between the different cell types of odontogenesis on the basis of their microscopic morphology and location in the developing tooth germ and adjacent structures. For the purpose of this study the IEE cells were defined as short columnar cells on a basal

membrane with centrally placed nuclei, opposite undifferentiated ectomesenchymal cells of the dental papilla without any signs of odontoblast differentiation or hard tissue formation. Presecretory ameloblasts were defined as tall columnar cells with reversed polarity (nuclei aligned adjacent to the stratum intermedium), but without the presence of enamel product adjacent to these cells. Secretory ameloblasts were defined as tall columnar cells, with prominent reversed polarity and located adjacent to formed enamel and dentin. The cells of the outer enamel epithelium were defined as low cuboidal cells with little cytoplasm at the periphery of the enamel organ. Enlarged ectomesenchymal cells of the dental pulp or papilla, directly adjacent to the IEE, where predentin was not visible, were referred to as undifferentiated odontoblasts or pre-odontoblasts, while elongated, polarized ectomesenchymal cells of the dental pulp adjacent to formed dentin were defined as odontoblasts. The oral epithelium on the alveolar ridge area from where the tooth germ originated, was referred to as the overlying alveolar epithelium. The term dental lamina was used when continuity of the lamina could be observed between the overlying oral epithelium and the developing tooth germ. When only discrete islands of epithelial cells were evident it was termed Serres rests.

5.3   Results

As the exact time of gestation of the developing dog foetuses were unknown, the foetuses were classified into 2 groups based on the odontogenic development stage. Foetuses 1-8, designated as group 1, only had tooth germs in the bud and cap stages of development with no ameloblast differentiation or dental hard tissue formation. The tooth germs of foetuses 9-24, designated as group 2, were already in the bell stage of development with visible cell differentiation and hard tissue formation. Many presented with more than one section that conformed to the requirements of the inclusion criteria and in 15 cases a rostral (anterior) and a caudal (posterior) section of the same foetus were used for comparison. In the remaining 9 cases, only one section was included in the study. A total of 39 sections were therefore harvested from the 24 foetuses for immunohistochemical investigation of p75NTR expression. The number of tooth germs on a single section varied from 1 to 4 and as the whole section was stained with the antibody there were sometimes more than one tooth germ that could be used in the

evaluation process of some sections. Only one tooth germ defined as the best representation of the bell or cap stage in the given section was chosen for analysis of the immunohistochemical stain. No discrepancies were however found between amelogenin stains of the tooth germs on the same section. No apparent general differences were observed when the staining pattern for p75NTR in group 1 foetuses was compared to the group 2 foetuses.

Table 5.1 lists the results found for p75NTR staining in dog odontogenic tissue.

p75NTR in epithelial cells

In bud stage dental organs no staining of the epithelium was observed (Fig. 5.2). Focal positivity for p75NTR was observed in cap stage IEE (Fig. 5.3 and 5.4) as well as in some bell stage tooth organs as the IEE in the region of the cervical loop stained positive for p75NTR (Fig. 5.5 and 5.6). No staining was observed in OEE, stellate reticulum, stratum intermedium, differentiated ameloblasts, dental lamina, Serres rests or overlying alveolar epithelium (Fig. 5.3 and 5.5).

p75NTR in mesenchymal tissue

In most cases some pre-odontoblasts stained positive for p75NTR (Fig. 5.5 and 5.7). As soon as the odontoblasts differentiated, these cells were devoid of p75NTR staining. Faint positivity was observed in the pulp during the cap stage (Fig. 5.3) and during the bell stage staining was restricted to the apical region of the pulp (Fig. 5.8). The dental follicle cells stained diffusely positive throughout the evaluated sections (Fig. 5.9).

Interesting observations

Positive staining for p75NTR was observed in developing muscles of the face, tongue (Fig. 5.10) and blood vessels (tunica media) (Fig. 5.1). The tunica adventitia of blood vessels also revealed positivity, probably as a result of immunoreactivity of nerve bundles to p75NTR (Fig. 5.1).

Table 5.1 – *p75NTR expression in dog odontogenic tissue.*

| Foetus nr.	AMB	IEE	OEE	ODB	Pre-ODB	Papilla / Pulp	Lamina / Serres Rests	Alv. Epith.	Dental Follicle
NGFR p75									
1	NA	F+	-	NA	NA	F+	-	-	D++
2	NA	S+	-	NA	NA	-	-	-	D++
3	NA	S+	-	NA	NA	-	-	-	D++
4	NA	S+	-	NA	NA	S+	-	-	D++
5	NA	NA	NA	NA	NA	NA	-	-	F+
6	NA	NA	NA	NA	NA	NA	-	-	F+
7	NA	F+	-	NA	NA	F+	-	-	D++
8	NA	NA	-	NA	NA	NA	-	-	D++
9a	-	-	-	-	D++	D+	-	-	D++
9b	-	F++	-	-	D++	D++	-	-	D++
10a	-	F++	-	-	D+	D++	-	-	D++
10b	-	F+	-	-	D+	D+	-	-	D++
11a	-	-	-	-	F+	D+	-	-	D++
11b	-	-	-	-	F+	F+	-	-	D+
12	-	-	-	-	S+	F+	-	-	D+
13a	-	-	-	-	D+	F+	-	-	D++
13b	-	-	-	-	F+	D+	-	-	D++
14a	-	-	-	-	F+	F+	-	-	D++
14b	-	F++	-	-	F+	F+	-	-	D++
15a	-	D+	-	-	D+	D+	-	-	D++
15b	-	-	-	-	D+	F+	-	-	D++
16a	-	-	-	-	F+	F+	-	-	D++
16b	NA	F+	-	NA	F+	F+	-	-	D++
17a	NA	-	-	-	F++	F+	-	-	D++
17b	-	F++	-	-	F++	F+	-	-	D++
18a	-	-	-	-	S+	S+	-	-	D+
18b	-	F+	-	-	S+	F+	-	-	D+
19a	-	-	-	-	NA	-	-	-	D++
19b	-	-	-	-	F+	F+	-	-	D++
20a	NA	N	-	NA	NA	NA	-	-	D++
20b	-	-	-	-	F+	-	-	-	D++
21a	-	-	-	-	-	-	-	-	D+
21b	-	-	-	-	-	-	-	-	D+
22a	NA	NA	-	NA	NA	NA	-	-	D++
22b	NA	-	-	NA	-	-	-	-	D+
23a	-	-	-	-	-	-	-	-	D+
23b	-	F+	-	-	-	-	-	-	D+
24a	NA	NA	NA	NA	NA	NA	-	-	NA
24b	-	-	-	-	F+	F+	-	-	D++

AMB – Ameloblasts, **IEE** – Inner Enamel Epithelium, **OEE** – Outer Enamel Epithelium, **ODB** – Odontoblasts, **Stel. Ret.** – Stellate Reticulum, **Strat. Interm.** – Stratum Intermedium, **Alv. Epith.** – Alveolar Epithelium, **NA** – Not present in section evaluated, **-** No positive staining observed, **S** – Single, **F** – Focal, **D** – Diffuse, **+** Weak positive, **++** Intense positive, **a** – Anterior section and **b** – Posterior section.
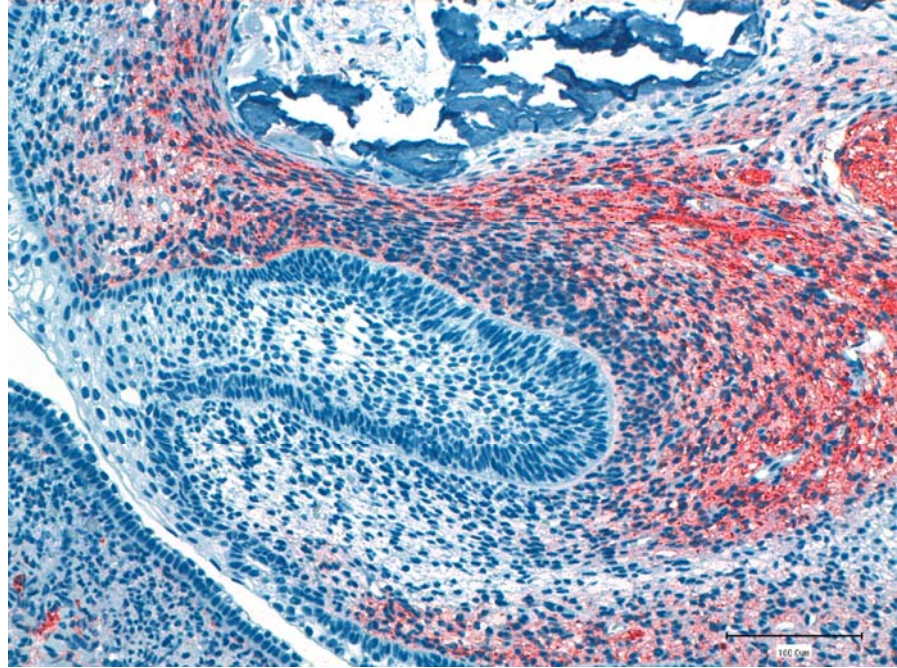
Fig. 5.2: *Positive staining for p75NTR in the ectomesenchyme associated with the developing tooth bud with no staining of the odontogenic epithelium (scale bar 100μm, original magnification 200x).*
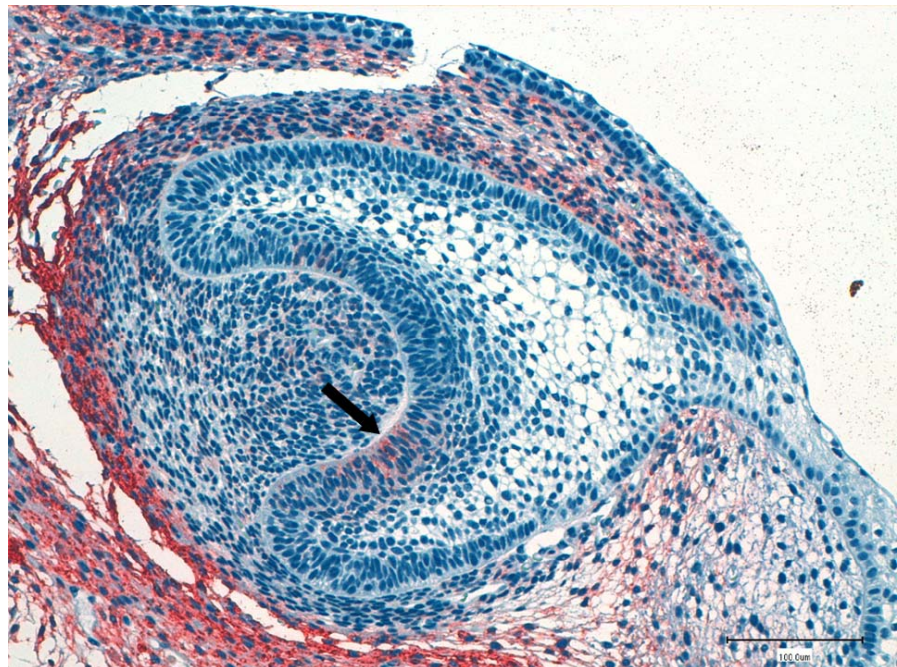
Fig. 5.3: *Focal positivity for p75NTR in the IEE of a cap stage tooth germ (arrow) (scale bar 100μm, original magnification 200x).*
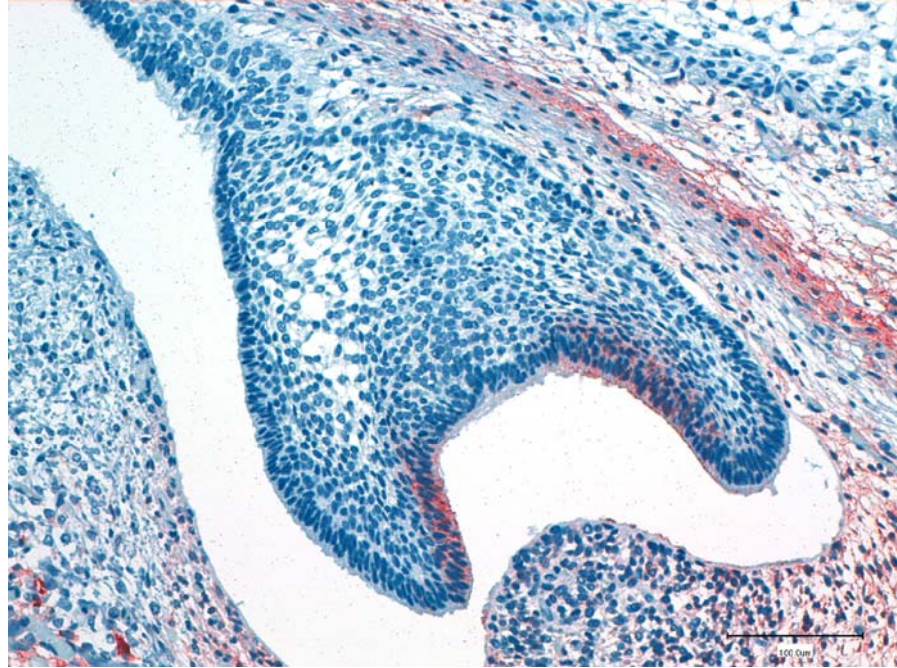
Fig. 5.4: *p75NTR expression in the lateral aspects of the cap stage IEE (scale bar 100µm, original magnification 200x).*
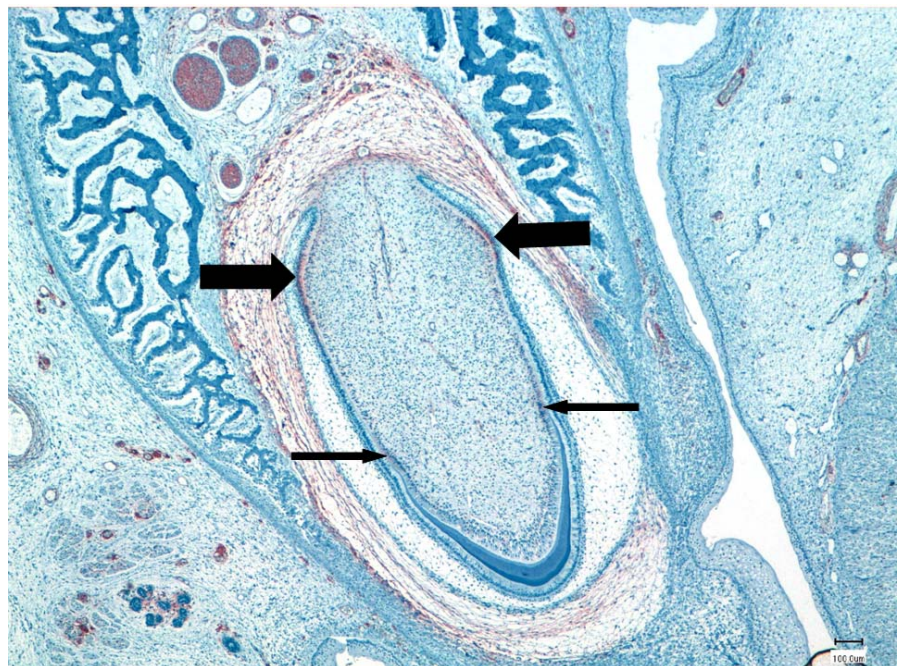
Fig. 5.5: *p75NTR expression in a bell stage tooth indicating the positive staining observed in the IEE of the cervical loop region (thick arrows) as well as differentiating odontoblasts (thin arrows) (scale bar 100µm, original magnification 40x).*
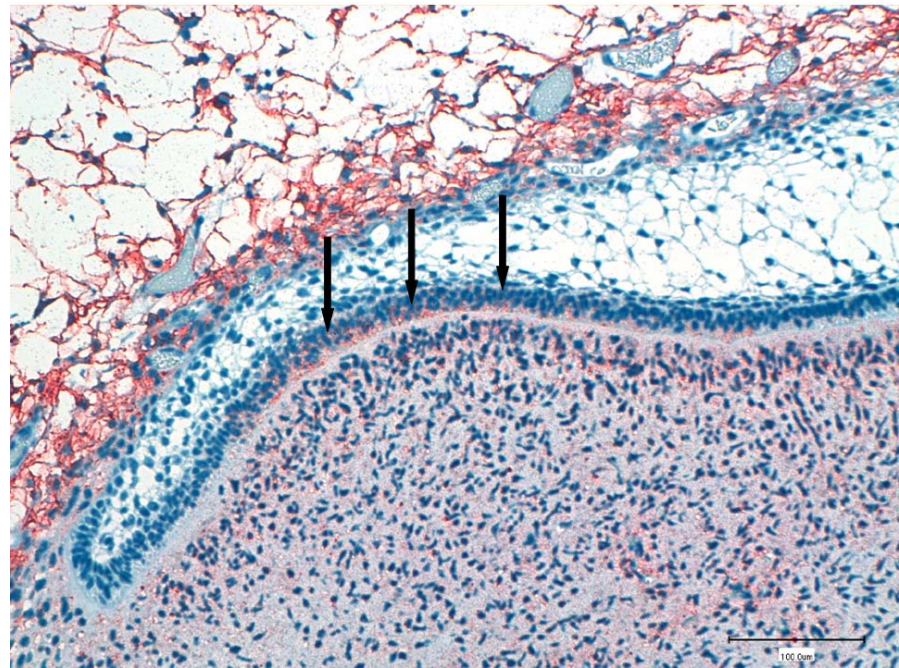
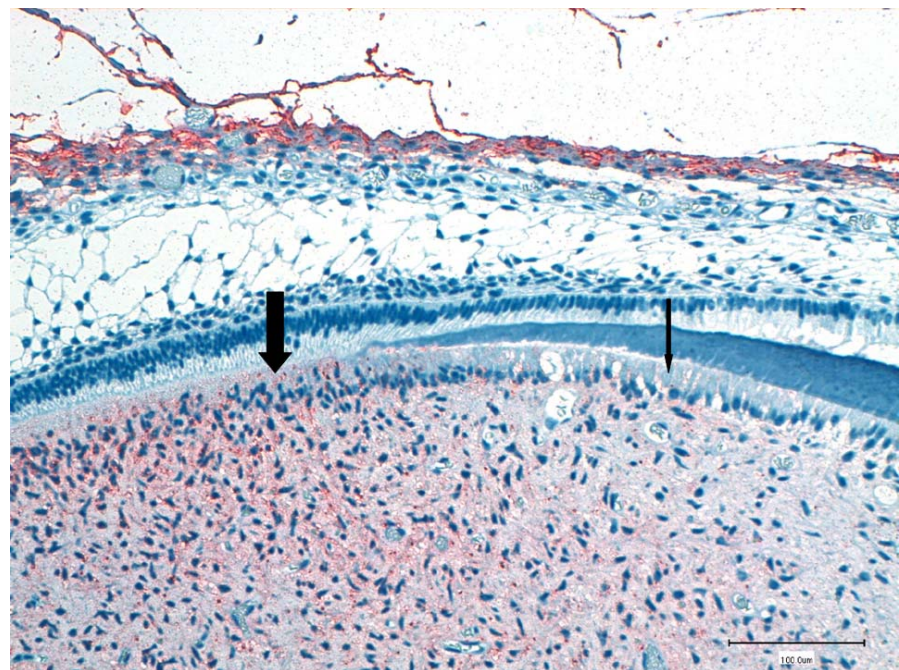**Fig. 5.6:** *Cervical loop IEE cells that revealed positive staining for p75NTR (arrows) (scale bar 100µm, original magnification 200x).*

Fig. 5.7: *Positive staining of differentiating odontoblasts (thick arrow), as opposed to no staining observed in the functional odontoblasts associated with dentin (thin arrow) (scale bar 100µm, original magnification 200x).*
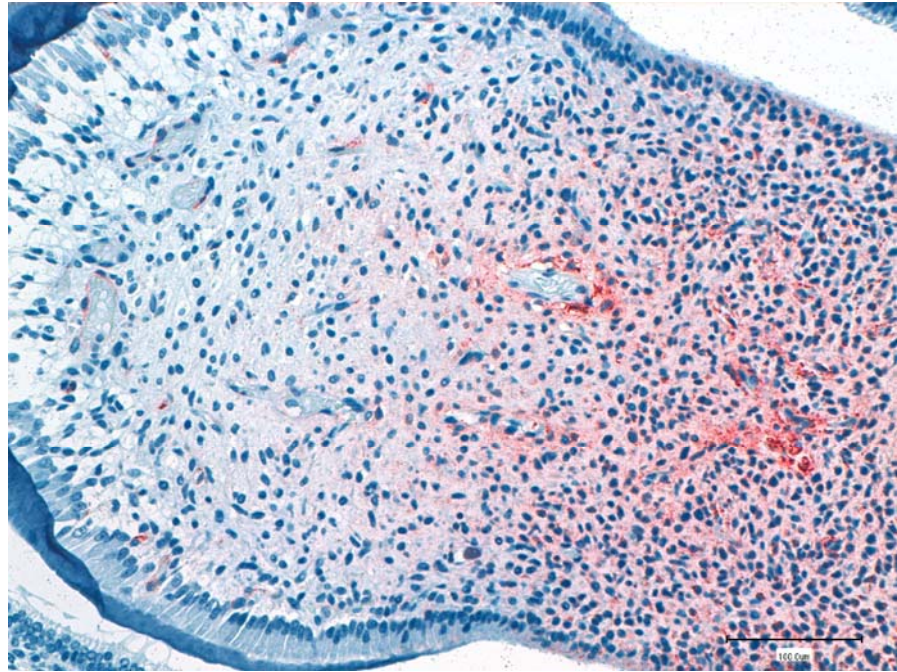
Fig. 5.8: *A section of a group 2 foetus revealing a polarized staining pattern towards the apical end of the developing pulp for p75NTR (scale bar 100µm, original magnification 200x).*
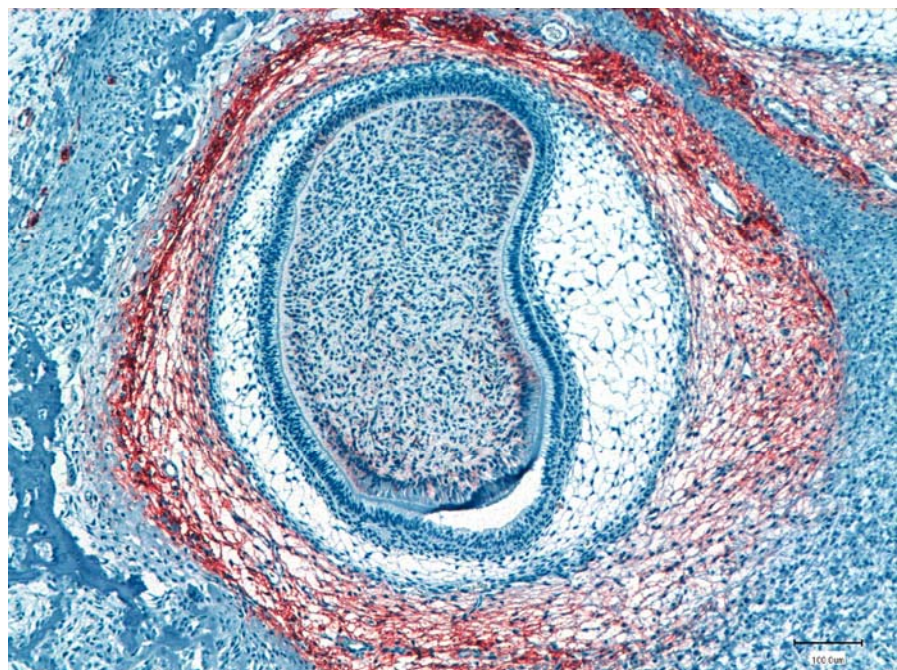
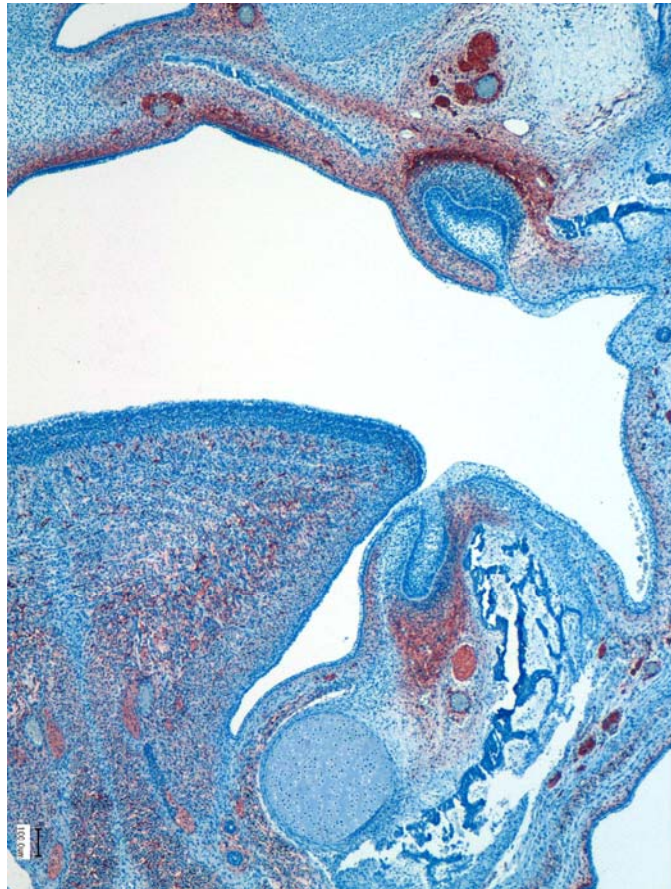**Fig. 5.9:** *Positive staining for p75NTR was observed in the dental follicle tissue throughout the evaluated sections (scale bar 100µm, original magnification 100x).*

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Fig 5.10:

p75NTR staining observed in the tongue muscle and ecto-mesenchyme associated with tooth development in a group 1 foetus (original magnification 40x).

5.4 Discussion

p75NTR expression in development

The results of this study support previous reports that indicated that p75NTR is widely expressed during embryological development as p75NTR expression was observed in forming muscle tissue of the face, tongue and blood vessels [216, 238]. The observation that p75NTR is expressed in non-neuronal tissue like teeth and cranial muscle has been proposed to be the result of these tissues containing cellular components derived from neural crest origin and that these tissues may retain the ability to produce p75NTR even after differentiating into non-neural tissues [222]. The diffuse staining that was observed in dog embryonic tissue examined in this study also supports the proposal that the

distribution of p75NTR at various embryonic stages could account for developmental survival decisions [185].

<u>p75NTR expression in epithelial cells</u>

In agreement with the study done by Mitsiadis and Luuko (1995), no staining for p75NTR was detected in epithelial cells of bud stage dental organs in the current study, but evident staining was observed in the adjacent dental mesenchyme [221]. This is in contrast to the study done in 1990 by Byers and co-workers who reported positive staining of the epithelial cells at the tip of the growing tooth bud of rat molars [220]. Although the study by Byers *et al.* and Mitsiadis and Luuko were done on rat molars with the monoclonal antibody designated 192-IgG developed by Chandler *et al*. in 1984 [220, 221, 241, 242], different results were found. As most of the studies on p75NTR expression in odontogenic tissue differed form the results found by Byers and colleagues, it is possible that improved techniques resulted in more specificity towards the p75NTR antigen in more recent studies. When the current study is compared to the one of Byers *et al.* various factors could have resulted in the different staining patterns observed, as the method of fixation, decalcification and antigen retrieval differed. The use of frozen sections as opposed to formalin fixed paraffin embedded tissue and different detection kits that were used in the respective studies could also have influenced the results found.

The results from this study are in agreement with previous studies as focal positivity for p75NTR was observed in cap stage IEE [35, 36, 214, 220, 221]. Primary enamel knots can be detected histologically during the cap stage within the internal epithelium [5], but as the plane of sectioning did not always permit clear visualization of a prominent enamel knot one can merely speculate on the presence thereof in most cases. The staining pattern for p75NTR was generally restricted to the IEE at the lateral aspects of the presumed primary enamel knot (Fig. 5.3 and 5.4). The enamel knot consists of non-dividing epithelial cells located in a region where active proliferation takes place (the IEE) [4, 5]. This may explain the basic mechanism of folding of the enamel organ in the early stages of odontogenesis as the enamel knot has been implicated as an organizational centre for cuspul morphogenesis [4, 5]. The current study

therefore support the proposal that p75NTR expression in the IEE is associated with active proliferating cells as no staining was observed in the enamel knot cells (Fig. 5.4) [214]. The positive staining for p75NTR that was observed in the cervical loop IEE of some sections of the current study (Fig. 5.6) also contributes to this postulate as it also represents a region of rapid proliferating cells. Similar staining patterns were observed in the cervical loop region in 1993 by Mitsiadis *et al.* [214].

Although p75NTR expression has been observed in OEE, stratum intermedium and stellate reticulum cells in rat teeth [214, 220, 221], the current study and the ones done by Kawano *et al.* (2004) and Morotomi *et al.* (2005) done on rat tissue, revealed no such staining [35, 36]. This could also have resulted from the various factors mentioned above.

To the best of the author's knowledge, p75NTR expression has not ever been described in differentiated ameloblasts and the current study supports this finding [35, 36, 214, 220, 221].

<u>p75NTR expression in mesenchymal tissue</u>

The current study confirmed previous findings as pre-odontoblasts stained positive for p75NTR in most cases but as soon as the odontoblasts differentiated and dentinogenesis began, single cell or focal staining was only observed in the sub-odontoblastic layer and not in the odontoblasts themselves [214, 220]. It was proposed that expression of p75NTR in the pre-odontoblasts correlates with the onset of odontoblast differentiation [221] and the results of this study support the conclusion that functional odontoblasts never revealed p75NTR staining [35, 36, 214, 220, 221].

Although various authors reported p75NTR staining of the pulp, it has not been confirmed what aspects of the pulp specifically results in the positive staining that was observed [35, 36, 214, 221]. This may be because the staining pattern for p75NTR, as observed in the current study, is very difficult to explain. In the cap stage faint staining was observed in the dental papilla (Fig. 5.3) that resembled background staining, and during the bell stage staining was mostly restricted to

the apical aspect of the developing pulp (Fig. 5.8) It has previously been proposed that neural tissue as well as non-neuronal cells in the dental pulp, such as fibroblasts, are immunoreactive for p75NTR during odontogenic morphogenesis [220]. From the results of the current study it is proposed that the observed staining in the pulp is most probably the result of p75NTR immunoreactivity of nerves and developing blood vessels. Nerve fibres of the sub-odontoblastic plexus has been shown to show positive staining for p75NTR [214] and forming blood vessels have also been described to express p75NTR in the tunica adventitia and muscle wall of the tunica media [222]. The observed staining of p75NTR in mesodermal structures like the adventitia of blood vessels [222] has also led to the proposal that a truncated form of the p75NTR circulates in the developing animal, which decreases in adulthood and that this truncated receptor is sequestered or bound to tissues like blood vessel walls [222]. From the results of the current study it is rather suggested that the positive staining observed in blood vessel walls was indeed nerve fibres in the tunica adventitia or developing muscle in the tunica media. The postulation regarding sequestered portions of p75NTR in blood vessels seems unlikely as such a mechanism would have resulted in p75NTR expression in the tunica intima and not the outer tunica adventitia.

The polarized expression of p75NTR in the bell stage pulp could be ascribed to various aspects. The diffuse staining observed in the apical region of the pulp could be regarded as background staining, but this is unlikely as it was restricted to the pulp. No background staining could be observed in any other regions of the evaluated sections. Another factor that could result in the observed staining pattern is the large arterioles found in the more apical region of the pulp, receiving a dense neural supply, while the small arterioles, in the coronal aspects, have much scarcer innervation networks [243]. As p75NTR has been proposed to stimulate axon growth and migration and thereby modulates innervation patterns [215, 216], it is possible that the diffuse expression thereof in the apical region could indicate the position of the pulp after odontogenesis has been completed, and thereby it dictates the position where future nerve bundles (such as Raschkow's plexus) should form. The vague p75NTR expression observed in the cap stage papillae could also result from p75NTR's role in signalling for neural innervation.

The dental follicle stained diffusely throughout the evaluated sections (Fig. 5.9) as also found previously [35, 36, 214, 221]. It is proposed that it could be the ectomesenchymal cells as well as neurovascular structures that stained positive for p75NTR as it has been proposed that the immunoreactivity for p75NTR in the dental follicle of rat incisors and molars correlates with sites of dense neural innervation [214] and the follicle is also well vascularized [4].

Proposed functional aspects of p75NTR

The expression pattern of p75NTR seemed to be restricted to areas of proliferating cells as it was found in the IEE but never in the ameloblasts and also in undifferentiated odontoblasts (pulp cells adjacent to the IEE), but not in the functional odontoblasts [35, 36, 214, 220, 221]. Therefore p75NTR expression in IEE and undifferentiated odontoblasts may indicate a role in the proliferation and terminal differentiation of these cells [214, 221].

An up-regulation of p75NTR expression has been described after injury of corticospinal neurons [233] and it has even been proposed that NGF can promote p75NTR re-expression in odontoblasts during pulp repair [244]. Such findings could have huge implications in future therapeutic treatment strategies, but a contradicting observation was made that, except for nerve fibres, little or no p75NTR immunoreactivity was detected in the pulp tissue after injury through drilling and etching [220]. Light was shed on this process when Woodnutt *et al*. proposed that fibroblasts in the sub-odontoblast zone of the crown normally express NGF and p75NTR and that p75NTR expression was lost in these fibroblasts after injury. As it is not possible to define a cell as a fibroblast on the basis its structure observed through a light microscope, the authors identified these cells as fibroblasts with the aid of an electron microscope. The decrease in p75NTR expression was postulated to result in an effort to avoid the apoptotic pathway and thereby enhancing pulpal healing and survival [245].

It has been proposed that NGF may be one of the diffusible factors regulating gene expression in the epithelium and ectomesenchyme during odontogenesis [220]. Therefore it is possible that p75NTR could also play some part in epithelium-mesenchymal interaction as it is one of the receptors for NGF [171,

175]. From the results of the current study we considered p75NTR in the process of epithelial-ectomesenchymal interaction, but could not find sufficient evidence to validate such a proposal. In line with previous studies, it can therefore be proposeed that apart from p75NTR's function in regulation of innervation patterns [215, 216], p75NTR could contribute to other functional aspects during development [215] as it is detected in mesenchymal cells that have instructive roles in the development of embryological systems [215]. The current study supports the former (as already described) and the latter as the odontogenic potential resides within the ectomesenchymal tissue since bud formation [3, 4] and p75NTR expression was found in the ectomesenchyme from that stage.

5.5 Conclusion

With regard to epithelial cells in the developing dog, p75NTR stained the IEE in some aspects thereof, but no other epithelial cells stained positive for p75NTR. It may therefore be regarded as a possible marker for odontogenic epithelium. Positive staining was however also observed in certain developing mesenchymal tissue such as the dental pulp and follicle cells and p75NTR does therefore not stain epithelial cells exclusively.

CHAPTER 6

CONCLUSION

Table 6.1 gives a simplified, summarized overview on the observed expression patterns of the different markers used in this study.

Table 6.1 *Expression patterns of various markers observed in developing dog teeth (odontogenic tissue).*

Expression patterns in dog odontogenic tissue					
	Calretinin	**Keratin 14**	**Keratin 19**	**Amelogenin**	**p75NTR**
IEE	-	D++	F+	-	F+
Presecretory Ameloblasts	-	-	D++	F+	-
Secretory Ameloblasts	-	D++	D++	D++	-
Dental Lamina	D++	D++	D++	-	-
Serres Rests	D++	D++	D++	-	-
Alveolar Epithelium	D++	D++	F+	-	-
Oral Epithelium	-	D++	F+		
Pre-Odontoblasts	-	-	-	-	F+
Odontoblasts	-	-	-	S+*	-

- IEE – Inner enamel epithelium
- -   No positive staining observed
- S – Single
- F – Focal
- D – Diffuse
- +   Weak positive
- ++ Intense positive
- *   Single odontoblasts stained in 20% of sections in association with mantle dentin formation
- Alveolar epithelium – epithelial cells directly overlying the developing tooth germ

The aim of the current study was to find a specific marker to identify odontogenic epithelium in developing dog teeth. For such a marker to be regard as "specific" it would need to stain the odontogenic epithelium exclusively. Therefore, keratin 14 and keratin 19 cannot be regarded as suitable markers, as staining was also observed in the various other epithelial cells. The expression of K19 in alveolar

epithelial cells of the oral epithelium diminished from diffuse full thickness staining observed in group 1 foetuses, to staining being restricted to the superficial layers of the alveolar epithelium in the group 2 foetuses. The pattern of staining for K14 and K19 appeared to change with development and it is possible that yet another profile will be seen in adult tissues.

Calretinin could be a useful marker for odontogenic epithelial cells as staining was observed in the alveolar epithelial cells (directly overlying the developing tooth germ and proposed as the oral epithelium where the dental lamina takes origin from), as well as the dental laminae and Serres rests. No staining was observed in the rest of the oral epithelium and the results of this study propose that calretinin could be a useful marker to distinguish between tumours originating form odontogenic epithelial cells as apposed to those from other epithelial origins.

Amelogenin seems to be a promising marker to distinguish between odontogenic tumours and non-odontogenic tumours as it was only detected in odontogenic tissues in this study. Although it has been observed in other tissues like dog and rat osteoblasts, osteoclasts, in some osteocytes and also in articular cartilage chondrocytes [146], the current study could not support these findings and further studies on this protein could assist in the process of elucidating the expression profile of amelogenin in odontogenic and non-odontogenic tissues.

p75NTR expression was restricted to certain regions of the IEE and no staining was observed in other epithelial cells. It therefore seems to be a promising marker to differentiate between odontogenic epithelium versus non-odontogenic epithelium, but the widespread staining observed in the mesenchymal tissue does not permit p75NTR to be a specific marker for odontogenic epithelium. The precise function of p75NTR expression in the IEE cells remains a speculative topic, but future research could elucidate the exact role thereof in odontogenesis.

Proposals for future research

Odontogenic tumours may originate from the epithelial or ectomesenchymal cells of the developing tooth germ or its remnants [246]. Most of the odontogenic

tumours are found in adult dogs and it is important to realize that all that is left of the odontogenic epithelium in adult tissue is the Serres rests and Malassez rests. Therefore it is likely that the odontogenic tumours in adult tissue would originate from remnants of odontogenic tissue namely the Serres rests in the gingiva or the Malassez rests in the periodontal ligament space [247]. We therefore propose that the expression of the same markers that were used in this study be tested in adult dog tissue, specifically the Serres rests, Malassez rests and associated gingiva.

As it was shown in this study that the expression profile of certain markers was altered in group 2 compared to group 1 foetuses in many respects, this may change even more in post-natal and adult tissue as previously indicated [55, 215, 216, 222]. Therefore, it would be possible that a marker could retain its expression in Serres- and Malassez rests, but could loose its expression in oral epithelial cells that lost its odontogenic potential. It is proposed that the oral epithelial cells would gain more mature differentiated characteristics in adult tissue as opposed to the Serres- and Malassez rests that may retain their original foetal characteristics and expression profiles, as they are quiescent remnants of foetal developmental cells. Therefore, finding a suitable marker that will stain the odontogenic rests without staining the remainder of the oral epithelial cells in adult tissue could aid in finding a marker to distinguish between tumours originating from odontogenic tissue compared to those taking origin from the adjacent oral epithelium.

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