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The relationship between cholesterol crystals, foamy macrophages and haemosiderin in odontogenic cysts

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DECLARATION

I certify that this treatise does not incorporate any material previously submitted for a Degree or Diploma in any University; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference has been made in the text.

Md. Firoz Iqbal

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SUMMARY

Odontogenic cysts often contain cholesterol clefts, foamy macrophages and haemosiderin deposits. Earlier studies demonstrating correlation between haemosiderin and cholesterol clefts in odontogenic cysts suggest that cholesterol may accumulate in these lesions from degenerate erythrocytes. The amount of cholesterol in erythrocytes, however, is quite small, so that an alternative source of cholesterol from plasma derived low density lipoprotein, similar to that described for atherosclerosis, seems more likely. While foamy macrophages have been proposed as important in the generation of cholesterol clefts in atherosclerosis, no studies have investigated the relationship between foamy macrophages and haemosiderin in odontogenic cysts, while there is also a paucity of information on the relationship between cholesterol clefts and foamy macrophages in such lesions. This thesis describes a histological survey of radicular cysts, dentigerous cysts and odontogenic keratocysts in which the occurrence of haemosiderin, cholesterol clefts and foamy macrophages was recorded and correlated. Contrary to expectations, but nonetheless consistent with the literature, the association between cholesterol clefts and haemosiderin was confirmed. Novel observations were a similar correlation between haemosiderin and foamy macorphages, as well as foamy macorphages and cholesterol clefts in odontogenic cysts. Also, this thesis describes the first spatial analysis of such deposits in odontogenic lesions. Intracellular cholesterol clefts in foamy macorphages were also described for the first time in this thesis. Although data demonstrate a relationship between haemosiderin and lipid deposits, further work is suggested to determine if this is a causal relationship.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

1.1. a. Cysts defined as epithelial lined pathological cavities

A cyst is an epithelial-lined pathological cavity containing fluid, semi-solid or gaseous contents and which is not created by the accumulation of pus (Kramer, 1974; Shear and Speight, 2007). Separate to epithelial-lined cysts, some cystic lesions do not have epithelial linings including solitary bone cysts and extravasation mucoceles, although the great majority of pathological cavities other than abscesses and haematomas do have an epithelial lining (Shear and Speight, 2007). Cysts lacking epithelium are described by many authorities as pseudocysts and in this thesis the term cyst will be confined to those lesions having an epithelial lining (Shear and Speight, 2007).

1.1. b. Radicular cysts are common dental inflammatory lesions

1.1. b. i. The aetiology and pathogenesis of radicular cysts

Radicular cysts are the most common inflammatory jaw cyst and develop as a sequel of untreated dental caries with pulp necrosis and periapical infection (Jones et al., 2006). The pathogenesis of radicular cysts has been described as comprising three distinct phases: the phase of initiation, the phase of cyst formation and the phase of enlargement (Shear and Speight, 2007).

In the first phase, the epithelial cell rests of Malassez proliferate in response to inflammatory signals within the inflamed tissues of an established chronic periapical periodontitis lesion. The precise identity of the stimulating factors

driving this initial epithelial proliferation is not known, but inflammatory mediators and bacterial toxins from the necrotic pulp are thought to play a central role (Meghji et al., 1996; Nair, 2003).

During the second phase, a cyst cavity develops which is lined by the proliferating epithelium. The exact mechanism of cyst cavity formation is a matter of debate. There are three main hypotheses for the mechanism of cavity formation. One hypothesis proposes that the epithelium proliferates to cover the connective tissue surface of an abscess cavity. Another widely accepted mechanism which may cause initial cyst formation is stromal degeneration, in which a cavity may form as a result of breakdown of connective tissue surrounded by sheets of epithelium (Summers, 1974). A further hypothesis is based on the idea of epithelial degeneration, in which microcysts are formed at the centre of large epithelial masses by epithelial cell degeneration and autolysis, resulting from nutritional deficiency (Nair, 2003; Ten Cate, 1972).

Microcysts may increase in size by coalescence with adjacent microcysts, but also likely undergo inflation through the accumulation of fluid under the influence of intra-cystic osmotic pressure from degenerate intra-luminal cells. This third inflationary phase is driven primarily by osmotic forces, and is thought to be enhanced by a lack of lymphatic drainage (Nair, 2003; Shear and Speight, 2007).

1.1. b. ii. The epidemiology of radicular cysts

Radicular cysts are the most common cysts in the jaws, and in one recent study comprised 3724 cases of 7121 (52.3%) separate jaw cysts in a UK population (Jones et al., 2006). Similar results are reported in other large recent series (Shear and Speight, 2007) consistent with earlier studies (Daley et al., 1994). Radicular cysts occur more commonly between the third and fifth decades of life in the UK population, which is similar to findings in the South African series (Jones et al., 2006; Shear and Speight, 2007). In both of the recent UK and South African series, radicular cysts were more common in males than females, while lesions were also more frequently seen in the anterior maxilla than other parts of the mouth.

1.1. b. iii. Clinical and radiographic features of radicular cysts

Many small radicular cysts are asymptomatic and discovered only during routine radiographic examination, such that the common clinical presentation is of an asymptomatic, slowly enlarging jaw swelling. The initial swellings are usually bony hard, but as cysts increase in size, the covering bone may become very thin despite initial subperiosteal bone deposition. Finally, with progressive bone resorption, the swellings exhibit 'springiness' or 'egg shell crackling' (Shear and Speight, 2007). With complete loss of the overlying bone, the overlying mucosa appears bluish and cysts become fluctuant. The associated teeth are always non-vital, and may show discolouration. Occasionally,

radicular cysts become painful but no clear correlation between infection and clinical symptoms has been found (Vier and Figueiredo, 2002).

Radiographic examination of radicular cysts usually shows well-defined round or ovoid radiolucencies. Radiolucencies are often surrounded by narrow radiopaque margins, which extend from the lamina dura of involved teeth (Shear and Speight, 2007). Although the associated teeth usually show no root resorption, there may be smooth resorption of root apicies. In addition to apically placed radicular cysts, these lesions may also occasionally arise from accessory root canals in non-vital teeth, creating confusion with lateral periodontal cysts.

It is difficult to differentiate radicular cysts from the obligatory pre-existing chronic periapical periodontitis lesions radiographically. A number of studies have shown poor correlation between the size of radiolucencies and histological findings of radicular cysts and periapical granulomas (Shear and Speight, 2007; Stockdale and Chandler, 1988). However, it is apparent that there is a greater likelihood of radiolucencies being radicular cysts rather than chronic periapical periodontitis lesions with increasing size of radiolucencies, particularly those over 2 cm in size (Natkin et al., 1984).

1.1. b. iv. Histological features of radicular cysts

Macroscopically, intact radicular cysts presented for histopathological evaluation may be spherical or ovoid, but are often collapsed during removal. The pathology laboratory often receives such lesions as fragmented or irregular curettage specimens. The luminal surfaces of enucleated radicular cysts may be smooth or corrugated, while yellow mural nodules of cholesterol may project into the cyst cavities (Shear and Speight, 2007). When cysts are intact, cyst cavities may be filled with brown or straw-coloured fluid, while the cyst fluid may have a shimmering gold appearance when light passes through it.

Almost all radicular cysts are lined partially or completely by non-keratinized stratified squamous epithelium. Keratinisation is seen in approximately 2% of cases, and when present orthokeratinization is more common than parakeratinization. The nature of the epithelial lining depends on the stage of development of the cyst, and also the severity of inflammation. In the majority of cases the epithelium is from 6 to 20 cell layers thick, but may be up to 50 cell layers thick in some areas.

The early stage of radicular cyst formation usually shows a proliferative epithelial lining, associated with an intense inflammatory infiltrate and marked intercellular oedema, while the epithelium may show an arcading pattern penetrating into the underlying capsule. The epithelium may also show spongiosis and be permeated by neutrophils (Cohen, 1979; Shear and Speight,

2007). As radicular cysts increase in size, the epithelial linings become more regular and quiescent in appearance.

The epithelium of radicular cysts may also show metaplastic changes in the form of mucous or ciliated cells (Browne, 1972; Slabbert et al., 1995; Takeda et al., 2005). Mucous cells usually present in the superficial layers of the epithelium, either as scattered cells or as a continuous layer. In addition, in up to 10% of cases the epithelium may contain linear, straight, curved or hairpin-shaped hyaline bodies (Dewey, 1918; Rushton, 1955; Shear and Speight, 2007; Takeda, 1985). Occasionally these hyaline bodies are concentrically laminated circular or polycyclic in the form. Although these hyaline structures were first described by K.W. Dewey in 1918, they are often referred to as Rushton's hyaline bodies. They are usually found in the superficial layers of epithelium, but can be found in the cyst wall and are often calcified. The origin of these hyaline structures remains unknown, although there is general agreement that they are of epithelial origin and represent a secretory product of odontogenic epithelium.

Radicular cyst walls usually contain an intense chronic inflammatory infiltrate, composed of lymphocytes, plasma cells, histiocytes and neutrophils. As these cysts enlarge, cyst capsules become less inflamed and more fibrous. Fibrous capsules are composed of collagen fibres interspersed with fusiform fibroblasts. The cyst walls may also contain needle-shaped cholesterol clefts, surrounded

by multinucleated giant cells. These clefts are often seen extending from the cyst wall into the epithelium and cyst cavity (Dewey, 1918). Radicular cyst walls may also contain haemosiderin pigment in inflamed areas.

1.1. c. Dentigerous cysts are common lesions associated with unerupted teeth

1.1. c. i. The definition of dentigerous cysts

Dentigerous cysts are common developmental lesions that enclose the crowns of unerupted teeth, and are attached to the cervical margins of affected teeth (Jones et al., 2006; Shear and Speight, 2007). An alternative and widely used name for these lesions is the "follicular cyst", but the term dentigerous cyst is preferable as "follicular cyst" implies an origin from the tooth follicle and may also result in confusion with hair or ovarian follicles. The literal meaning of dentigerous is 'tooth bearing', and seems most appropriate for this cyst (Shear and Speight, 2007).

1.1. c. ii. The aetiology and pathogenesis of dentigerous cysts

Although dentigerous cysts are common lesions, their precise origin is uncertain. It has been suggested that dentigerous cysts may develop by fluid accumulation either between the reduced enamel epithelium and the enamel, or alternatively between individual layers of the reduced enamel epithelium (Shear and Speight, 2007). The pressure exerted by an erupting tooth on the surrounding connective tissue follicle has been suggested as obstructing the

venous outflow to increase transudation of fluid across the capillary wall, so that the increased hydrostatic pressure of this fluid separates the follicle from the crown with or without the epithelium. It has also been argued that over time, the capillary permeability is altered and protein-rich exudate accumulates within this newly formed space, which causes more fluid accumulation by osmosis and enlargement of the cyst (Atkinson, 1972; Atkinson, 1976; Atkinson, 1977; Shear and Speight, 2007; Stanley et al., 1965). An inflammatory aetiology has also been proposed in the pathogenesis of some dentigerous cysts. It has been suggested that the crowns of permanent teeth may erupt into radicular cysts or other periapical inflammatory lesions at the apices of its deciduous predecessors. This theory is supported by clinical and radiographic evidence of inflammation of deciduous teeth in cases with histologically confirmed dentigerous cysts associated with permanent successors. Such lesions have been described as “inflammatory follicular cysts” distinguishing them from the more common lesions not associated with primary precursors (Shear and Speight, 2007; Shibata et al., 2004).

1.1. c. iii. The epidemiology of dentigerous cysts

In a recently reported large series of odontogenic cysts in a UK population collected over a 30-year period, dentigerous cysts were the most common developmental odontogenic cysts and comprised 18.1% of all odontogenic cysts. The reported frequency is similar to the 24.08% of odontogenic cysts reported in a similarly large and recent South African series collected over a 46-year

period (Jones et al., 2006; Shear and Speight, 2007), as well as when compared with the result of a Canadian series (Daley et al., 1994).

Dentigerous cysts are more common in second to fourth decades of life, with a peak prevalence in the third decade in the South African series (Shear and Speight, 2007), while the peak prevalence in the UK population was in the fifth decade (Jones et al., 2006). Dentigerous cysts are more common in males than females, while in the majority of cases, the cysts involve mandibular third molars, followed by maxillary permanent canines, mandibular premolars and maxillary third molars (Shear and Speight, 2007). Dentigerous cysts can also be associated with unerupted supernumerary teeth.

1.1. c. iv. The clinical and radiographic features of dentigerous cysts

The majority of dentigerous cysts are asymptomatic and are often found on radiographic examination when patients present with a missing tooth or failure of tooth eruption. Dentigerous cysts associated with unerupted teeth in edentulous patients, may present with as slowly enlarging, painless jaw swellings. When dentigerous cysts become infected, they may also present with pain (Shear and Speight, 2007).

Dentigerous cysts commonly present as unilocular radiolucencies associated with the crowns of unerupted teeth, and often have well-defined and sclerotic borders. Three radiographic presentations of dentigerous cysts have been

described, being the central, lateral and the circumferential forms (Shear and Speight, 2007). In central cysts, crowns of the unerupted teeth are enveloped symmetrically by cysts and the teeth are usually displaced away from their direction of eruption. In the lateral form, cysts develop form one aspect of partially erupted or unerupted teeth. This variant is commonly associated with a partially erupted mandibular third molar. In the circumferential type of dentigerous cyst, the teeth are entirely enveloped by cyst cavities.

Radiographically, a hyperplastic dental follicle can be confused with a small dentigerous cyst. Most authorities believe that a pericoronal radiolucency of more than 4 mm is suggestive of a small dentigerous cyst (Daley and Wysocki, 1995; Shear and Speight, 2007). It is accepted that the final diagnosis of dentigerous cyst depends on the combined radiographic, intra-operative and histological findings.

1.1. c. v. The histopathological features of dentigerous cysts

Gross examination of enucleated dentigerous cysts, usually reveal cysts to be attached to the cemento-enamel junction of associated teeth. Epithelial linings of dentigerous cysts resemble the reduced enamel epithelium, and consist of flat or cuboidal cells of 2 to 4 cell layers thickness. Localized proliferation of the epithelial lining has been reported in areas of inflammation. The epithelium may also contain mucous producing or ciliated cells representing metaplastic change. Occasionally dentigerous cysts contain Rushton's hyaline bodies

similar to those reported for radicular cysts (Browne, 1972; Rushton, 1955; Shear and Speight, 2007).

Dentigerous cysts usually have a thin fibrous capsule with widely separated fibroblasts and stroma. The stroma is often loose in places, with accumulation of glycosaminoglycans, while isolated cords and islands of inactive odontogenic epithelium are also often seen in the cyst walls. The stroma is usually uninflamed, although inflammation is seen when dentigerous cysts become infected (Benn and Altini, 1996). Inflamed dentigerous cyst walls occasionally contain cholesterol clefts and haemosiderin pigments (Shear and Speight, 2007).

1.1. d. Odontogenic keratocysts are important benign neoplasms

1.1. d. i. The definition of odontogenic keratocysts

The term 'odontogenic keratocyst' was introduced by Philipsen in 1956 and used to describe jaw cysts filled with keratin material (Shear and Speight, 2007). Odontogenic keratocysts were recently recognized as benign odontogenic tumours by the World Health Organization, and as a consequence these lesions have been re-named as "keratocystic odontogenic tumours (KCOT)" (Philipsen, 2005), while another suggested name for these lesions has been "keratinizing cystic odontogenic tumour" (Reichart and Philipsen, 2004). To date, no international consensus has been achieved regarding the terminology for these cysts. Nonetheless, the terms 'odontogenic keratocyst' or 'keratocyst' are widely

used by clinicians, and for the purposes of the current thesis these lesions will be referred to as odontogenic keratocysts.

1.1. d. ii. The aetiology and pathogenesis of odontogenic keratocysts

Several studies have shown that odontogenic keratocysts result from mutation of PTCH tumour suppressor gene, located in the chromosome 9q22.3-q31 (Barreto et al., 2000; Cohen, 1999; Lench et al., 1996; Lench et al., 1997; Philipsen, 2005; Shear and Speight, 2007). The PTCH gene encodes a trans-membrane protein that affects the *Sonic Hedgehog* signalling pathway. This ultimately affects cellular growth and pattern formation in numerous tissues, including odontogenic epithelium (Barreto et al., 2000; Cohen, 1999). It is now widely accepted that odontogenic keratocysts arise from odontogenic epithelium, and that the two major sources of this are the dental lamina or its remnants, and basal cells from the overlying oral epithelium (Philipsen, 2005; Shear and Speight, 2007).

Similar to other tumour suppressor genes, deficient PTCH gene function is the basis for a heritable autosomal dominant syndrome associated with the formation of specific neoplasms, namely nevoid basal cell carcinoma syndrome (NBCCS). This syndrome is inherited with variable penetrance and expressivity, and is associated with both developmental deficiencies and neoplasms (Shear and Speight, 2007; Woolgar et al., 1987b).

1.1. d. iii. The epidemiology of odontogenic keratocysts

The true frequency of odontogenic keratocysts is difficult to determine as the terminology for these lesions differs throughout the literature, ranging from descriptions of the cysts as a developmental lesions to recognition as a benign tumours (Philipsen, 2005; Reichart and Philipsen, 2004; Shear and Speight, 2007). There are also two different histological variants of this entity, being the orthokeratinized and parakeratinized forms of odontogenic keratocyst (Shear and Speight, 2007). Some authorities include both of these variants under the same name, but others believe that the orthokeratinized variant is a separate entity (Shear and Speight, 2007). In one recently published and large UK series of odontogenic cysts, odontogenic keratocysts were the second most common developmental odontogenic cysts identified, comprising 11.6% of all odontogenic cysts over a 30-year period (Jones et al., 2006). The reported frequency in the UK study is similar to that of a major South African series collected over a 46-year period (10.2%) (Jones et al., 2006; Shear and Speight, 2007).

The age of presentation of odontogenic keratocysts ranges from the first to the ninth decades of life, with a peak frequency in the second and third decades (Jones et al., 2006; Shear and Speight, 2007). Approximately 40% to 60% of cases are diagnosed in these age groups, while the age of patients with NBCCS having odontogenic keratocyst is significantly lower than those presenting with sporadic lesions (Shear and Speight, 2007; Woolgar et al., 1987b). Odontogenic

keratocysts are more frequently seen in males than females (Shear and Speight, 2007), while these cysts are more common in the mandible than the maxilla. In the lower jaw, odontogenic keratocysts most commonly involve the angle of the mandible and extend anteriorly as well as posteriorly (Jones et al., 2006; Philipsen, 2005; Shear and Speight, 2007; Woolgar et al., 1987a).

1.1. d. iv. The clinical and radiographic features of odontogenic keratocysts

In many cases, patients with odontogenic keratocyst are asymptomatic until cysts achieve a significantly large size. Occasionally, the cysts are associated with swelling of the affected jaws, pain or discharge (Philipsen, 2005; Shear and Speight, 2007). Odontogenic keratocysts may be associated with unerupted teeth or malocclusion, and occasionally patients may complain of paraesthesia of dependant areas of the affected nerves.

Patients with NBCCS usually have: multiple recurrent odontogenic keratocysts; multiple nevoid basal cell carcinomas; and numerous skeletal deformities including ocular hypertelorism; cervical ribs; bifid ribs; and a shortened fourth metacarpal bone; as well as palmer and or planter pits (Gorlin, 1987). An important clinical property of odontogenic keratocysts is a high propensity for recurrence, and this is consistent with the recent recognition of these lesions as benign neoplasms (Philipsen, 2005; Shear, 2002a; Shear, 2002b).

Small odontogenic keratocysts usually present as unilocular round or ovoid radiolucent areas. The radiographic margins of the radiolucencies in early odontogenic keratocysts may be well-defined or diffuse. Some of these unilocular radiolucencies have scalloped margins, reflecting an invasive growth pattern. Odontogenic keratocysts may also present as multilocular radiolucencies, particularly when advanced. Occasionally the cysts present with expansion of the cortical plates. Any associated teeth may be displaced, and may also show root resorption. There may be downward displacement of the inferior alveolar canal in mandibular odontogenic keratocysts (Philipsen, 2005; Shear and Speight, 2007). Radiographically, four different types of odontogenic keratocyst have been described being the: replacement, envelopmental, extraneous and collateral variants. Radiographs of patients with NBCCS may show calcification of falx cerebri, bifid or fused ribs and other skeletal abnormalities (Gorlin, 1987; Manfredi et al., 2004).

1.1. d. v. The histological features of odontogenic keratocysts

Macroscopically the cyst cavities of odontogenic keratocysts contain pale-white granular keratinous material. Histologically, the cyst cavity contents of odontogenic keratocysts are usually composed of desquamated keratinous cells, while the cyst linings are typically comprised of a parakeratinized, stratified squamous epithelium. Some cysts are lined by orthokeratinized epithelium, and these have been considered separate to the parakeratinizing odontogenic keratocyst (Philipsen, 2005; Shear and Speight, 2007). The epithelium of these

lesions is usually 5 to 8 cell layers thick, while the parakeratinized surface is usually corrugated or folded and presents as an abrupt transition from the underlying spinous layer. The junction between the epithelium and connective tissue stroma is usually flat and devoid of rete ridges, and the epithelium often separates readily from the capsule. There is usually a well-defined and often palisaded basal cell layer of columnar cells which are hyperchromatic and display reverse nuclear polarity, reminiscent of pre-ameloblasts. Mitotic figures are frequently found amongst the basal cells, and are also often seen in the supra-basal cells. It is noteworthy that the number of mitoses is higher in odontogenic keratocysts from patients with NBCCS compared with odontogenic keratocysts from people not suffering this syndrome (Philipsen, 2005; Shear and Speight, 2007; Woolgar et al., 1987a).

The epithelial linings of odontogenic keratocysts may also show features of epithelial dysplasia (Philipsen, 2005; Shear and Speight, 2007). In the presence of an intense inflammatory infiltrate, the epithelium loses its characteristic cellular and architectural features, so that diagnosis of odontogenic keratocysts becomes difficult when there is infection. The cyst walls are usually thin and composed of uninflamed, mature fibrous connective tissues, while when infected the stroma may contain an intense chronic inflammatory infiltrate (Shear and Speight, 2007). Rarely, the cyst walls of odontogenic keratocysts contain cholesterol clefts and haemosiderin deposits.

1.1. e. Haemosiderin pigment is derived from haemoglobin

Haemoglobin is the major oxygen carrying protein, and is contained within erythrocytes. Haemoglobin synthesis begins in pro-erythroblasts and continues into the reticulocyte stage of erythrocyte development. The haemoglobin tetramer consists of two pairs of globin polypeptide chains. Each haemoglobin molecule has two alpha protein chains and two non-alpha chains, while each of these four protein molecules is associated with a heme group (Hoffman, 2005). Succinyl-CoA formed in the Krebs cycle of carbohydrate metabolism binds with glycine to form pyrrole molecules. Four such pyrrole molecules combine to form a single protoporphyrin IX ring and this then combines with iron to form the heme group (Guyton and Hall, 2006).

Erythrocytes have a life span of approximately 120 days. By this time, erythrocyte membranes become fragile and prone to rupture upon passage through capillary networks, while the spleen plays a central role in removing effete erythrocytes. Erythrocytes collected in the spleen, or shed into the tissues during bleeding are phagocytised by macrophages and the haemoglobin protein and heme components are degraded by differing pathways. The protein globin component is converted into amino acids, and returned to the general amino acid pool for manufacture of new protein. The porphyrin component of degrading haemoglobin is converted into bile pigment through the formation of biliverdin and bilirubin, before excretion. Released iron may be bound to transferrin for transport to the bone marrow and synthesis of new haemoglobin,

or alternatively the iron may be taken up by the liver and other tissues for storage in the form of ferritin. Ferritin is an intracellular protein which forms micelles trapping iron, and as such plays a critical role in iron storage in cells. Large quantities of ferritin form aggregates in cells, to produce haemosiderin granules readily appreciated in paraffin sections as golden-brown intracellular deposits . Extensive haemosiderin deposits are seen in circumstances where there has been appreciable iron overload, or alternatively when there has been haemorrhage into tissue sites. Such haemosiderin deposits usually appear as intracellular pigments within macrophages (Stevens and Chalk, 1997; Stevens and Wilson, 1997).

Haemosiderin contains iron in the ferric hydroxide form, bound to a protein framework. In tissue sections stained with standard haematoxylin and eosin, these haemosiderin deposits are readily identified as golden brown, usually intra-cellular, granular deposits. When tissue section are stained by the Perl's Prussian blue reaction, haemosiderin deposits are appear as blue granular sediments (Stevens and Chalk, 1997; Stevens and Wilson, 1997).

1.1. f. Haematoxylin and eosin stain is routinely used for paraffin section histology

The most widely applied staining system for paraffin sections in histopathology combines haematoxylin and eosin (H&E). These stains label almost all tissue structures, and demonstrate clearly the outlines of cells, location of nuclei, and most elements of the extracellular matrix (Stevens and Wilson, 1997).

Haematoxylin is initially extracted from the heartwood ('logwood') of the *Haematoxylin campechianum* tree. Haematoxylin itself is not a stain but its oxidative product haematein is a natural dye. Haematein is anionic, and has a poor affinity for tissue, however, when it is used in combination of several different types of mordant, haematin is a highly effective histological dye. Haematoxylin solutions have been sub-classified according to the type of mordant used, such as alum haematoxylin, iron haematoxylin, and tungsten haematoxylin. The most widely used form of haematoxylin is Myer's haematoxylin, which is an alum form of the dye (Stevens and Wilson, 1997). Since haematoxylin is a basic dye with a net positive charge, it is attracted to the negative charge of nucleic acids to label DNA and RNA, as well as negatively charged carbohydrates. The appearance of haematoxylin in paraffin sections is an intense blue, which contrasts well with the pink stain of eosin (Stevens and Wilson, 1997).

Eosin is an acidic dye with a net positive charge, so that this pink dye labels negative groups, primarily of proteins in cells as well as the extracellular matrix. The intensity of eosin labelling varies, largely according to the amount of protein present, so that eosin stains in paraffin sections range in appearance from pink to orange and red (Stevens and Chalk, 1997; Stevens and Wilson, 1997).

1.1. g. Perl's Prussian blue reaction for haemosiderin in paraffin sections

To aid identification of haemosiderin pigments, as well as to confirm deposits identified as haemosiderin, it is convenient to exploit the chemical properties of ferric hydroxide in haemosiderin in the Perl's Prussian blue reaction. Haemosiderin is insoluble in alkaline solutions, but soluble in strong acidic solutions. In Perl's Prussian blue reaction, the tissue sections are treated with an acid ferrocyanide, resulting in release of ferric iron as ferric hydroxide. The ferric iron then reacts with potassium ferrocyanide to produce an insoluble blue compound of ferric ferrocyanide (Prussian blue) (Stevens and Chalk, 1997; Stevens and Wilson, 1997). In this way, it is possible to confirm granular brown deposits in the paraffin sections as haemosiderin, and this approach was used in the current thesis.

1.1. h. Cholesterol crystals in tissues

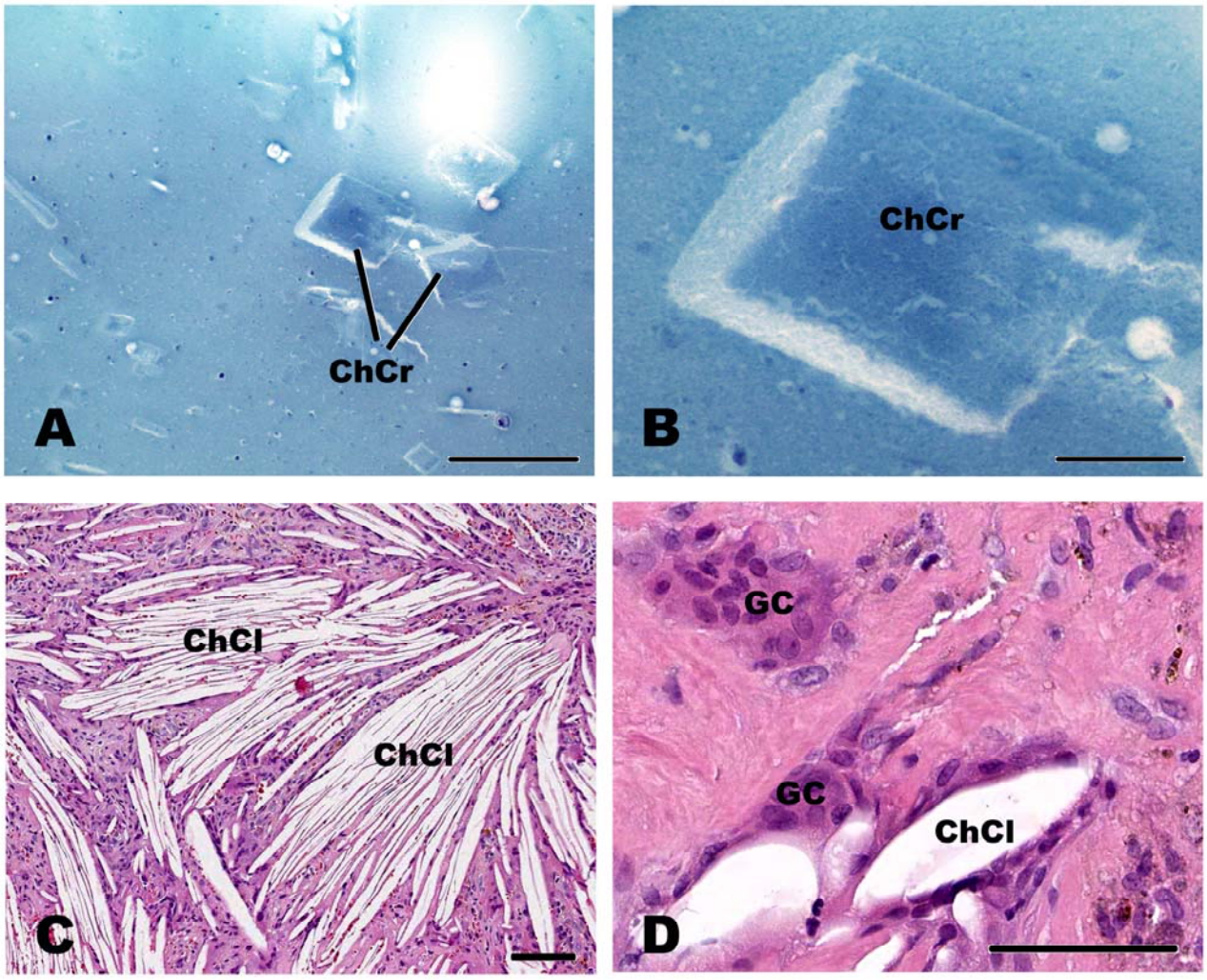
Cholesterol crystals are found in many odontogenic cysts including radicular cysts, dentigerous cysts, and odontogenic keratocysts. The presence of

cholesterol crystals in radicular cyst fluid has in the past been recognized as a characteristic feature (Browne, 1971; Shear, 1963), although this is no longer accepted as a diagnostic criterion. When such cyst fluid is examined macroscopically with transmitted light, cholesterol crystals impart a shimmering appearance to the gold or straw coloured fluid (Shear and Speight, 2007). If an unstained smear of cyst fluid is examined using a light microscope, cholesterol crystals are seen to have a typical rhomboid shape (**Fig. 1.1 A&B**). In paraffin sections processed for routine H&E staining, these crystals are dissolved by the fat solvents used in dehydration and infiltration, leaving needle-shaped clefts known as "cholesterol clefts" within cyst walls and cavities(Shear, 1963). Notably, once cholesterol crystals have been deposited in the cysts wall, they behave as foreign bodies and elicit a foreign body giant cell reaction (Shear and Speight, 2007). In consequence, cholesterol clefts in histological sections are seen surrounded by multinucleated foreign body type giant cells (**Fig. 1.1 C&D**) (Shear and Speight, 2007).

The clinical significance of cholesterol clefts in odontogenic cysts is yet to be determined. The presence of cholesterol crystals in aspirated cyst fluid was once used for the diagnosis of inflammatory odontogenic cysts and radicular cysts in particular (Browne, 1971; Shear and Speight, 2007). However, not all radicular cysts have cholesterol crystals and some developmental odontogenic cysts also have cholesterol crystals in their cysts cavities, so that the presence of cholesterol crystals in aspirated fluid is no longer accepted as a diagnostic criterion (Browne, 1971; Shear and Speight, 2007). It is important to note that

cholesterol crystals are frequently seen in atherosclerotic plaques (Ross, 1999) and this may have implications for the origin of such crystals in odontogenic lesions. Similarly, it is interesting that cholesterol crystals are also occasionally seen in non-odontogenic lesions, including lesions from breast (**Fig. 1.1 A&B**) (Rosen, 1997).

Figure 1.1. Photomicrographs of cholesterol crystals in smears taken from an aspirated breast lesion (A, B) and cholesterol clefts in a radicular cyst (C, D). (A, B) The typical rhomboid shape of cholesterol crystals (ChCr) is seen at both low (A) and high (B) magnification in cyst fluid aspirated from a breast lesion. (C) In paraffin sections, such crystals appear as needle-shaped cholesterol clefts (ChCl), while high magnification (D) reveals the presence of multinucleated giant cells (GC) surrounding cholesterol clefts. (A = Papanicolaou stain, size bar 200 μm; B = Papanicolaou stain, size bar 50 μm; C = H&E stain, size bar 100 μm; D = H&E stain, Bars = 50 μm)


A
ChCr
B
ChCr
C
ChCl
ChCl
D
GC
GC
ChCl

1.1. i. The origin of cholesterol crystals in odontogenic cysts is uncertain but often considered related to erythrocyte degeneration

Cholesterol clefts are reported in many but not all cases of radicular cysts (Browne, 1971; Shear and Speight, 2007). In one series, cholesterol clefts were seen in 57 of 200 dental cysts (28.5%) (Shear, 1963), while in a separate study such clefts were seen in 216 of 537 odontogenic cysts (40.2%) (Browne, 1971). The incidence of cholesterol crystals is reported as highest (43.5%) in inflammatory cysts, particularly in radicular cysts, while the lowest incidence (17.1%) is reported for cysts of non-inflammatory origin such as odontogenic keratocysts (Browne, 1971). This suggests that the inflammatory process plays an important role in the pathogenesis of cholesterol clefts.

The origin of the cholesterol crystals in odontogenic lesions is still controversial and largely based on speculation. Some authorities suggest that cholesterol clefts accumulate in the tissues as a result of degeneration and disintegration of epithelial cells (Thoma et al., 1970). M. Shear and others have on the other hand, suggested circulating plasma lipids as a more likely source, as cholesterol clefts are common in atherosclerotic plaques and circulating lipids have been identified as the origin of cholesterol in atherosclerosis (Shear, 1963; Skaug, 1976).

R.M. Browne analysed 535 odontogenic cysts, including 402 radicular cysts (Browne, 1971). In his study, cholesterol clefts were more prevalent in cases

where there was haemosiderin pigment. He postulated that the main source of cholesterol crystals was disintegrating erythrocytes. Approximately 55% of the erythrocyte membrane lipid is phospholipid, while 42% is cholesterol and 3% comprise glycolipids. Cholesterol in the erythrocyte membrane is present in its free and non-esterified form (Best et al., 1985). Since erythrocytes have no internal membranes, and the plasma membrane is the only possible source of erythrocyte cholesterol, it would appear that the amount of cholesterol present in the bimolecular lipid layer of erythrocytes membrane is negligible, so that it is difficult to see how the large volume of cholesterol crystals seen in odontogenic cysts could be derived from erythrocyte membranes alone.

Notably, cholesterol clefts are also reported in chronic periapical periodontitis (Neville et al., 2002), consistent with an inflammatory basis. While the earlier study (Browne, 1971), recognized erythrocytes as a possible source of cholesterol, no mention was made of other cell types potentially contributing to cholesterol deposits including degenerating epithelial cells, connective tissue cells, inflammatory cells and plasma lipoproteins.

1.1. j. Foamy macrophages appear to be the origin of cholesterol clefts

Foamy macrophages are seen in atherosclerosis, where they are known to form by the unregulated uptake of oxidized-low density lipoprotein (LDL) via scavenger receptors. Macrophages phagocytising large numbers of degenerate

cells may also acquire a “foamy” appearance from accumulated lipid in their cytoplasm (Osterud and Bjorklid, 2003; Ross, 1999).

While foamy macrophages are noted as present in odontogenic cysts (Browne, 1971; Shear and Speight, 2007), no numerical analysis has been performed correlating these lipid laden cells with haemosiderin, while there is also little clear published data relating foamy macrophages to cholesterol crystals in odontogenic cysts (Browne, 1971). Cholesterol crystals have been suggested as forming both within foamy macrophages , as well as in the extracellular matrix in atherosclerosis. Separately, intracellular cholesterol crystal formation has been observed in cultured cells (Kellner-Weibel et al., 1998; Kellner-Weibel et al., 1999). However, the possible intracellular origin of cholesterol crystals in odontogenic lesions has not been studied.

1.1. k. The origin of odontogenic cyst cholesterol may have implications for understanding the pathogenesis of these lesions

Most plasma cholesterol is bound in LDL, which are lipid particles solubilised by apo-lipoprotein B, demonstrated by immunohistochemistry in atherosclerotic plaques and also more recently in chronic periapical periodontitis lesions (Yamazaki et al., 2004).

LDL in the extracellular matrix undergoes oxidation, to produce oxidized LDL, known to have cytokine-like inflammatory properties . If cholesterol clefts were

derived from LDL in odontogenic cysts, then this would suggest an important role for LDL and remnant cholesterol clefts in the development of these lesions. Also, if cholesterol clefts in foamy macrophages in odontogenic cysts were derived from oxidized LDL, then the cytokine-like activation of the oxidized LDL suggests a role for foamy macrophages in persistence of the inflammatory status of odontogenic cysts.

1.2 Aims and Hypothesis

The origin of cholesterol crystals in odontogenic cysts is still uncertain. The hypothesis, that degenerating erythrocytes are the origin of cholesterol clefts in odontogenic cysts, is based primarily on the presence of haemosiderin in lesions with cholesterol clefts. Work described in this thesis was to extend the earlier reported studies demonstrating association between haemosiderin and cholesterol clefts, and to also determine if there is co-localization of such deposits in individual odontogenic cysts, an aspect of histological evaluation not previously reported. In addition, the presence of foamy macrophages was studied, an aspect of odontotgenic cysts for which little data is published.

In light of the large amount of cholesterol in LDL, relative to the relatively small amount in erythrocytes, it was hypothesized that there would be poor correlation between haemosiderin and either cholesterol clefts or foamy macrophages in odontogenic cysts.

CHAPTER 2

MATERIALS AND METHODS

2.1. Ethical approval was obtained for this study

The Human Research Ethics Committee of the Sydney West Area Health Service and the University of Sydney Australia approved this study. The Scientific Committee of the Sydney West Area Health Service based at Westmead Hospital also approved this study.

2.2. Selection of routine histopathology sections for study

Archival H&E stained paraffin sections of all radicular cysts, dentigerous cysts and odontogenic keratocysts presenting in the Institute of Clinical Pathology and Medical Research (ICPMR) at Westmead Hospital New South Wales Australia between January 2005 and May 2008 were included in this study, with the exception of very occasional cases where relevant histological sections were missing from the archive. This archival material was identified using the CERNER PATHNET database system at ICPMR and the SNOMED diagnostic retrieval tool.

Among the radicular cysts identified with both cholesterol clefts and haemosiderin, 6 cases were selected at random for further analysis of co-localization of these deposits within sections. Of these six cases, three cases were further analysed following preparation of near serial paraffin sections and staining by Perl's Prussian blue reaction.

2.3. Haematoxylin and eosin staining

Archived sections were all stained with H&E using routine laboratory procedures established at the ICPMR, using a Tissue-Tek® DRS™ (Sakura, Japan) automatic tissue processor. In brief, tissues fixed in 10% neutral buffered formalin were dehydrated with graded alcohols (LOMB scientific, Australia) and infiltrated with paraffin (LOMB scientific, Australia) for preparation of paraffin blocks. 4µm sections were prepared using a Leica microtome (Leica Microsystems, Australia) and disposable knives (Arthur Bailey Surgico, Australia). Sections were collected onto glass slides (H-D scientific, Australia) and Dewaxed by baking at 65^0C followed by treatment with xylol (LOMB scientific, Australia) and graded alcohols. Staining was firstly with Myers haematoxylin (Australian Biostain) followed by treatment with in house prepared blueing solution and followed by staining with eosin (Australian Biostain). Sections were then dehydrated with graded alcohols followed by xylol and finally cover slipped using Tissue-Tek® Glas™ (Sakura, Japan) for examination.

2.4. Perl's Prussian blue reaction

Sections were stained manually by Perl's Prussian blue method using procedures established at the ICPMR. Near serial 4µm paraffin sections of 3 radicular cysts with known haemosiderin and cholesterol clefts as observed in H&E sections, were prepared as described above (2.3). Staining was done manually with in-house prepared Perl's solution containing equal parts of

potassium ferrocyanide (2% w/v) and hydrochloric acid (2% v/v) (Crown scientific, Australia) in a coplin jar. This was followed by counterstaining with nuclear fast red ("Gurr" Certistain®). Sections were then dehydrated with graded alcohols followed by xylol and finally cover slipped using Tissue-Tek® Glas™ coverslips (Sakura, Japan) for examination.

2.5. Determination of the incidence of cholesterol clefts, foamy macrophages and haemosiderin deposits in odontogenic cysts

All archival sections were examined by light microscopy using an Olympus microscope at magnifications varying from x2 to x40. Diagnoses recorded for odontogenic cysts were confirmed on the basis of well recognized histological criteria (1.1.b.vi, 1.1.c.v, 1.1.d.v). The presence or absence of cholesterol clefts, foamy macrophages, and haemosiderin deposits was recorded for each specimen studied. Cholesterol clefts in sections were recognized on the basis of their distinctive shape, as well as the presence of multinucleated giant cells. As a further internal control, we compared these clefts with the artefactual spaces formed during sectioning as well as with small vascular spaces. Foamy macrophages were identified as cells with multiple fine clear granular spaces and centrally placed nuclei, often in close relation to cholesterol clefts. We recognized haemosiderin as a golden to brown granular pigment, and this was further confirmed in three cases stained by the Pearl's Prussian blue method.

2.6. Analysis of the spatial distribution of cholesterol clefts and haemosiderin deposits in individual odontogenic cysts

As indicated in section 2.2, six cases of radicular cyst with deposits of both haemosiderin and cholesterol clefts were selected at random for detailed analysis of the spatial distribution of these deposits throughout tissues. H&E sections were scanned by a digital slide scanner at the Royal College of Pathologists Australasia (RCPA) Quality Assurance Programme (QAP), Melbourne Australia, to produce digital images. These high resolution digital images were then analysed using ImageScope® software provided by Aperio technologies. Analysis of the spatial distribution of haemosiderin and cholesterol clefts was performed by superimposition of a rectangular grid over digital images, and assignation of each grid square to one of five groups being: no tissue, tissue with no haemosiderin or cholesterol clefts, tissue with haemosiderin alone, tissue with cholesterol clefts alone, or tissue with both haemosiderin and cholesterol clefts. The number of grid rectangles corresponding to each of these groups was recorded for each specimen, and provided a means of objectively assessing co-localization or otherwise of cholesterol clefts with haemosiderin deposits in individual specimens. To confirm that the analytical approach used was sufficiently specific for haemosiderin, this analysis was repeated for 3 of these cases using near parallel sections stained by Perl's Prussian blue, and results compared with those obtained by analysis of H&E sections.

2.7. Statistical analysis

The chi square test for differences in proportion was used throughout in the current study . A *P* value of less than 0.05 in a two tailed test was considered statistically significant.

CHAPTER 3

RESULTS

3.1. The cysts studied displayed the characteristic histopathological features expected for radicular cysts, dentigerous cysts, and odontogenic keratocysts

297 separate cases of odontogenic cystic lesions were examined in the current study, comprising 135 cases of radicular cyst, 108 cases of dentigerous cyst, and 54 cases of odontogenic keratocyst. All lesions studied had histopathological features typical for the respective cyst diagnosis.

Radicular cysts had cavities lined by non-keratinized stratified squamous epithelium, varying in thickness from 6 to 50 cell layers. A proliferative and arcading pattern of epithelium with an intense inflammatory infiltrate and marked intercellular oedema was seen in cysts at an early stage of development, while the epithelium was often permeated by neutrophils (**Fig. 3.1**). Some of these cysts were lined by more regular and quiescent epithelium suggestive of a later stage of radicular cyst development. Also cyst linings occasionally showed mucous or ciliated cell metaplasia. Curved and concentrically laminated circular Rushton's hyaline bodies were also occasionally seen in the superficial layers (**Fig. 3.2**).

Radicular cyst walls were composed of fibrous connective tissue with areas of intense inflammatory infiltrate comprising plasma cells with Russel's bodies, lymphocytes and histiocytes. Cyst walls also often contained needle-shaped cholesterol clefts surrounded by multinucleated cells, as well as areas with

golden to brown granular haemosiderin pigments (**Fig. 3.1**). Foamy macrophages were also occasionally seen.

Dentigerous cysts studied had cavities lined by flat or cuboidal epithelial cells of 2 to 4 cell layers in thickness, resembling the reduced enamel epithelium (**Fig. 3.3**). Dentigerous cyst cavities were also lined focally by proliferative epithelium, particularly in areas of inflammation.

Cyst walls were usually thin and composed of loosely arranged fibrous connective tissue with scanty or no inflammatory infiltrate. Where there was inflammation, however, a moderate to intense inflammatory infiltrate composed of lymphocytes, histiocytes and plasma cells were seen. Very occasionally, mural nodules of cholesterol clefts were found, projecting from the capsules of dentigerous cysts through the epithelium into cyst lumina (**Fig. 3.3**).

Figure 3.1. Photomicrographs of paraffin sections of radicular cysts showing the histopathological features typical for these lesions. *(A)* Cyst cavities were lined by stratified squamous epithelium (E), while significant inflammatory infiltrate was seen at low magnification. *(B)* At higher magnification, the epithelium (E) and adjacent inflamed cyst wall were clearly infiltrated with inflammatory cells, which at yet higher magnification *(C)* were revealed to be primarily neutrophils (PMNL) in the epithelium (E), and plasma cells (PC), lymphocytes and histiocytes in the capsule *(D)*. Russel bodies (RB) were also occasionally found amongst the plasma cells *(D)*. (E) Golden brown granular haemosiderin (H) pigment was frequently seen, as were cholesterol clefts (ChCl) surrounded by multinucleated giant cells. The extent of cholesterol clefts formation varied greatly from only occasional crystals *(E)*, to quite extensive deposits occupying most of the tissue volume examined in some areas *(F)*. (H&E stain, Bars for A = 250 μm; B, C, D, E & F = 50 μm).

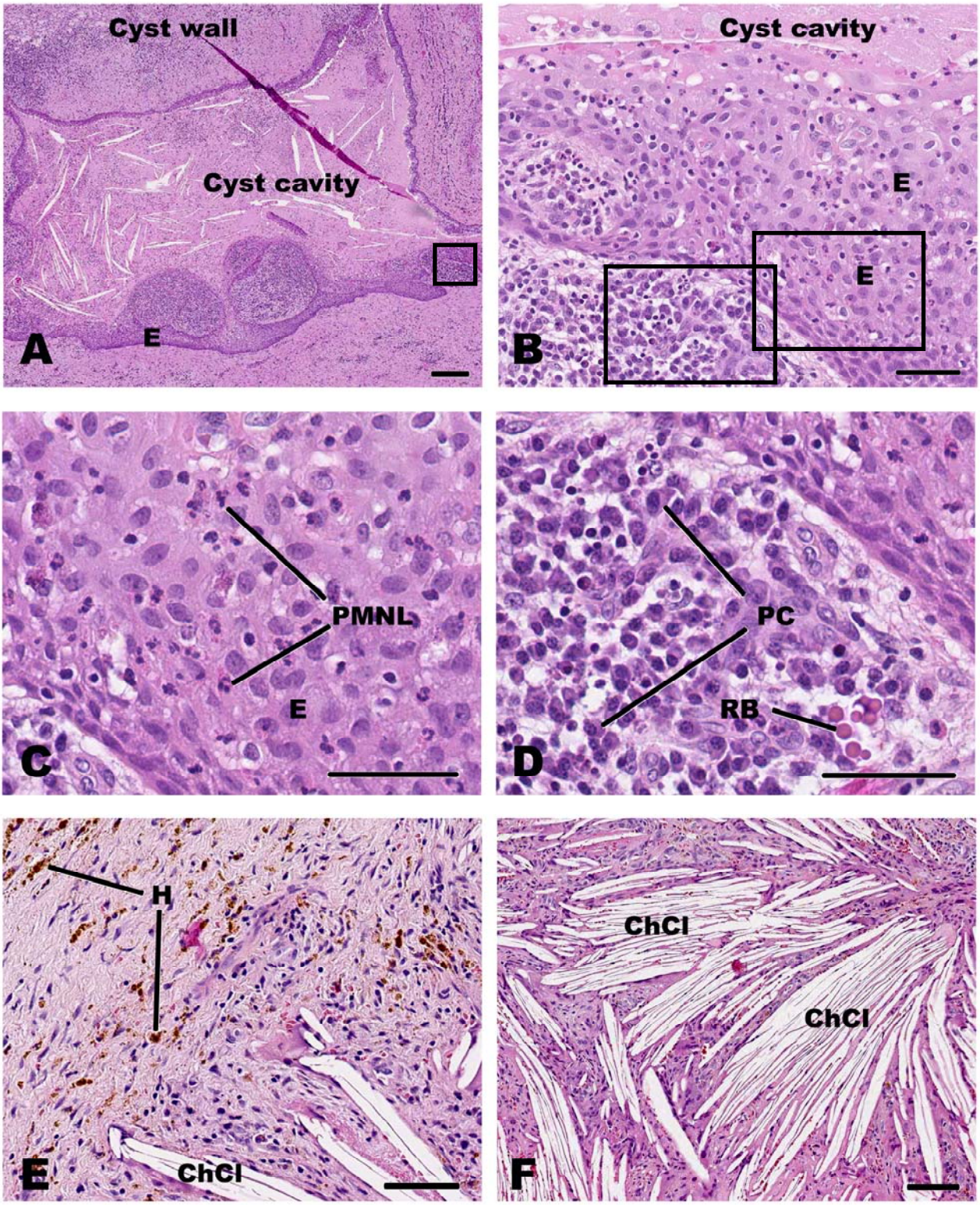

Cyst wall
Cyst cavity
E
A
Cyst cavity
E
E
B
PMNL
E
C
PC
RB
D
H
ChCl
E
ChCl
ChCl
F

Figure 3.2. Photomicrographs of paraffin sections of a radicular cyst containing Rushton's hyaline bodies. *(A)* Arcading cords of epithelium (E) were seen infiltrating the cyst wall, whilst scattered throughout the epithelium, eosinophilic Rushton's hyaline bodies were occasionally seen (arrows). *(B)* Higher magnification revealed the laminated curvilinear form of Rushton's bodies (arrows). *(C)* Many of the Rushton's bodies observed were concentrically laminated and circular in appearance (arrows). (H&E stain, Bars for A = 100 μm; B&C = 50 μm).

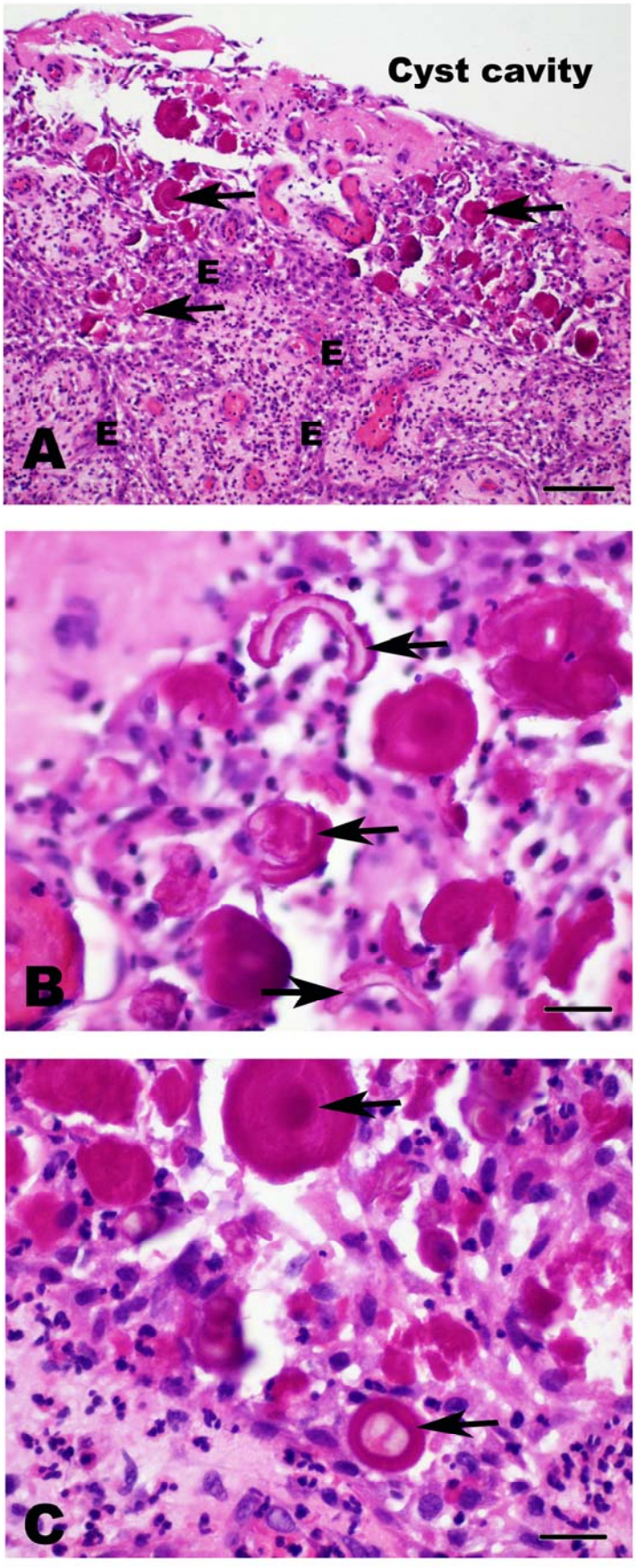
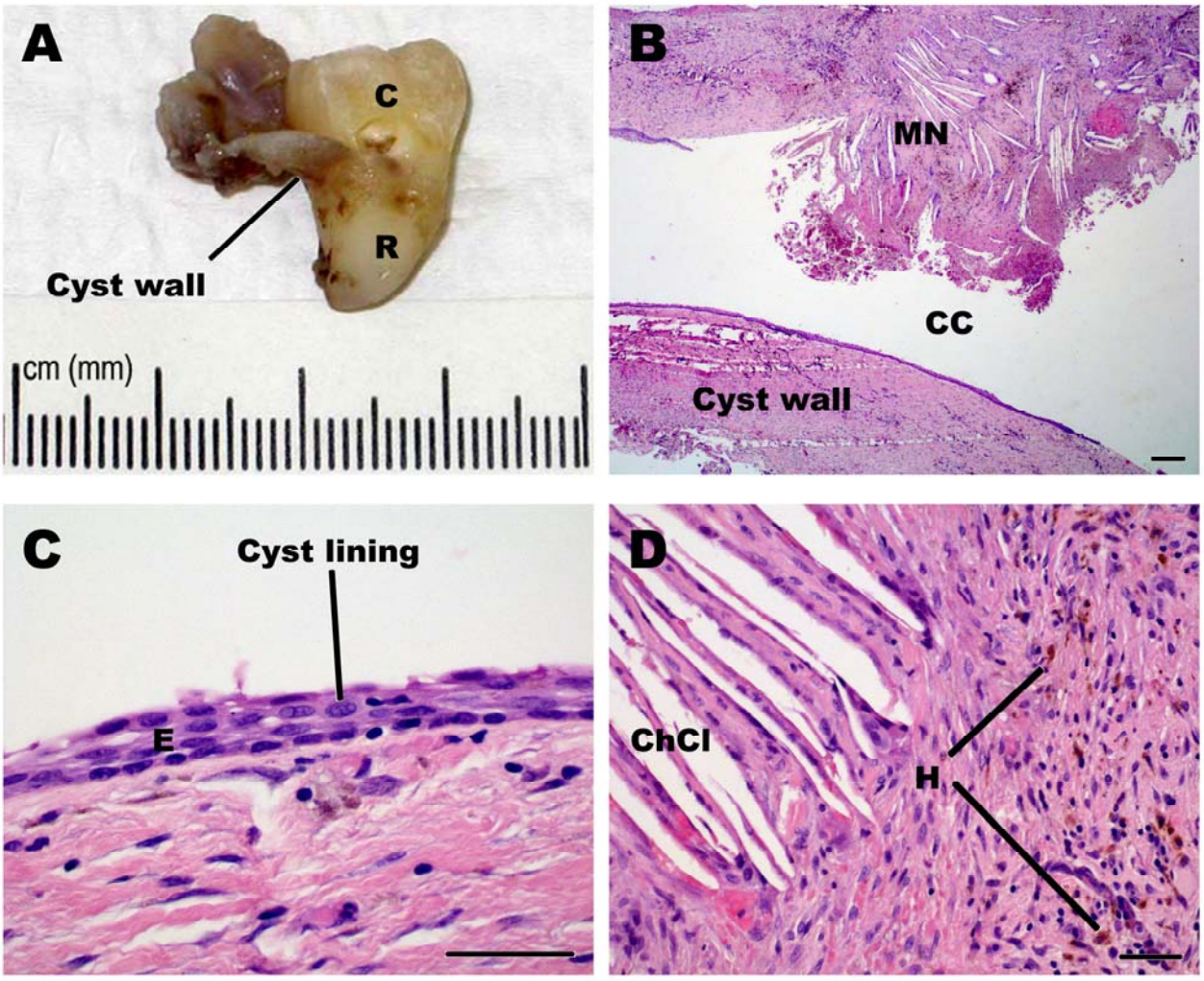
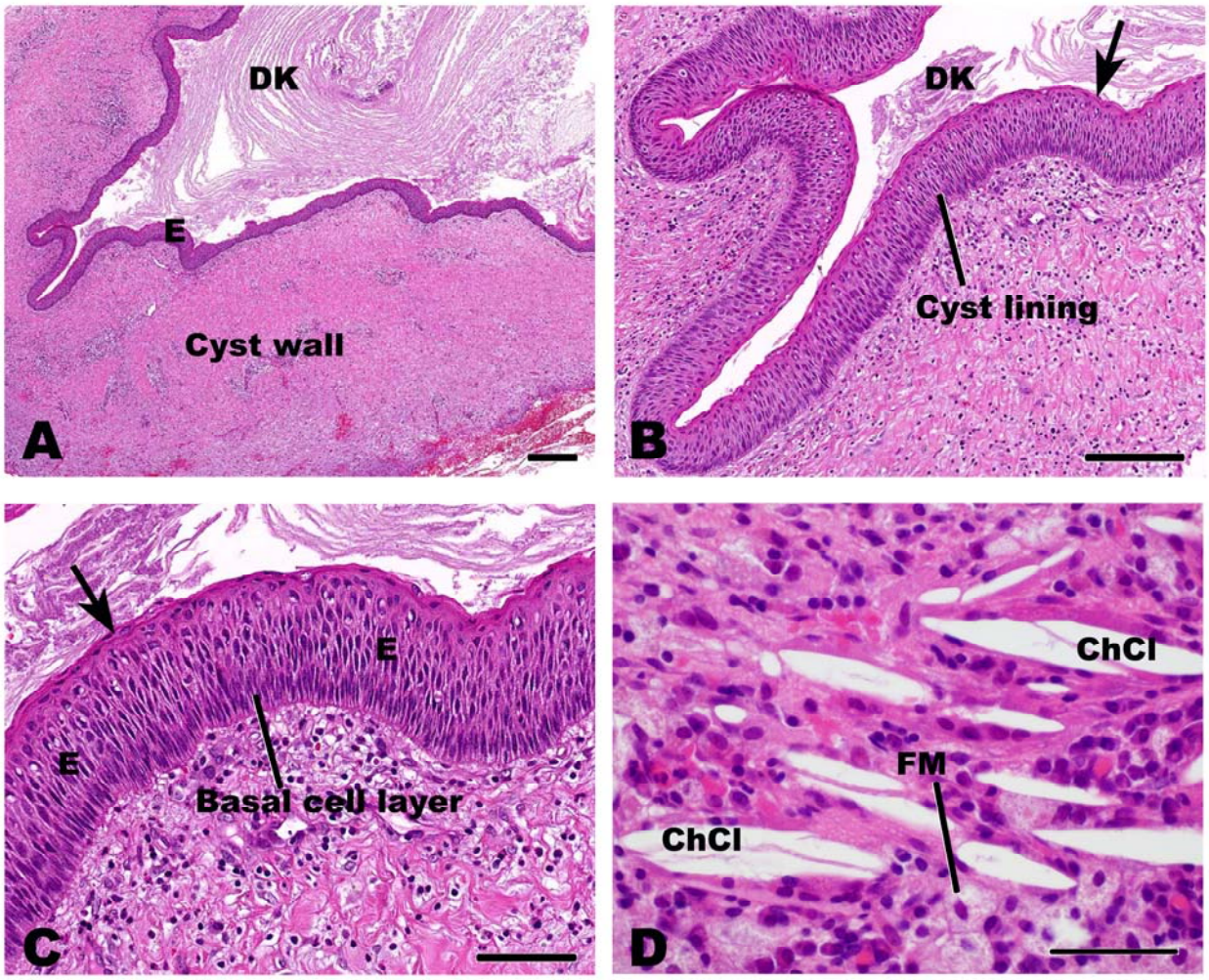

Cyst cavity
E
E
E
E
A
B
C

Figure 3.3. Photograph of a surgically removed dentigerous cyst with attached tooth *(A)*, as well as photomicrographs of paraffin sections showing typical histopathological features of dentigerous cysts *(B-D)*. *(A)* Examination of the removed tooth reveals the cyst wall attached to the cemento-enamel junction separating the tooth crown (C) from the root (R). *(B)* Mural nodules (MN) were occasionally seen comprised of cholesterol clefts penetrating from the cyst wall into the cyst cavity (CC). *(C)* Dentigerous cysts were characteristically lined by a characteristic non-keratinized flattened epithelium (E) approximately 4 cell layers thick, with the cyst wall composed of uninflamed fibrous connective tissue. *(D)* The cyst capsules of inflamed dentigerous cysts, however, showed cholesterol clefts (ChCl), haemosiderin pigments (H) and a chronic inflammatory infiltrate (H&E, Bars for B = 250 μm; C&D = 50 μm).


A
C
R
Cyst wall
cm (mm)
B
MN
CC
Cyst wall
C
Cyst lining
E
D
ChCl
H

Odontogenic keratocyst cavities were filled with desquamated keratinous material, and were lined by parakeratinized stratified squamous epithelium. The basal layers of epithelial linings were palisaded while the parakeratinized surface layers were corrugated in profile. The connective tissue capsule was usually flat and devoid of rete ridges (**Fig. 3.4**). Where inflammation was seen, the lining epithelia lost their characteristic features, although there were always some areas within these specimens with histopathological characteristics typical of keratocysts. Cyst walls were composed of fibrous connective tissue and usually devoid of any inflammation. In a single case only, cholesterol clefts were found in the cyst wall. Also, there were few cases where the cyst wall contained haemosiderin pigment or foamy macrophages (**Fig. 3.4**).

Figure 3.4. Photomicrographs of paraffin sections of odontogenic keratocysts. *(A)* At low magnification, the luminal contents of keratocysts were seen to comprise desquamated keratin material (DK), while the lining epithelium (E) had a typically flat interface with the underlying capsule. *(B)* At higher magnification, the parakeratinized surface epithelium (arrow) of odontogenic keratocysts was more clearly apparent, as was the desquamated keratinized material (DK) in cyst cavities. *(C)* The corrugated parakeratinized epithelial (E) surface layer (arrow) was more readily appreciated at still higher magnification, while the palisaded columnar basal cells with reverse nuclear polarity characteristic of these lesions was also seen. *(D)* In a single case of odontogenic keratocyst, the cyst contained both cholesterol clefts (ChCl) and foamy macrophages (FM). (H&E, Bars for A = 250 μm; B = 100 μm; C&D = 50 μm).

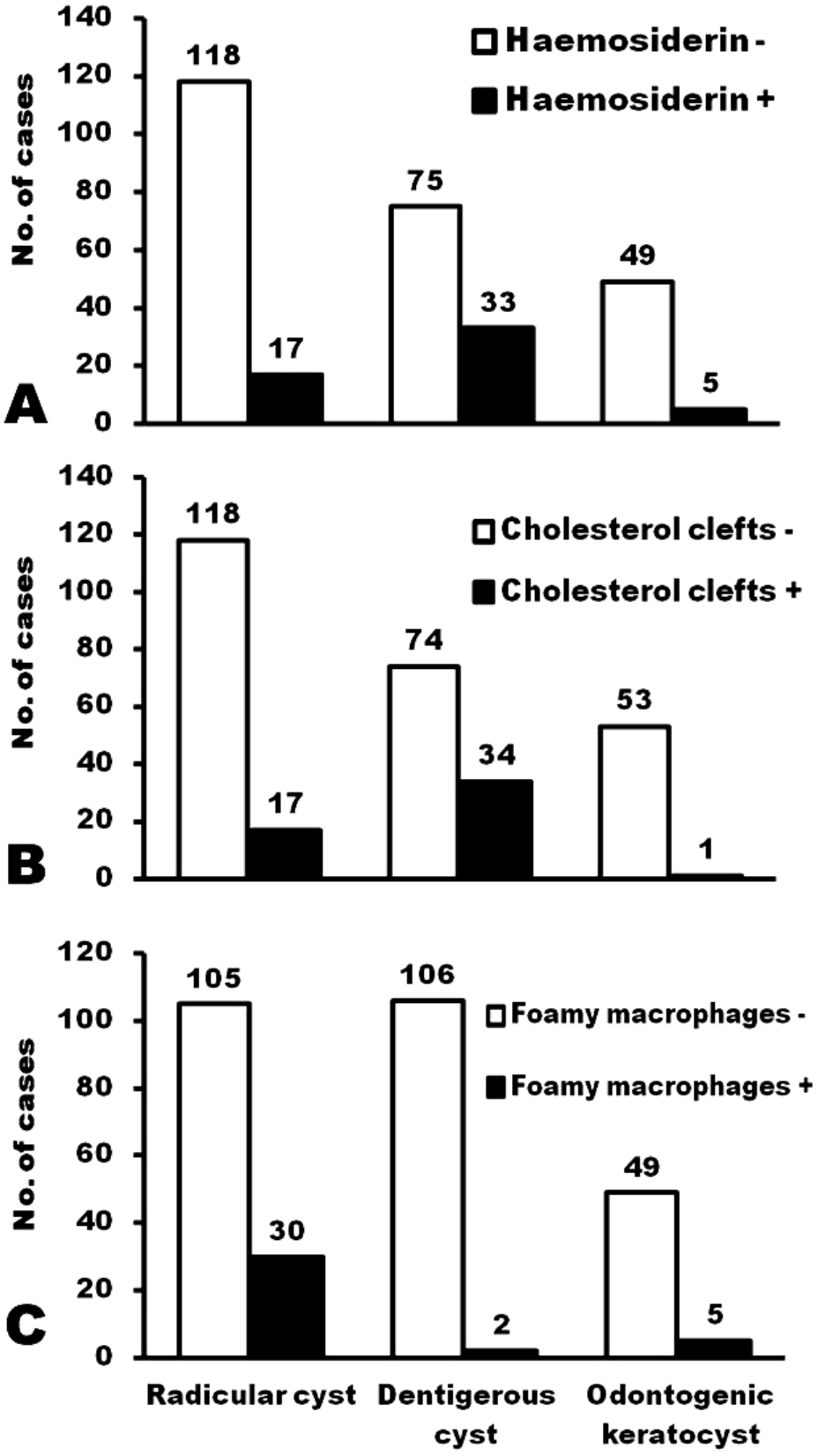

DK
E
Cyst wall
A
DK
Cyst lining
B
E
E
Basal cell layer
C
ChCl
FM
ChCl
D

3.2. Haemosiderin and cholesterol clefts were proportionately more frequently seen in dentigerous than radicular cysts

The proportional frequency of cases with haemosiderin pigments in radicular cysts, dentigerous cysts and odontogenic keratocysts was 12.6%, 30.55% and 9.26% respectively (**Fig. 3.5 A**). There were proportionately more cases with haemosiderin in dentigerous cysts as compared with radicular cysts ($p < 0.001$), as well as compared with odontogenic keratocysts ($p < 0.01$). Separately, there was no statistically significant difference between radicular and keratocysts with regard to the relative occurrence of cases with haemosiderin.

Cholesterol clefts were seen in 12.6% of radicular cysts, 31.5% of dentigerous cysts and 1.8% of odontogenic keratocysts (**Fig. 3.5 B**). There were proportionately more cases with cholesterol clefts amongst dentigerous cysts as compared with radicular cysts ($p < 0.001$), as well as compared with odontogenic keratocysts ($p < 0.001$). There were also proportionately more cases with cholesterol clefts amongst radicular cysts as compared with odontogenic keratocysts ($p < 0.05$).

Figure 3.5.: Histograms showing the number of cases of radicular, dentigerous and odontogenic keratocysts presenting with haemosiderin deposits *(A)*, cholesterol clefts *(B)* and foamy macrophages *(C)*. Proportionally more dentigerous cysts had haemosiderin deposits ($p < 0.01$), as well as cholesterol clefts ($p < 0.01$), compared with both radicular and odontogenic keratocysts. Foamy macrophages on the other hand, were seen in proportionally more cases of radicular cyst compared with both dentigerous and odontogenic keratocysts ($p < 0.05$).


A
No. of cases
140
120
100
80
60
40
20
0
118
17
75
33
49
5
Haemosiderin -
Haemosiderin +
B
No. of cases
140
120
100
80
60
40
20
0
118
17
74
34
53
1
Cholesterol clefts -
Cholesterol clefts +
C
No. of cases
120
100
80
60
40
20
0
105
30
106
2
49
5
Foamy macrophages -
Foamy macrophages +
Radicular cyst
Dentigerous cyst
Odontogenic keratocyst

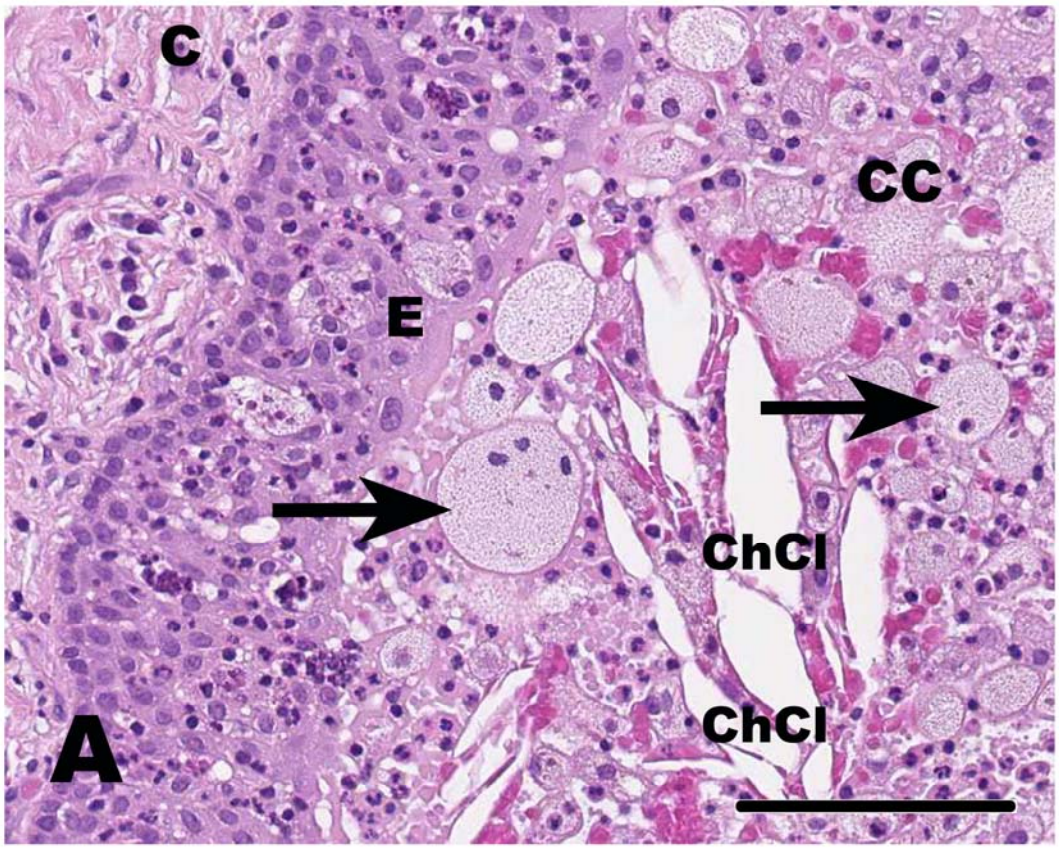
3.3. Foamy macrophages were proportionately more frequently seen in radicular than dentigerous cysts

Foamy macrophages were seen in each type of cyst studied, with proportionately more cases of radicular cyst (22.2%) having foamy macrophages compared with both dentigerous cysts (1.85%) ($p < 0.001$), and odontogenic keratocyst (9.26%) ($p < 0.05$). There were also proportionately more cases with foamy macrophages amongst odontogenic keratocysts compared with dentigerous cysts ($p < 0.05$) (**Fig. 3.5 C**).

3.4. Cholesterol clefts were usually extracellular but also occasionally found inside foamy macrophages

In some cases, cholesterol clefts and foamy macrophages were found in the same specimens and also in the same areas. While cholesterol clefts were usually present in the intercellular areas including cyst cavities (**Fig. 3.6 A**) and cyst capsules (**Fig. 3.6 B**), occasional small slit-like cholesterol clefts were seen within the cytoplasm of foamy macrophages (**Fig. 3.7 A&B**)

Figure 3.6. Photomicrographs of paraffin sections of radicular cysts with foamy macrophages and cholesterol clefts in a cyst cavity *(A)* and cyst wall *(B)*. *(A)* The epithelium (E) was seen to separate the capsule (C) from the cyst cavity (CC). Foamy macrophages (arrows) were readily identified within cyst cavities, as were cholesterol clefts (ChCl). *(B)* Cholesterol clefts (ChCl) were readily identified, while foamy macrophages (arrows) were sometimes found within the cyst capsule. (H&E, Bars = 50 μm).


C
CC
E
ChCl
ChCl
A

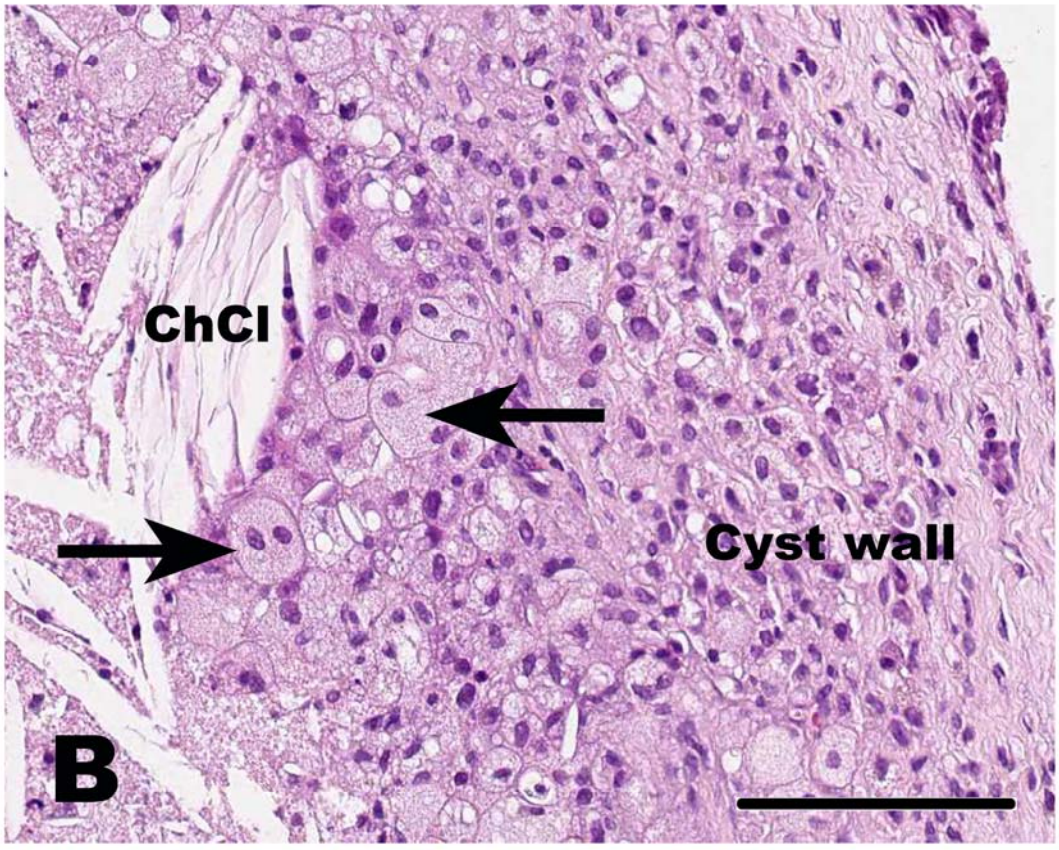
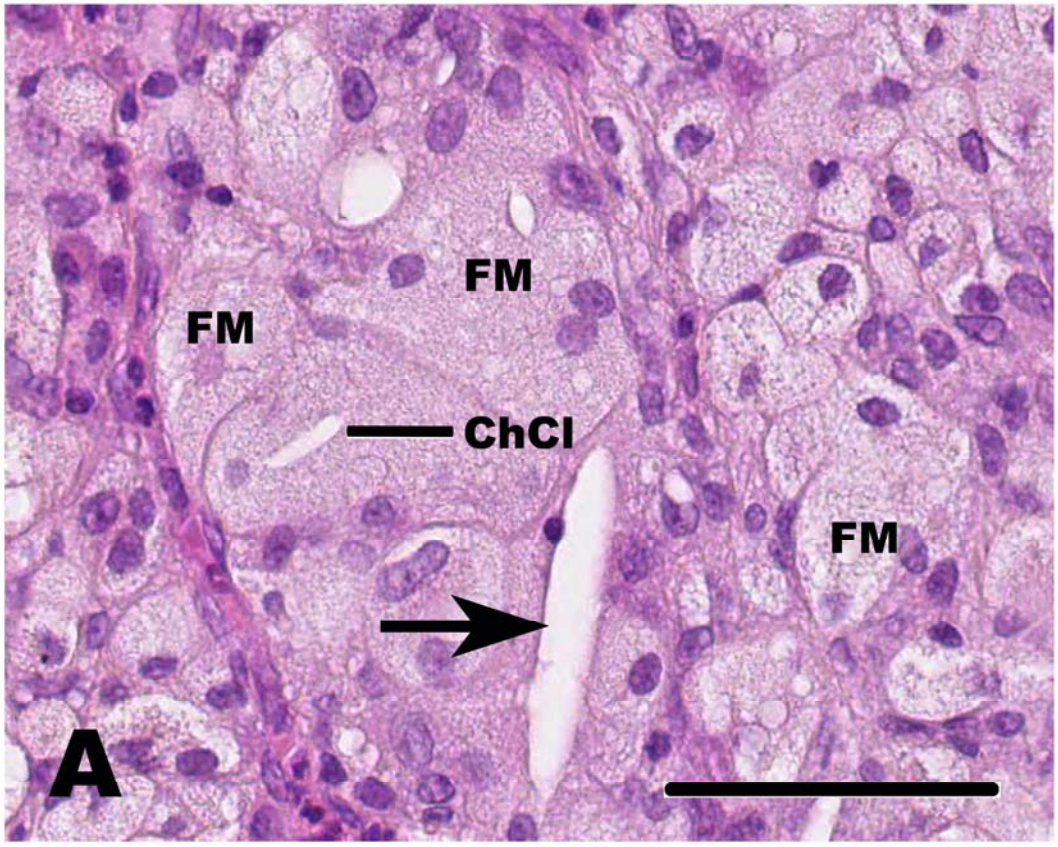
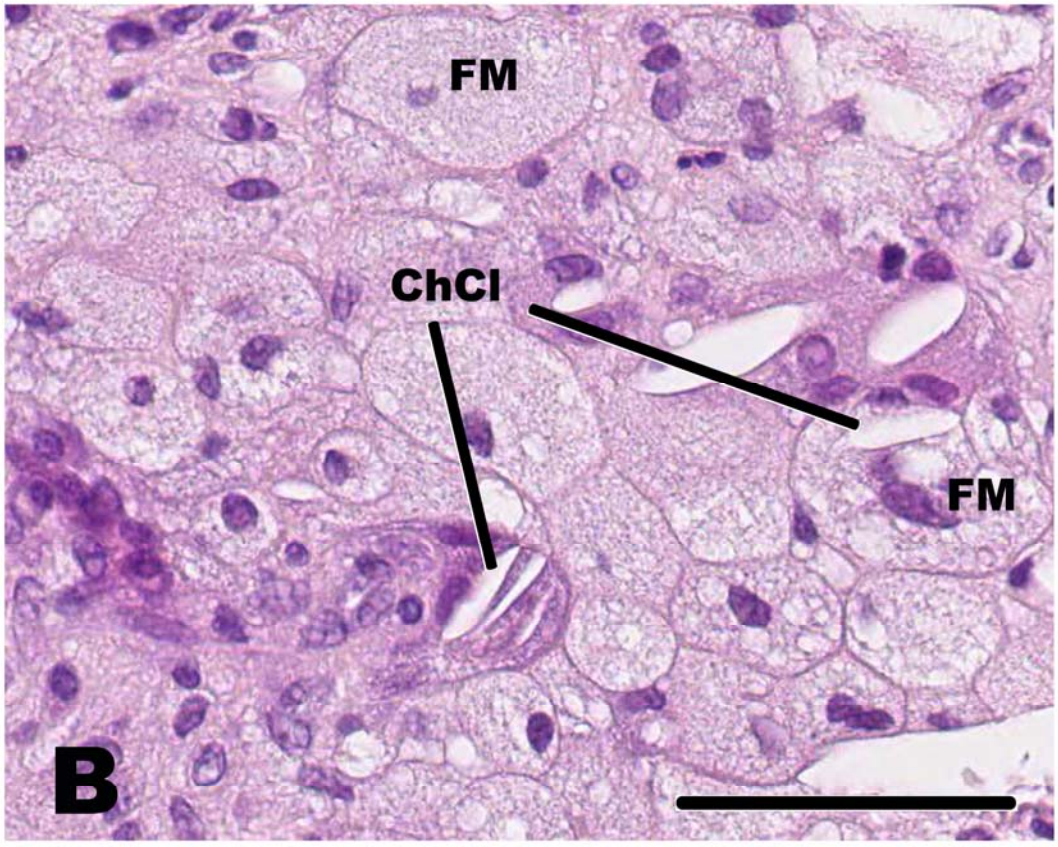

ChCl
Cyst wall
B

Figure 3.7. Photomicrographs of intracellular cholesterol clefts in foamy macrophages. Occasionally foamy macrophages (FM) were found with small cholesterol clefts (ChCl) within their cytoplasm, while larger cholesterol clefts (arrows) appeared outside of foamy macrophages. (H&E, Bars A&B = 50 μm).


FM
FM
ChCl
FM
A


FM
ChCl
FM
B

3.5. Odontogenic cysts with haemosiderin deposits were more likely to have cholesterol clefts and foamy macrophages

As illustrated in **Fig. 3.8**, there was a strong correlation between the presence of haemosiderin and both cholesterol clefts and foamy macrophages. In radicular and dentigerous cysts, haemosiderin was proportionately more prevalent in the presence of cholesterol clefts ($p < 0.001$), and this was more pronounced in dentigerous cysts as compared with radicular cysts ($p < 0.05$). Cholesterol clefts were observed in only one case of odontogenic keratocyst. Further, in radicular cysts haemosiderin was proportionately more prevalent in the presence of foamy macrophages ($p < 0.05$), and this was also the case in odontogenic keratocysts ($p < 0.001$) (**Fig 3.8**). Only two dentigerous cysts were found to have foamy macrophages, so that no clear relationship between haemosiderin deposits and foamy macrophages could be clearly demonstrated in these particular odontogenic cysts. Nonetheless, foamy macrophages were more prevalent in the absence of haemosiderin in radicular cysts as compared with dentigerous cysts ($p < 0.001$).

Since there appears to be an aetiological relationship between foamy macrophages and cholesterol clefts, we also investigated the presence of either foamy macrophages or cholesterol clefts combined with regard to haemosiderin deposits (**Fig. 3.8**). These features of lipid dysregulation were more prevalent in the presence of haemosiderin than without in radicular cysts ($p < 0.001$), dentigerous cysts ($p < 0.001$), and keratocysts ($p < 0.05$). While there was no

statistically significant difference between radicular and dentigerous cysts in this regard, the association between lipid deposits and the presence of haemosiderin was more pronounced in dentigerous cysts than in keratocysts ($p < 0.05$).

The stronger association between haemosiderin deposits and pooled data for cholesterol clefts and foamy macrophages than for either of these lipid deposits alone supports a causal relationship between foamy macrophages and cholesterol clefts.

3.6. Foamy macrophages were more frequently seen in cases where there were cholesterol clefts

Cholesterol clefts were more commonly seen in cases with foamy macrophages than without in radicular cysts ($p < 0.001$), and odontogenic keratocysts ($p < 0.01$) (**Fig. 3.9**). Consistent with the paucity of dentigerous cysts with foamy macrophages, this was not a statistically findings in dentigerous cysts. In the absence of foamy macrophages, however, cholesterol clefts were more prevalent in dentigerous cysts as compared with radicular cysts ($p < 0.001$) and keratocysts ($p < 0.05$) (**Fig. 3.9**). These data support a possible causal relationship between cholesterol clefts and foamy macrophages.

Figure 3.8. Histograms showing the relationship between the presence of haemosiderin deposits and cholesterol clefts and foamy macrophages considered alone or together, in radicular cysts, dentigerous cysts and odontogenic keratocysts. Haemosiderin deposits were more frequently seen in cases where there were cholesterol clefts in both radicular and dentigerous cysts ($p < 0.001$), as well as where there were foamy macrophages in radicular cysts and odontogenic keratocysts ($p < 0.05$). Considering foamy macrophages and cholesterol clefts together, these features of lipid dysregulation were more prevalent in the presence of haemosiderin than in without in radicular cysts (p, 0.001), dentigerous cysts ($p < 0.001$), and odontogenic keratocysts ($p < 0.05$). The association between combined lipid deposits and the presence of haemosiderin was more pronounced in dentigerous cysts than in keratocysts ($p < 0.05$).

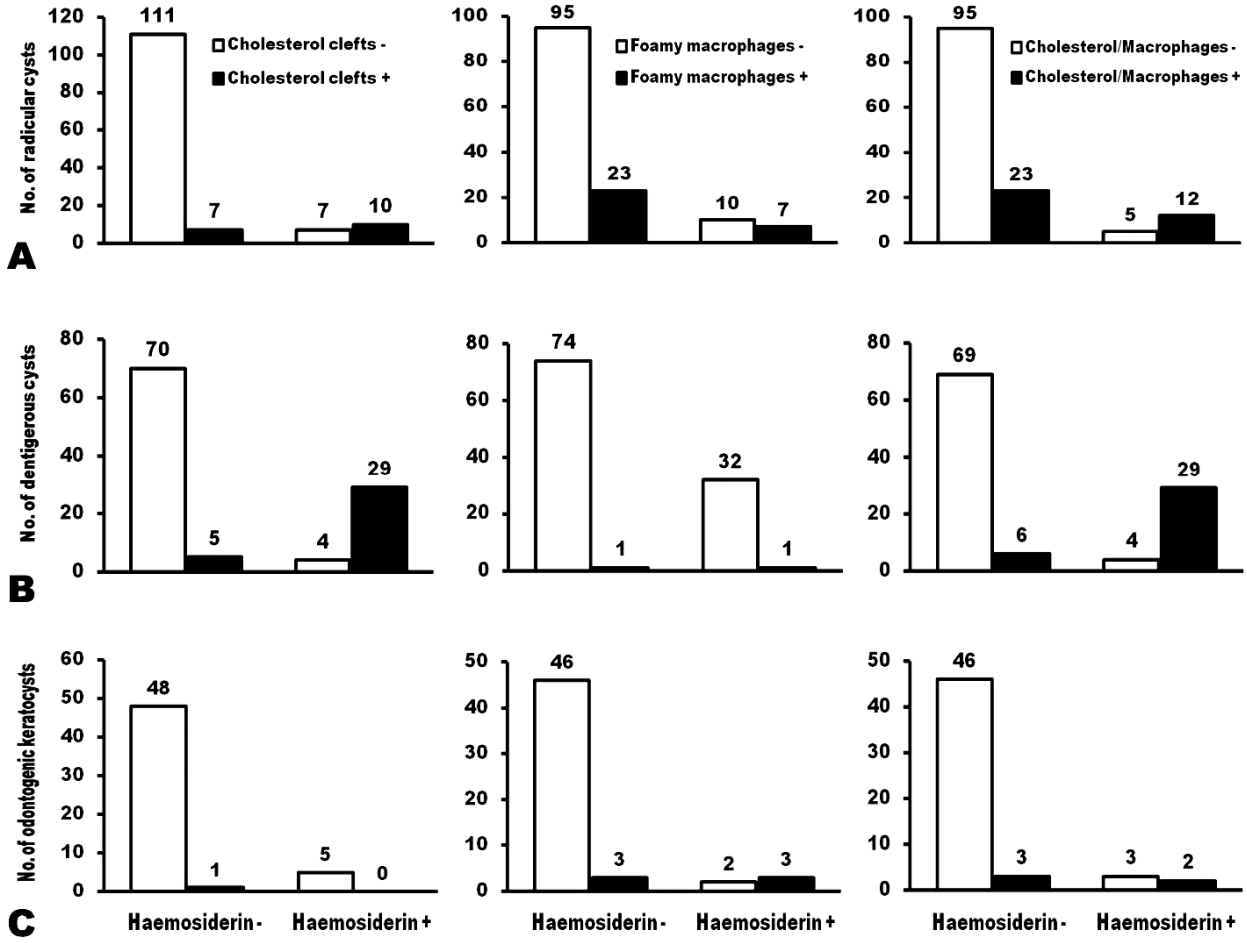
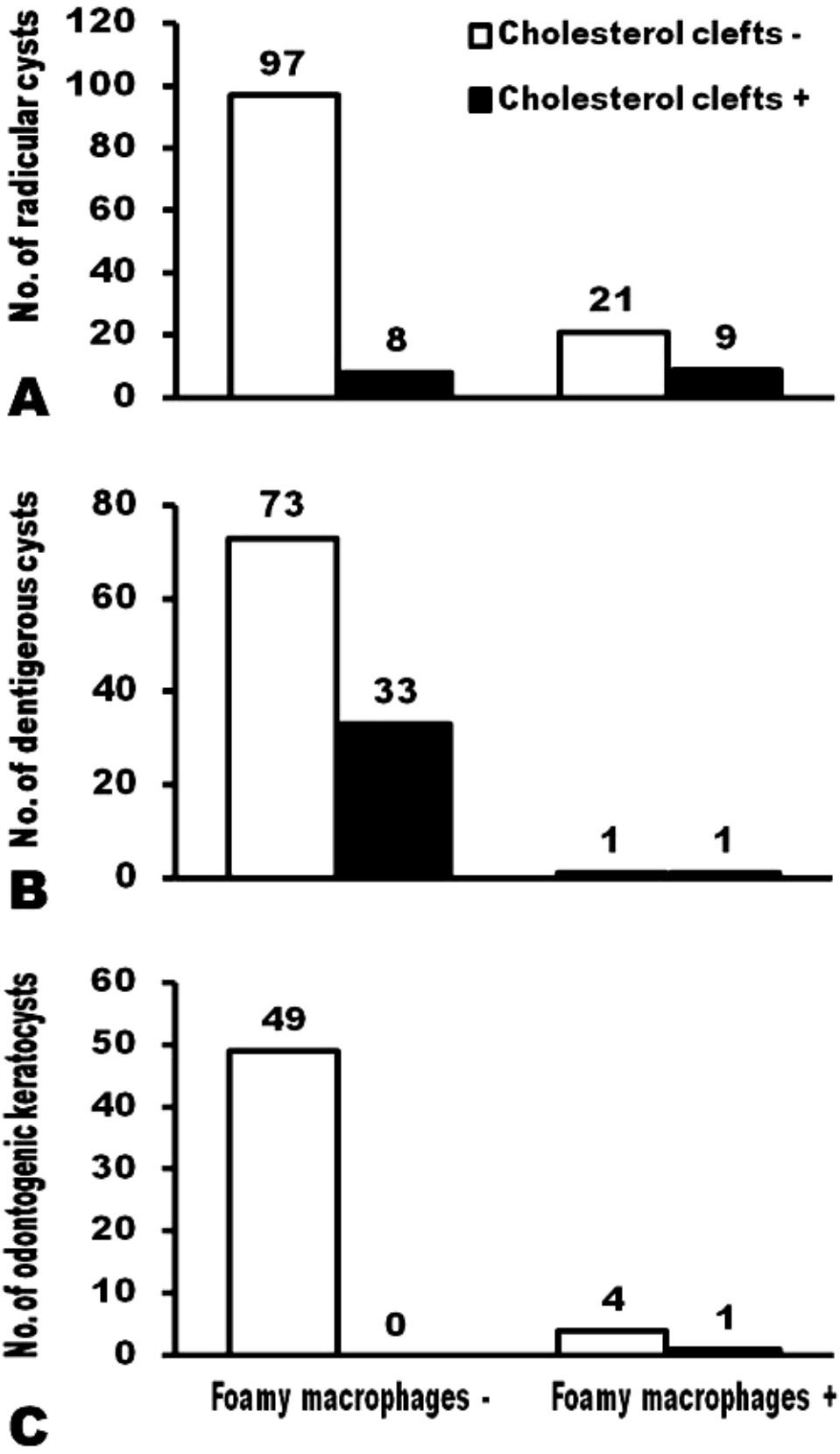

A
No. of radicular cysts
Cholesterol clefts -
Cholesterol clefts +
111
7
7
10
Foamy macrophages -
Foamy macrophages +
95
23
10
7
Cholesterol/Macrophages -
Cholesterol/Macrophages +
95
23
5
12
B
No. of dentigerous cysts
70
5
4
29
74
1
32
1
69
6
4
29
C
No. of odontogenic keratocysts
48
1
5
0
46
3
2
3
46
3
3
2
Haemosiderin -
Haemosiderin +

Figure 3.9. Histogram showing the relationship between foamy macrophages and cholesterol clefts in radicular cysts, dentigerous cysts and odontogenic keratocysts. Cholesterol clefts were more commonly seen in cases with foamy macrophages than without in radicular cysts and odontogenic keratocysts ($p < 0.001$). In the absence of foamy macrophages, cholesterol clefts were more prevalent in dentigerous cysts as compared with radicular cysts and keratocysts ($p < 0.05$).

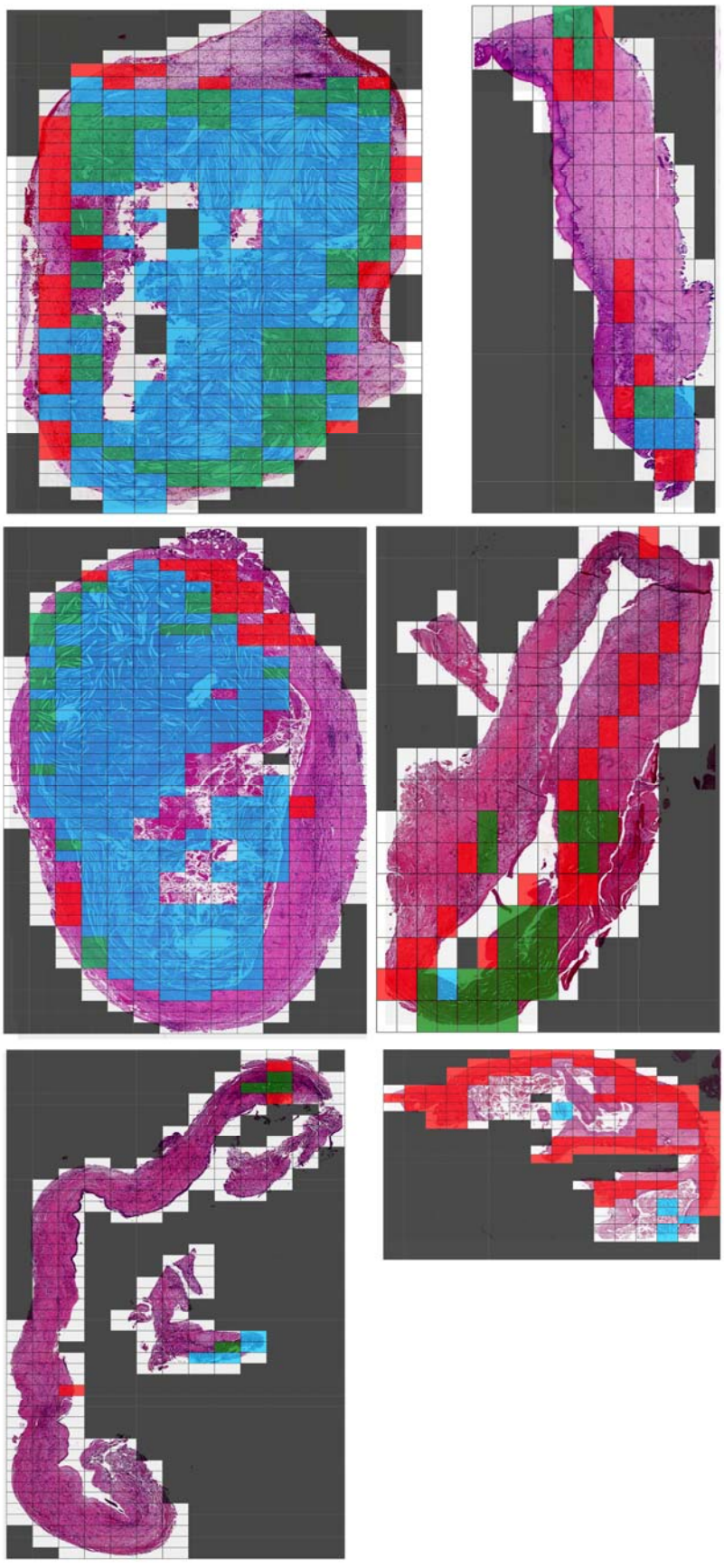

No. of radicular cysts
120
100
80
60
40
20
0
97
8
21
9
Cholesterol clefts -
Cholesterol clefts +
A
No. of dentigerous cysts
80
60
40
20
0
73
33
1
1
B
No. of odontogenic keratocysts
60
50
40
30
20
10
0
49
0
4
1
Foamy macrophages -
Foamy macrophages +
C

3.7. There was no consistent pattern in the spatial distribution of haemosiderin or cholesterol clefts

While the above described data indicate a relationship between haemosiderin deposition and cholesterol clefts, we investigated the spatial relationship between haemosiderin and cholesterol clefts within individual specimens in a further analysis using tissues from six separate radicular cysts. Figure 3.10 shows composite photomicrographs with superimposed grids indicated the location of domains with or without cholesterol clefts and or haemosiderin deposits.

In one specimen, there appeared to be a zonal distribution, with areas predominated by haemosiderin alone merging with separate areas having both cholesterol clefts and haemosiderin, and these merging in turn with separate areas of having only cholesterol clefts. However, this apparent zonal pattern of haemosiderin and cholesterol deposits was not seen in any of the other specimens studied, so that it is not possible to conclude that there is any zonal patterning of haemosiderin or cholesterol deposition.

Figure 3.10. Composite photomicrographs of paraffin sections from six separate radicular cysts with superimposed grids showing the location of areas with haemosiderin alone (red), both haemosiderin and cholesterol clefts (green), cholesterol clefts alone (blue), and areas without tissue (grey). Appreciable areas of sections were occupied by haemosiderin and cholesterol clefts. However, with the exception of a single case (upper left pannel), no clear zonal distribution of the deposits studied was seen.



3.8. There was correlation in the spatial location of cholesterol clefts and haemosiderin deposits in most radicular cyst specimens analysed

The histogram in figure 3.11 shows the number of grid domains identified with or without cholesterol deposits, haemosiderin deposits or both, in six separate radicular cysts. Although in two specimens, there was a negative correlation between the location of haemosiderin and cholesterol clefts (**Fig. 3.11 A & B**) ($p < 0.001$), cholesterol clefts were more commonly seen in areas containing haemosiderin in all four remaining specimens (**Fig. 3.11 C,D,E & F**) ($p < 0.001$). When data from all six specimens were pooled (**Fig. 3.12**), the positive correlation between location of haemosiderin deposits and cholesterol clefts was still apparent ($p < 0.001$).

Confirming the approach used to identify haemosiderin deposits in H&E sections, was a repeated analysis of near serial paraffin sections of three cases stained with Perl's Prussian blue specific for haemosiderin (**Fig. 3.13**). No significant difference was seen in data derived from sections stained with Perl's Prussian blue compared with data where haemosiderin was identified in H&E sections (**Fig. 3.14**).

Figure 3.11. Histograms showing the co-location or otherwise of cholesterol clefts and haemosiderin in H&E stained paraffin sections of six radicular cysts. While in two separate cases *(A,B)*, a negative relationship was seen between the location of haemosiderin deposits and cholesterol clefts, there were strong correlation between the spatial position of cholesterol clefts and haemosiderin deposits in the remaining 4 cases (C,*D,E, F)* ($p < 0.001$).

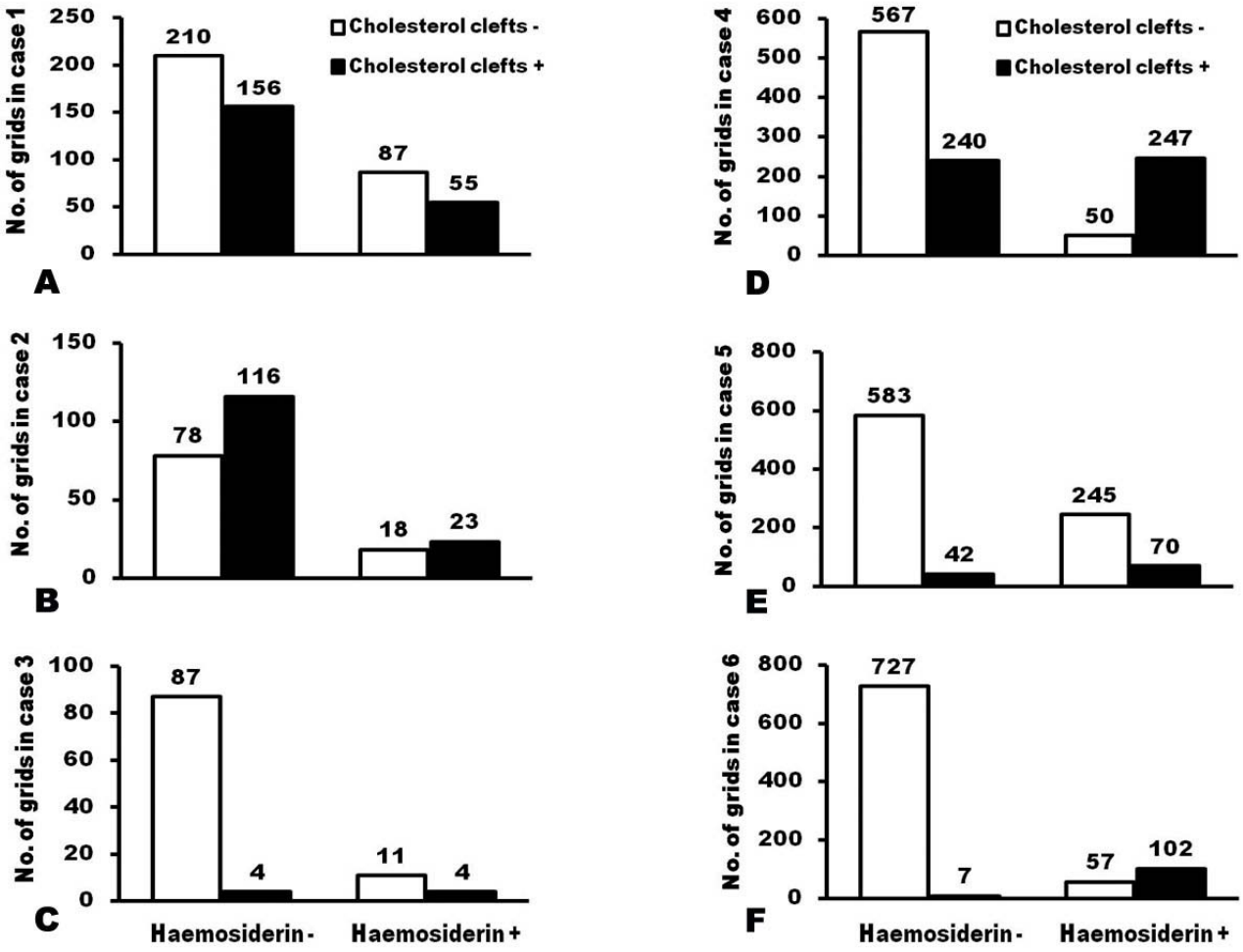

No. of grids in case 1
250
200
150
100
50
0
210
156
87
55
Cholesterol clefts -
Cholesterol clefts +
A
No. of grids in case 4
600
500
400
300
200
100
0
567
240
50
247
Cholesterol clefts -
Cholesterol clefts +
D
No. of grids in case 2
150
100
50
0
78
116
18
23
B
No. of grids in case 5
800
600
400
200
0
583
42
245
70
E
No. of grids in case 3
100
80
60
40
20
0
87
4
11
4
Haemosiderin -
Haemosiderin +
C
No. of grids in case 6
800
600
400
200
0
727
7
57
102
Haemosiderin -
Haemosiderin +
F

Figure 3.12. A histogram showing pooled data for the co-location or otherwise of cholesterol clefts and haemosiderin in H&E stained paraffin sections of six radicular cysts. There was strong correlation between the spatial position of cholesterol clefts and haemosiderin deposits ($p < 0.001$).

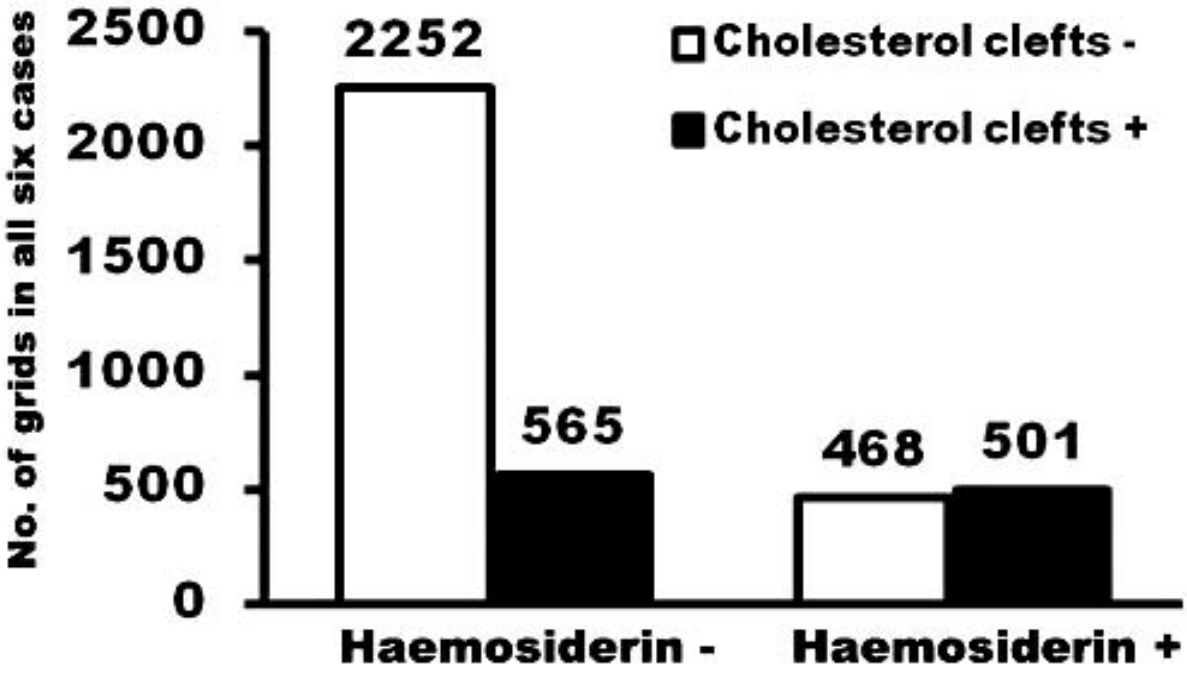
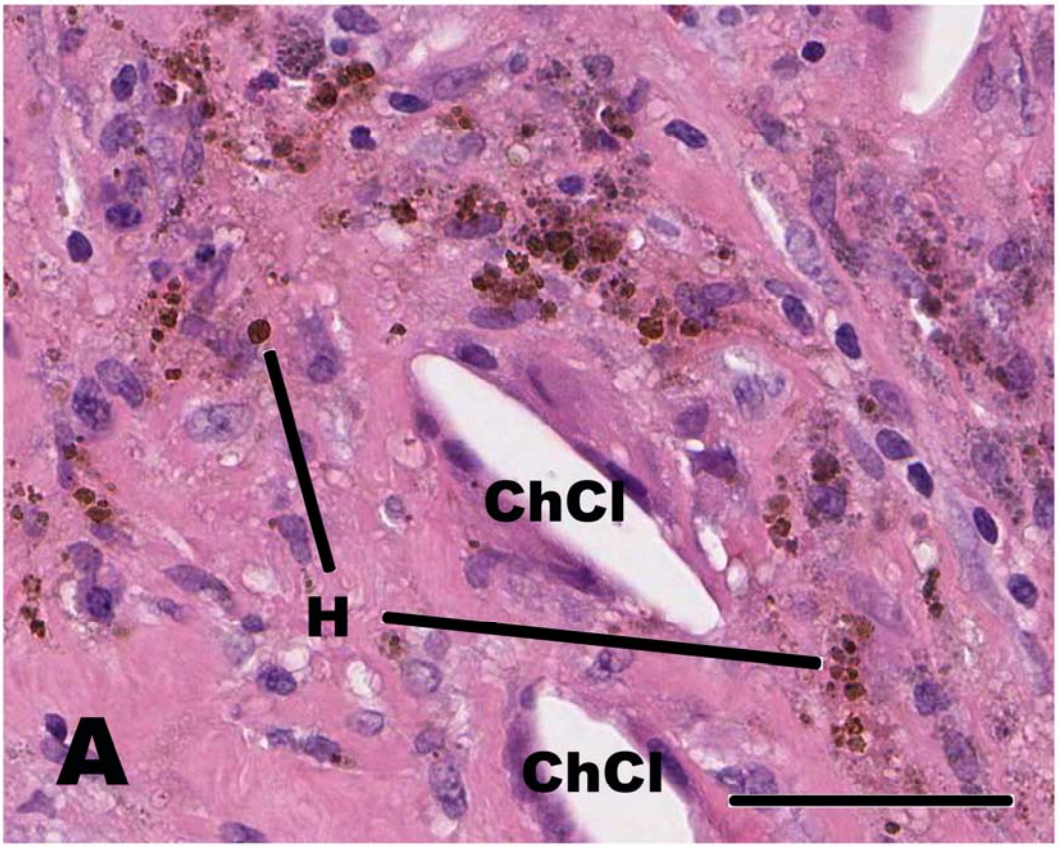

No. of grids in all six cases
2500
2000
1500
1000
500
0
2252
565
468
501
Cholesterol clefts -
Cholesterol clefts +
Haemosiderin -
Haemosiderin +

Figure 3.13. Photomicrographs of near serial paraffin sections of a radicular cyst stained by H&E (A) and Perl's Prussian blue (B). The location of golden brown haemosiderin pigment (H) in the H&E stained section (A) correlates well with that of the strong Prussian blue stain (B). Cholesterol clefts (ChCl) were readily identified in sections stained by both methods. (H&E, size bar A = 50 μm; Prussian blue, size bar B = 50 μm).


ChCl
H
A
ChCl

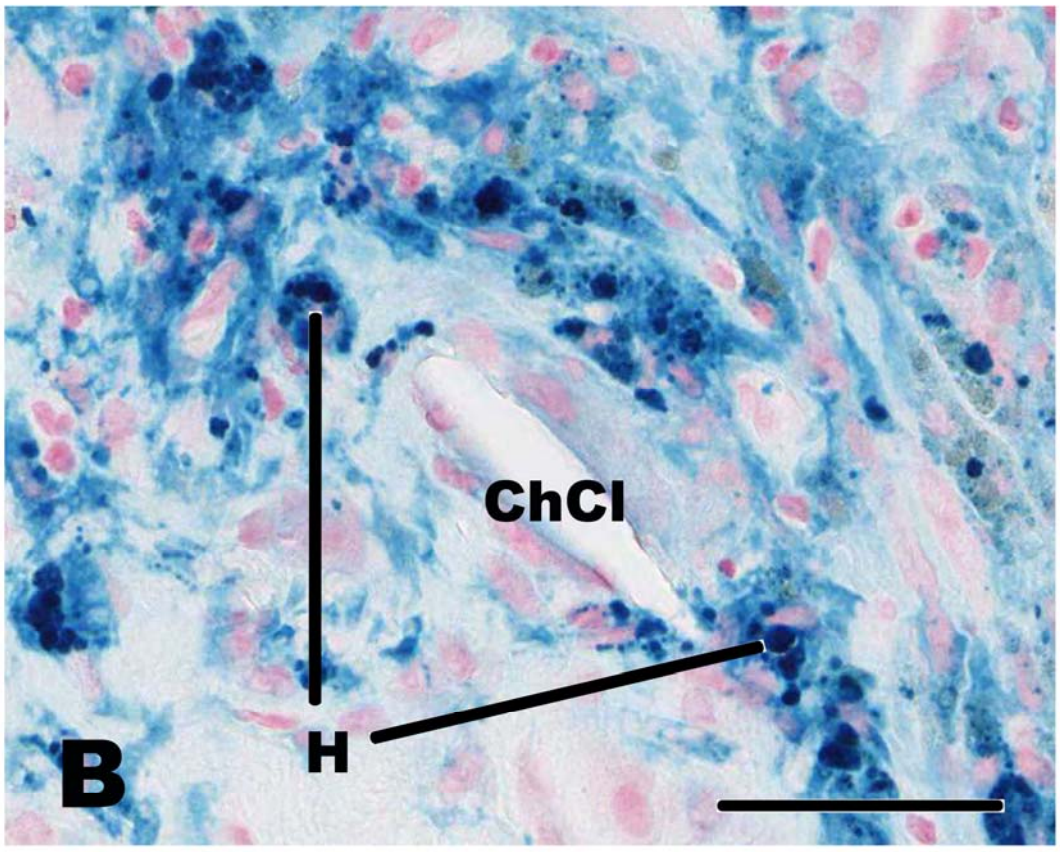
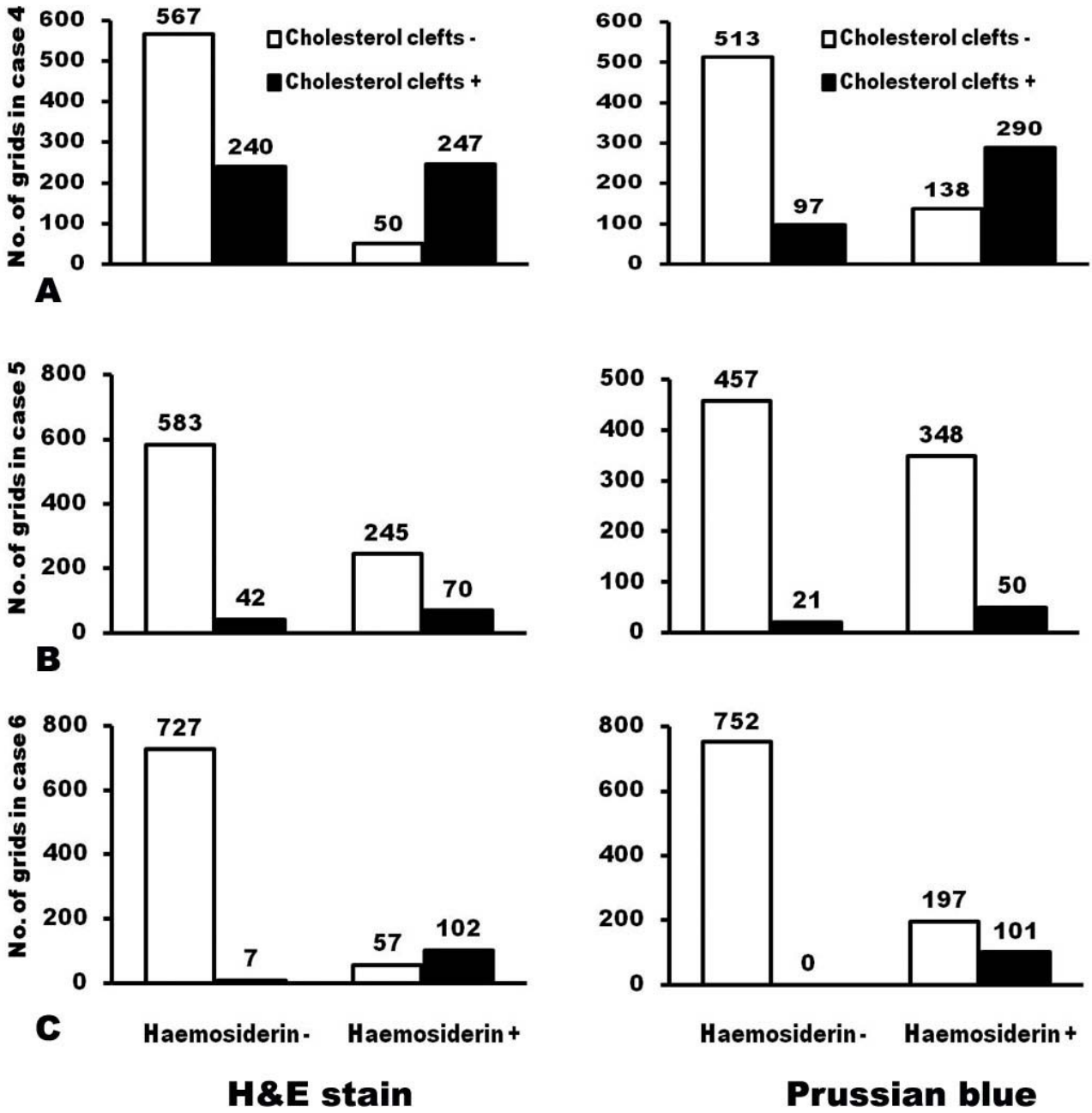

ChCl
H
B

Figure 3.14: Histograms showing the distribution of cholesterol clefts and haemosiderin in H&E as well as Perl's Prussian blue stained paraffin sections of three radicular cysts. While in each of the three cases examined by Prussian blue staining, there was strong correlation between cholesterol clefts and the location of haemosiderin ($p < 0.001$), there was no clear difference in the data obtained from H&E as compared with Prussian blues stained sections.


No. of grids in case 4
600
500
400
300
200
100
0
567
240
50
247
Cholesterol clefts -
Cholesterol clefts +
A
513
97
138
290
No. of grids in case 5
800
600
400
200
0
583
42
245
70
B
500
400
300
200
100
0
457
21
348
50
No. of grids in case 6
800
600
400
200
0
727
7
57
102
C
752
0
197
101
Haemosiderin -
Haemosiderin +
H&E stain
Prussian blue

CHAPTER 4

DISCUSSION

4.1. Discussion

Routine H&E stained histological sections were used throughout this thesis for identification of both haemosiderin deposits and cholesterol crystals, and it is acknowledged that not all brown stains in tissues are necessarily haemosiderin, formalin deposits for example occasionally creating similar appearing artefacts . Nonetheless, the granular golden-brown appearance of intracellular deposits seen in the current study are widely accepted as characteristic for haemosiderin in H&E sections (Iancu, 1992). Although it was not possible to perform the more specific Perl's Prussian Blue stain for haemosiderin in all sections studied (Stevens and Chalk, 1997; Stevens and Wilson, 1997), when this specific stain was used in three specimens, the strong correlation between Prussian Blue positive material and brown deposits identified as haemosiderin in H&E sections, supported the approach used in the current study.

Similarly, loss of cholesterol by routine paraffin tissue processing precluded confirmation of the specific composition of cholesterol clefts and foamy macrophages in the current study . Nonetheless, histological features with an identical appearance have been carefully characterized at the chemical level by others , and in light of the extensive literature accepting these structures in odontogenic cysts as lipid materials (Browne, 1971; Shear, 1963; Shear and Speight, 2007; Skaug, 1976; Thoma et al., 1970), it seems reasonable to conclude that cholesterol clefts and foamy macrophages in the current study are essentially similar to those described elsewhere.

The numerical and statistical approach used to analyse the relationship between haemosiderin deposits, cholesterol clefts and foamy macrophages used the widely accepted Chi-squared test for differences in proportion, and this statistical strategy is accepted as appropriate for non-parametric, independent nominal level data , considering each case studied as independent of the others. This approach for numerical analysis can be justified on first principals, and has been used by others in similar studies (Browne, 1971). However, it is recognized that pooled data from six separate specimens shown in Figure 3.12 may breach the requirement for independence of samples, in that areas within individual specimens are differently related to each other as compared with areas in separate specimens. Nonetheless, on balance the approach used seems reasonable, providing confidence in the conclusions drawn from the current study.

This project was initiated with the expectation that no clear correlation between haemosiderin deposits and either cholesterol clefts or foamy macrophages would be found. However, this expectation was not supported by the data collected, so that the earlier reported correlation between cholesterol clefts and haemosiderin is supported for both radicular and dentigerous cysts (Browne, 1971).

One difference from the earlier study by Browne (1971), is that this correlation between haemosiderin and cholesterol clefts was not seen in odontogenic

keratocysts in the current study. It is interesting that although the current study investigated 54 odontogenic keratocysts compared with 41 in the earlier study (Browne, 1971), that only one case in the current series had cholesterol clefts compared with 6 recorded by Browne (1971), and this may reflect differences in the incidence of infection of odontogenic keratocysts between the two studies.

There are no published reports of the spatial relationship between cholesterol clefts and haemosiderin deposits, as described in data of the current thesis (Figs 3.10, 3.11, 3.12). No clear zonal relationship between cholesterol clefts and haemosiderin deposits was apparent in sections, although the correlation between haemosiderin and cholesterol clefts was supported in terms of co-location in 4 out of 6 separate radicular cysts studied. One deficiency of the current study is the absence of an identical spatial analysis for the distribution of foamy macrophages, and it would be interesting to repeat the current analysis including this important criterion.

Despite the absence of a spatial analysis of the distribution of foamy macrophage in the current study, one novel aspect of this thesis not reported elsewhere, is clear correlation between the presence of foamy macrophages and cholesterol clefts. Also, intracellular cholesterol clefts have not been described in odontogenic cysts, and are here recorded similar to intracellular clefts in atherosclerosis .

Similarly, a further novel aspect of the current study not previously reported, is that there is correlation between the presence of foamy macrophages and haemosiderin in odontogenic cysts.

It would be interesting to extend the current study to include more specimens in the spatial analysis of cholesterol clefts, haemosiderin deposits and foamy macrophages, as the current sampling of 6 radicular cysts clearly fails to include dentigerous cysts. Also, despite the striking statistical significance of results shown, a sample of only six specimens seems insufficient to make robust conclusions.

Although the current work confirms an association between haemosiderin and lipid deposits, it seems reasonable to remain sceptical of a causal relationship between these two as earlier inferred (Browne, 1971), since it is also possible that both of these deposits arise independent of one another in response to inflammation. It would be interesting to further probe the chemical nature of lipid deposits in cholesterol clefts and foamy macrophages in odontogenic cysts using frozen sections as described by others. Similarly, it would be interesting to characterize the distribution of LDL, oxidized LDL and lipoprotein lipase in such sections by immunohistochemistry, as well as by Western blot analysis. It is strongly suspected that such further study would reveal an origin for these lipid deposits from plasma components, similar to atherosclerosis .

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