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MASTER THESIS

IMMUNOHISTOCHEMICAL STUDY ON THE EXPRESSION OF NOTCH-1, NOTCH-2 RECEPTORS, JAGGED-1 LIGAND AND HERP-1 TRANSCRIPTION FACTOR ON HUMAN PERIAPICAL CYSTS

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To my family

Table of Contents

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2. Review of Literature

2.1. Apical periodontitis

2.1.1. Introduction

2.1.2. Bacterial portals of entry
i. Root canal microflora

2.1.3. Classification

2.1.4. Diagnosis

2.1.5. Pathogenesis of apical periodontitis

2.1.1. INTRODUCTION

Apical periodontitis is a general term used to describe an inflammatory process in the periapical tissues induced by the presence of microorganisms, especially their noxious byproducts, toxins and infected necrotic pulp remnants in the root canal system. (Kakehashi et al. 1965, Moller et al. 1981, Sundvist 1976)

More specifically, Kakehashi (1965) reported that no periapical lesion could be developed in germ-free rats whose pulps were exposed in the oral environment, in contrast to the control rats with the common microflora of the oral cavity, in which periapical lesions were developed. The important role of bacteria as the cause of apical periodontitis was further stated by Sundqvist in 1976. On a microbiology study on 32 traumatized anterior teeth Sundqvist reported that apical periodontitis can only be detected in teeth with bacteria present in canal systems. Necrotic, but sterile pulp cannot induce inflammatory reaction to periradicular tissues, and consequently apical periodontitis, in contrast to the necrotic and infected teeth. Additionally, Moller and colleagues (1981) found that the predominant species in the necrotic pulp are obligate anaerobes and reported the significance of asepsis during sampling and cultivation techniques.

2.1.2. BACTERIAL PORTALS OF ENTRY

Bacteria are not normally present into a normal and intact dentin-pulp complex. If dentin-pulp complex become exposed to the oral environment, bacteria may enter the root canal system, causing pulp inflammation, pulp necrosis and gradually apical periodontitis. Common routes of root canal infection are caries lesions, cracks, trauma, attrition, abrasion, deep cavity/crown preparations or leaking fillings. Deep periodontal pockets may also allow bacteria penetration to exposed dentinal tubules, accessory canals or even to the apical foramen (Langland et al. 1974). Furthermore, bacteria may be found in teeth with necrotic pulp and intact crowns due to previous trauma (Bergenholtz 1974). “Anachoresis” is a theory that has been proposed, but not scientifically established for endodontic infection. Specifically, the term “anachoresis” describes the infection of the necrotic pulp via blood circulation during transient bacteremia (Gier et al. 1968). However, this phenomenon could not be reproduced experimentally, when Delivanis and colleuagues (1985) infected the bloodstream, and tried to recover bacteria from the root canal system. A recent study (Love et al. 2002) suggests that macro- and micro-

cracks are more likely to be the possible mechanism of infection in traumatized teeth. A single crack may expose a large number of dentinal tubules to the oral environment, and therefore promote bacterial invasion into the previously necrotic pulp, considering the absence of the protective mechanism that the outward movement of the dentinal fluid offers to a vital pulp.

i. Root canal microflora

Sundqvist (1994) found a wide diversity of bacteria in infected root canals, predominately obligate anaerobes. Recent studies confirmed these results using newer molecular techniques (Sakamoto et al. 2006). In a primary infection common microbial composition includes gram-negative bacteria species, such as Fusobacterium, Porphyromonas, Prevotella, Tannerella Forsithia, Treponema, Dialister, Campylobacter, Veillonella and Selomonas, and gram-positive species, such as Streptococcus, Peptostreptococcus, Actinomyces, Lactobacillus, Bifidobacterium, Prorionibacterium and Eubacterium.

Yeasts of the genus Candida were also found in root canals exposed to the oral environment (Sen et al. 1995), as well as in root filled teeth with treatment-resistant apical lesions in up to 18% of the cases (Egan et al. 2002). Recent studies (Siqueira 2005, 2005) using new molecular techniques to detect microbial species associated with endodontic treatment failures revealed a wide range of diversity, with a significant proportion (55% of the taxa) of bacteria which are described as-yet-uncultivated. Bacterial taxa other than E. faecalis are, among others, Pseudoramibacter alatolyticus, Propionibaterium propionicum, Filifactor alosis, Dialister Pneumonosintes, Bacteriodetes clone X083, Streptococcus species and Tannerella forsythia.

Enterococcus faecalis is gram-positive facultative anaerobic, enteric bacterium which is rarely found in primary infections. However, it has been highly associated with failed endodontic treatment (Molander et al. 1998), teeth treated in multiple visits or left open for drainage (Siren et al. 1997). This microorganism has the ability to penetrate far into dentinal tubules and avoid contact with intracanal instrumentation and irrigants (Haapasalo et al. 1987). Also, it has been shown to be able to form biofilms in root canals, which is an important ability for bacterial resistance and persistence in the instrumented canals (Distel et al. 2002). E. Faecalis is able to resist to $Ca(OH)_2$, due to a functioning proton pump, which enables the cell to import protons in order to acidify its cytoplasm, thus become highly resistant to

high pH values (Evans et al. 2002). Another factor contributing to its resistance is the ability to survive in environments with insufficient nutrients by entering a viable but non-cultivable state and recover vigorously, even after 12 months of starvation. (Figdor et al. 2003)

The aim of the root canal treatment is to interrupt and eliminate bacteria densely accumulated and firmly attached to the root canal walls and dentinal tubules (Sen et al. 1995). In order to survive in the root canal system, these microorganisms tend to form communities (Costerton 2007). These heterogenic communities are known as biofilm and can be defined as a sessile multicultural community characterized by cells that are firmly attached to a surface (dentin walls), submerged into a matrix of extracellular polymeric substance of host and microbial origin (mainly polysaccharide), and separated by water channels or pores. These channels facilitate movement of nutrients, removal of metabolic waste products and transportation of the bacterial cells to new colonization sites (Costerton et al. 1999, Socranski et al. 1963, Costerton 2007). Biofilms are dynamically organized ecological communities which have evolved to support community as a whole though defense mechanisms against competing microorganisms, host defenses, antimicrobial agents and environmental stress. Contrary to biofilms, planktonic microorganisms, which are free-floating bacteria existing in an aqueous environment, can be easily eliminated. In addition, biofilms display metabolic cooperativity and as mentioned before, a primitive circulatory system via water channels.

Apical periodontitis lesion may be assumed as an inherent defense mechanism aiming to protect the surrounding alveolar bone and tissues from the infective material existed in the apical part of the root canal, acting like an effective barrier. Ricucci and colleagues (2006) proposed that bacteria are not normally found in the periapical lesions. In case of massive microbial extrusion, either in a free-floating state or inside phagocytes or both, in the periapical tissues through the apical foramen, an apical acute abscess may develop (Siqueira et al. 2001). Additionally, bacterial may be present in infected radicular cysts, especially when the cavity is in direct communication with the root canal system (pocket cysts) (Nair 1997), in apical root cementum due to colonization of bacteria biofilms (Lomcali et al. 1996) and yeasts (Tronstad et al. 1990), in periapical actinomycosis (Sundqvist et al. 1980), or in case dentinal chips, debris or materials are forced out of the apical foramen during root canal instrumentation (Holland et al. 1980). However, difficult-to-culture bacteria are often missed using traditional culture methods, resulting in an underestimation of the bacterial diversity in periapical lesions. Taking into consideration that apical

periodontitis has a heterogeneous etiology with multiple species implicated, novel molecular methods need to be used in order to shed light on the existence and composition of periapical tissues' microbial communities. Recently, towards this approach, Zakaria and colleagues (2014) used 16S rRNA gene with the clone library method, which gives the ability to detect and identify even difficult-to-culture bacteria down to species level, and they detected bacteria in all the examined periapical lesions.

2.1.3. CLASSIFICATION

The World Health Organization (WHO 1995) has classified apical periodontitis in five categories:

- Acute apical periodontitis of pulpal origin
- Chronic apical periodontitis of pulpal origin
- Periapical abscess with sinus
- Periapical abscess without sinus
- Radicular cysts.

Due to the fact that this classification does not take into account the histological and structural characteristics of periapical lesions, Nair (1997) proposed an alternative classification, which was strictly based on the histopathology criteria only, such as a) distribution of inflammatory cells, b) presence or absence of epithelial cells, c) cystic transformation of the lesion, and d) the relation of the cyst lumen to the root canal system. Nair's classification of periapical radiolucencies is as follows:

- Acute apical periodontitis – primary or secondary
- Chronic apical periodontitis
- Apical abscess – acute or chronic
- Periapical cyst – true or pocket

2.1.4. DIAGNOSIS

It is very well established that the clinical diagnosis of the true histological status of apical periodontitis cannot be made, unless biopsy is performed. Apical periodontitis lesions appear radiographically as radiolucent areas at the apex of the infected root apex. A periapical lesion cannot be differentially diagnosed into cystic or

not lesion in the basis of radiographs only (Priede et al. 1954, Lalonde 1970, White et al. 1994). Complete absence of symptoms and radiographic signs is not necessarily indicative of absence of apical periodontitis too. Many studies have attempted to correlate the clinical signs and symptoms with the histological findings but not with definite results. Mortensen and colleagues in 1970 suggested that cysts are larger than granulomas, but this statement was not supported by subsequent scientific evidence.

Recently, Ultrasound real-time imaging (echography), together with the application of Color Power Doppler (Aqquarwal et al. 2008), Ultrasound (Gundappa et al. 2006) and Cone- Beam Computed Tomography scans (Simon et al. 2006) are proposed for preoperative diagnosis between granulomas and cystic lesions based, but further investigation on this field is mandatory. Even these novel techniques are not able to distinguish different inflammatory cells or stages of inflammatory processes.

It is widely accepted that many other types of lesions in the jaws may present as ‘periapical pathosis’ and should be considered as part of the differential diagnosis, such as odontogenic keratocyst, nasopalatine duct cyst, dentigerous cyst, nasolabial cyst, lateral periodontal cyst, glandular odontogenic cyst, odontogenic and non-odontogenic neoplasms and tumors, foreign body reaction and wound healing with scar tissue.

2.4.5. PATHOGENESIS OF APICAL PERIODONTITIS

Apical periodontitis is a result of an inflammatory response in the periapical tissues, evoked by bacteria, bacterial toxins (lipopolysaccharide- LPS, lipoteichoic acid- LTA) and noxious metabolic by-products (Dahlén et al. 1981). Endotoxin (LPS) is released during disintegration of gram-negative bacteria after death or during multiplication, and can activate innate immune system. As gram-negative bacteria are predominantly found in infected root canals, it is not surprising that they may die or multiply in the apical area of the root canals, thus releasing lipopolysaccharide, which may exit the apical foramen and evoke or sustain an inflammatory process in the periradicular tissues. This process represents a protective and destructive inflammatory reaction which also involves the surrounding bone of the infected tooth apex.

The initial periapical response to bacterial presence within the canal or to bacterial invasion of the periapical region will be an acute inflammatory response,

known as primary acute apical periodontitis (Nair 1997). In acute apical periodontitis the pulp may be irreversibly inflamed or necrotic, and it contains PMNs and monocyte/macrophages-like cells within a localized area at the apex. The bone and root resorption is not yet extensive enough to be detected in radiographs. If the pathogens in the root canal space are not eliminated, acute apical periodontitis may progress to become into chronic apical periodontitis**.** The first line of host defense against the bacterial invasion through apical foramen is provided by PMNs, which, by active phagocytosis and by releasing their proteolytic enzymes, effectively keep the bacterial amount and penetration as low as possible. Monocytes/macrophages also participate in the primary phagocytic protective phase and secrete antigens to act as chemotactic stimuli and to recruit other cells to participate in the inflammatory reaction (Márton et al. 2000).

The host defense responds to the noxious by-products of the infected root canal space forming a defensive granulation zone. Histologically, chronic apical periodontitis is characterized by fibrous and granulation tissue, proliferating epithelium or cyst infiltrated by a variety of inflammatory cells (Gao et al. 1988)

Chronic apical periodontitis is predominantly infiltrated by lymphocytes, plasma cells and monocytes/macrophages, as well as PMNs. Occasionally mast cells, eosinophils and foam cells can be observed (Neville et al. 2002). Antigen presenting cells, especially dendritic cells (DCs) play critical role in the polarization of T helper (Th) immune responses towards Th1, Th2, Th17 or T regulatory cells (T regs). Cholesterol crystal deposits associated with multinucleated foreign body giant cells and red blood cell areas with hemosiderin pigmentation can often be found, while small abscess formation with acute inflammation may be present (Walton et al. 1996, Neville et al. 2002).

Furthermore, chronic apical periodontitis is characterized by bone and dental hard tissue resorption. This process is a consequence of a disturbed balance between osteoclasts and osteoblasts and the activity of multinucleated osteoclasts. (Bohne 1990). There are many factors that have a stimulating effect on bone resorption in the apical periodontitis, such as bacterial components, mainly LPS like endotoxin and short-chain fatty acids, released to the apical area, as well as host-derived substances. The osteoclasts' differentiation from their precursors is mediated by several cytokines and growth factors, such as RANKL, OPG, IL-1, IL-6, TNF and prostaglandins. More specifically, during phagocytosis the host immune system stimulates the tissue destructive reactions by releasing arachidonic acid derivates (prostaglandins and leukotrienes) and other cell-mediated inflammatory factors, such

as cytokines. High levels of immunoglobulins, secreted by plasma cells, are also found in periapical lesions (Torres et al. 1994). Other plasma proteins, such as complement components, bradykinin, kallikrein and thrombin and acute phase reactants, derived from dilated blood vessels are also present and may stimulate bone resorption. During periapical periodontitis, bone resorptive cytokines IL-1α and IL-1β, TNF-α and IL-6 can be released by a variety of cells e.g. monocyte/macrophage-like cells, plasma cells, fibroblasts, epithelial and endothelial cells, PMNs, as well as osteoblasts and osteoclasts when these cells triggered by external stimuli (Márton et al. 2000). Also, T-cells express IL-2, IL-4, IL-6, IL-10 and interferon-γ (IFN-γ), and B-cells, fibroblasts and monocyte/macrophage-like cells IL-10 (Walker et al. 2000). It has been proposed that, in chronic apical periodontitis, the immune cytokines, such as Interleukin- 1α (IL- 1α), IL- 1β, Tumor Necrosis Factor-α (TNF-α), TNF-β, interleukin-6 (IL-6), and IL-11, can accelerate bone resorption (Stashenko 1998). The tissue destructive process is interactive, and these cytokines can trigger other cells to express proteolytic enzymes, including MMPs, which are also present in periapical granulation tissue (Takahashi 1998).

Regarding the progression of periapical lesions, it has been proposed that Th1 immune responses, mediated by interferon-γ (IFN-γ), along with other pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α(TNF-α) promote bone resorption and further destruction of the periapical tissues. On the other hand, immunosuppressive mechanisms mediated by transforming growth factor-β (TGF-β) and Th2 cytokines (IL-4, IL-5, IL-5, IL-10) are responsible for healing processes and the restriction of the inflammatory/immune mechanisms (Lukic 2000). IL-17, by stimulating the production of IL-8, may play a role in exacerbating inflammation in periapical lesions (Colic et al. 2007). The role of T regs in these processes is unknown.

Periradicular granulomas and cysts represent two different stages in the development of chronic periradicular pathosis.

2.2. Periapical Cyst

2.2.1. Incidence

2.2.2. Histopathology

2.2.3. Pathogenesis

2.2.4. Formation theories

2.2.5. Expansion theories

2.2.6. Regression possibilities

2.2.7. Wound healing

Simon (1980) was the first to describe two distinctive types of periapical cysts, based on the relation of the cystic lumen to the root canal space. Nowadays bay cysts are also known as Pocket cysts (Nair et al. 1996) and their lumen is in direct communication with the root canal system. On the other hand, true cysts are totally enclosed epithelial-lined cavities. Periapical cysts are generally considered to be a direct sequel to chronic apical granulomas, yet not every granuloma present cystic transformation.

2.2.1. INCIDENCE

The reported incidence of periapical cysts varies from 6% (Sommer 1966) to 55% (Priede 1954), depending on the methodology of obtaining the samples and the histologic interpretation. Nair and colleagues (1996) brought in strict criteria as far as it concerns accurate histopathologic diagnosis, proposing serial-sectioning of the periapical lesion from one side to another as a mandatory requirement. Under these conditions, the prevalence of periapical cysts ranges from 15% to 20% (Sonnabend et al. 1966). Of all the examined cystic lesions, 9% fulfilled the criteria for true cysts and the remaining 6% was indicative for pocket cysts. Apical granulomas represent 50% of all periapical lesions, whereas apical abscesses the 35%. A more recent study (Ricucci et al. 2006) found that incidence of periapical cysts is 32%, higher than that reported by Nair et al. (1996). These results may not reflect their real incidence in the population, as these only reflect lesions attached to a sample of extracted teeth and not a randomized study of patients. Furthermore, in Ricucci's (2006) study, all of the teeth were untreated endodontically and the duration of the apical periodontitis was not assessable. As the teeth were largely unrestorable, the sample would be biased toward advanced lesions. Sections containing epithelial strands might be erroneously diagnosed as cysts. Nair and his colleagues (1996) examined 256 lesions, of which the 52% showed epithelial strands but only 15% could effectively be diagnosed as periapical cysts.

2.2.2. HISTOPATHOLOGY

True cysts are completely sealed, epithelial-lined cavities, completely separated from the infected root canal system. The epithelial wall of the cavity shows great regional variation in thickness and may be infiltrated by PMNs (Ricucci et al. 2006) and IgG, IgM, IgA immunoglobins (Toller et al. 1969). This cavity wall is surrounded by inflamed connective tissue forming a pseudo-capsule (Ricucci et al. 2006) and the cystic cavity contains necrotic cells and debris, cells at various stages of disintegration, neutrophil leukocytes and erythrocytes, due to hemorrhage. Cholesterol crystals can also be found in the cystic lumen, in the lining wall and fibrous connective tissue capsule. They appear histologically as elongated tissue clefts, which may act as irritant towards chronic inflammation mediated by activated macrophages and giant cells (Nair 1999). The tissue between epithelium and the fibrous capsule contains blood vessels, T- and B- lymphocytes, plasma cells, macrophages and rarely neutrophils.

Pocket cysts are also lined by non-keratinized stratified squamous epithelial wall. This wall encloses the foramen of the tooth, thus providing a communication of the root canal space to the cystic cavity. It grows on the outer surface of the root tip so as to seal off lumen from the rest of the periapex, forming epithelial collars (Nair et al. 1996). The epithelial wall often shows sighs of cell exfoliation and is infiltrated by PMNs (Ricucci et al. 2006).

2.2.3. PATHOGENESIS

During tooth development, after the completion of crown formation, the apical mesenchyme continuous to proliferate in order to form the developing periodontium. On the other hand, the inner and outer enamel epithelia fuse below the level of the crown cervical margin to form the cervical loop, which invaginates into the underlying connective tissue of the dental organ. The formation of this double-layered epithelial sheath is known as Hertwig's epithelial root sheath (HERS). After the completion of the root formation, the epithelial sheath disintegrates, and cells migrate away from the root into the region of the future periodontal ligament to form the epithelial cell rests of Malassez (ERM), not as isolated groups of cells but as a network, similar to a fishnet surrounding the root (Valderhaung et al. 1967)

Although the role of HERS cells in root formation is widely accepted, the exact function of epithelial cell rests of Malassez is still debatable.

Ultrastructural studies using electron microscopy has shown that epithelial cell rests have minimal amounts of rough endoplasmatic reticulum, high nuclear-cytoplasmatic ratio and absence or ribosomes, suggesting that are not metabolically active cells (Ten Cate 1965, 1972). Although, evaluation of the mitochondrial morphology and the scarcity of Golgi complexes (Valdeuhaung et al. 1966) indicate that they are rather in a resting state, with a potential of activation and division as a result of an appropriate extracellular signal (Lin et al. 2007). Furthermore, it has been recently reported that the epithelial cell rests of Malassez may contain unique stem-cell populations that are capable of undergoing epithelial-mesenchymal transition (Xjong et al. 2012)

It is believed that the epithelial cell rests of Malassez may regulate the maintenance of the periodontal ligament, periodontal space, alveolar bone remodeling and prevent ankylosis (Xiong et al. 2013). It has been suggested that the epithelial cell rests of Malassez might modulate osteogenesis in the periodontal ligament space by producing certain molecules to prevent alveolar bone from migrating to the cementum space. Some recent studies support this hypothesis, and that they likely participate in regeneration of periodontal tissues, especially of cementum, by secreting matrix proteins, such as bone morphogenetic protein 2, osteopontin and ameloblastin (Mizuno et al. 2005, Mouri et al. 2003, Rincon et al. 2005). Fujiyama and colleagues (2004), in an attempt to explore the roles of Malassez epithelium, evaluated the plausible changes in dento-alveolar tissues surrounding this epithelium by experimental denervation of the inferior alveolar nerve in rats. They reported that in the absence of the epithelial cell rests due to denervation of the inferior alveolar nerve, the number of osteoclasts and odontoclasts increased during ankylosis, proposing a possible inhibitory role of the epithelial cell rests of Malassez cells on the function of the latter. Moreover, epithelial cell rests are found in the vital periodontal ligament areas of replanted teeth, suggesting that they may play an important role in the maintenance of the periodontal ligament space (Loe et al. 1961). Additionally, the absence of the epithelial cell rests of Malassez cells in regenerated periodontal ligament is associated with the narrowed periodontal ligament space (Shimono et al. 2003). Another possible explanation is that production of collagenase by epithelial rest cells, and thus the destruction of collagen, might contribute to the maintenance of the periodontal ligament space and the prevention of ankylosis (Rincon et al. 2006). Finally, Brice and colleagues (1991)

in their ultrastructural study found epithelial cell rest of Malassez in areas repairing orthodontic root resorption, proposing their important role in mediation of repair cementogenesis.

The epithelial cell rest of Mallassez have also been proposed to participate in radicular cysts formation (Ten Cate 1972). The exact mechanism of epithelial cells activation is not clear, yet there is evidence that local changes in pH or carbon dioxide tension in the supporting connective tissue may be responsible (Grupe et al. 1967).

Proliferation of epithelial cells in periapical lesions is likely to be related not only to the inflammatory mediators, growth factors and pro-inflammatory cytokines released by host cells during periapical inflammation (Torabinejad et al. 1980) but also to microbial cytokines and endotoxins present in apical periodontitis (Meghi et al. 1996). It is suggested that PGE_2 induce proliferation of epithelial cell rests by raising the level of intracellular cAMP (Brunette et al. 1984). Furthermore, IL-1, IL-6 and KGF have been shown to play an important role in up-regulating the epithelial cell proliferation (Saunder 1989, Grossman et al. 1989, Chelid et al. 1994). It has been reported that these factors, as well as PGE_2, TNF and TNF-a (Irwin et al. 1991, Lin et al. 1996, Chang et al. 1996), may up-regulate EGF receptors gene expression by influencing transcriptional factors. It has been scientifically proven that epidermal growth factor - EGF receptors are expressed by epithelial cells in normal periodontal tissues and odontogenic cysts, such as odontogenic keratocysts, and dentigerous cysts (Thesleff 1897, Li et al. 1993), and are up-regulated during inflammatory processes (Irwin 1991, Lin 1996). Several other growth factors has been proposed as stimulators for the proliferation of epithelial cell rests, such as insulin-like growth factor (Götz et al.2003) fibroblast growth factor 2- FGF2 (So et al. 2001).

There are contradictory findings regarding the potential relationship between the presence of inflammation and the proliferation of epithelium in periapical cysts (Cury et al. 1998, Loyola et al. 2005). Atrophic epithelium seems to have less inflammatory cell infiltration than hyperplastic, whereas cysts lined by hyperplastic epithelium are more inflamed than those covered by atrophic epithelium, indicating that the degree of inflammation may be proportionally associated with the extent of epithelial proliferation of the cystic wall (Takahashi 1999, Suzuki et al. 2006, Lin et al. 2009, Martins et al. 2011**).** On the other hand, Moreira and colleagues (2000) found that there is no correlation between the structure of the lining epithelium (atrophic or hypertrophic) and the degree of inflammatory cell infiltration.

From another aspect of view, the previous findings propose a potential stimulation of the epithelial cell rest of Malassez under various stress conditions. Additional stress conditions, such as mechanical stretching, may stimulate proliferation (Yamasaki et al. 1989) via gap junctional communication pathways (Haku et al. 2011) and up-regulation of Heat Shock Protein 70 –HSP70, VEGF and OPN in vitro (Koshihara et al. 2010). Apart from this, it has been reported that epithelial cell rests which synthesize DNA are increased by mechanical stretching (Brunette 1984).

2.2.4. FORMATION THEORIES

There are several theories proposed about the formation of the periapical cysts.

a) The nutritional deficiency theory (Ten Cate 1972, Shear 1992, Summers 1974) postulates that the central cells of the continuously growing epithelial mass are removed from their source of nutrients and undergo necrosis and liquefaction degeneration. The necrosis byproducts attract neutrophilic granulocytes. Subsequently, microcavities are formed and fused to develop a cystic cavity which is surrounded by stratified squamous epithelium
b) The abscess theory (Nair et al. 2008, Summers 1974) assumes that the proliferating epithelium surrounds the pre-existing abscess cavity, based on the innate tendency of any epithelium to cover exposed connective tissue surfaces.
c) The merging of epithelial strands theory (Lin et al 2007) rests on the assumption that the proliferating epithelial strands merge in all directions to form a three-dimensional ball mass. Connective tissue can be trapped inside the developing epithelial ball mass and, as consequence, to degenerate, since the essential blood supply is absent. Eventually, a cystic cavity is formed.

2.2.5. EXPANSION THEORIES

Many attempts have been made in order to explain the possible expansion mechanisms of periapical cysts.

a) It has been suggested (Toller 1970, Shear 1983) that radicular cysts may expand as a result of the osmotic and hydrostatic pressure. Increased osmosis,

owing to the degradation of epithelial and inflammatory cell in the lumen, leads to an inward movement of exudate from the surrounding tissue into the cyst cavity. The pressure inside the cystic lesion is further increased, as well as the pressure to the surrounding tissues. However, this theory does not take into account the cellular aspects of the cyst and the complex array of immunologic mechanisms which take part in such pathologic responses

b) There is evidence in support of molecular mechanisms involvement in the expansion of periapical cyst. The expansion of the cystic epithelium has been associated with the degree of the surrounding bone destruction (Shear 1994). It has been suggested that activation of neutrophils, fibloblasts, mononuclear leukocytes and macrophages leads to secretion of matrix metalloproteinases and, as a consequence, to degradation of fibrous connective tissue capsule (Teronen et al. 1995) Additionally, epithelial cell rests of Malassez are found to secrete several proteins, prostaglandins E and F, IL-1 and IL-6 (Bando et al.1993), thus participating in bone resorption. Harris and colleagues (1973) had also reported the existence of PGs in the cystic wall. Macrophages and T-lymphocytes of the cystic wall secrete prostaglandins which are capable of inducing resorption of the surrounding bone (Formigli et al. 1995). Fibroblasts from human periodontal ligament have been shown to produce a large amount of prostaglandins as a respond to intermittent hydrostatic pressure stimuli and mechanical stress (Ngan et al. 1992). Additionally, many other inflammatory mediators and pro-inflammatory cytokines have been detected in the cystic fluid or the cystic wall, and are associated with the activation of osteoclastic activity and the enhancement of the surrounding bone destruction, such as IL-1, IL-2, IL-6 (Kasumi et al. 2004), IL-8, TNF-a, leukotrienes (Ohshima et al. 2000) IL-17, NFkB ligand RANK), (Andrade 2013), osteoprotegerin (Menezes et al. 2006) and the pre-angiogenic factor VEGF (Nonoka et al. 2008).

The current evidence highlights the complexity of the mechanisms underlying the immunopathogenesis, the maintenance and the concurrent slow expansion of periapical cystic lesions.

2.2.6. REGRESSION POSSIBILITIES

It is a general belief that most periapical inflammatory lesions may heal after proper non-surgical root canal treatment *(*Kerekes et al. 1979). Root canal infection is the primary cause for the subsequent periapical pathology, including granulomas, abscesses, pocket cysts as well as true cysts, regardless the size. As a

consequence, these defects are expected to regress after the effective elimination of the irritants in the root canal system. Even though these lesions share common pathogenetic mechanisms and have almost the same biological behavior, there is still a controversy about the healing potential of apical pocket cysts and true cysts.

It is widely accepted that true cysts and large cyst-like periapical lesions are not likely to heal after non-surgical root canal treatment, owing to their self-sustaining nature (Nair 1998), and their healing requires surgical intervention. True cysts are completely lined cavities, without any communication between the cystic lumen and the infected root canal space and as a result, bacteria and their by-products are not able to affect the defect. Thus, the elimination of these irritants accomplished by root canal treatment, is not expected to affect them *(*Bhaskar 1972, Simon 1980).

However, there is strong evidence that inflammatory cells are always present either in the cystic lumen or in the epithelial wall and the surrounding connective tissue (Ricucci et al. 2006), suggesting that there are irritants attracting these inflammatory cells and evoking immune response (Lin et al. 2009). Supporters of the assumption that true cysts may heal after non surgical root canal system argue that any disease caused by infection should be able to heal after the proper elimination of the irritants, unless the irritants are neoplasm-including agents or carcinogens (Lin et al. 2009.) They propose that the hyperplastic epithelium of periapical inflammatory cysts, either pocket or true, is likely to regress by the same mechanism of apoptosis or programmed cell death.

Taken together, the treatment of choice of periapical lesions is non-surgical root canal treatment. In case of failure, surgical endodontic therapy can be performed. The latter has reported to present faster healing potential than non-surgical treatment, owing to the fact that residual bacteria, necrotic cells and tissue remnants are completely removed by the surgeon via surgical debridement. This debridement is considered to be more efficient and fast (Kvist et al. 1999), comparing to biologic debridement, as it allows the immediate growth of fresh fibrovascular granulation tissue into the affected area, thus promoting wound healing. On the other hand, biologic debridement relies on the ability of the activated macrophages to kill and digest bacteria and remove dead cells and cellular debris, which is a time consuming procedure (Lin et al. 1996). Furthermore, the surgeon has the ability to perform new regenerative techniques in order to promote healing, such as bone grafts and barrier membranes.

2.2.7. WOUND HEALING

Wound healing is a complex process which requires interactions between different type of cells, interactions between cells and extracellular matrix and a wide variety of cytokines, growth factors, neuropeptides and apoptosis (Brain 1997, Greenhalgh 1998, Werner et al. 2003) This process in periapical pathosis follows the same fundamental mechanisms of wound healing of connective tissues elsewhere in body (Lin et al. 2007), including granulation tissue formation and activation of macrophages in order to digest necrotic tissue remnants and dead bacteria resulting in regeneration and/or repair of the involved tissue. After elimination of the root canal irritants by chemomechanical instrumentation and effective irrigation with antimicrobial agents, periapical inflammation gradually resolves. It has been assumed that this may lead to a pause of the epithelial proliferation (Gatanzaro-Guimaraes et al. 1973). It has been also suggested that disintegration of inflammatory cells, epithelial cells and fibroblasts is conducted by apoptosis or programmed cell death, rather than by cell necrosis (Majno et al. 1995), while extracellular matrix is remodeled by metalloproteinases (Greenhalgh 1998). The lack of survival factors, such as trophic factors, or favorable environmental conditions, will influence the balance between proliferation and cell death in favor of apoptosis, and, consequently, tissue repair. In case apoptosis fails, pro-inflammatory mediators are released from necrotic cell to the surrounding tissues, resulting in the persistence of the inflammatory reaction. Apoptotic cells do not become lysed, therefore they cannot evoke an inflammatory reaction (Majno et al. 1995).

Torabinejad (1983) suggested a theory which was based on the capability of the activated epithelial cells to engulf foreign materials from the infected root canal space by phagocytosis (Odland et al. 1968). Therefore, they can be recognized as an antigenic unit and destroyed by inflammatory responses though macrophages, cytotoxic cells or natural killer cells after root canal therapy.

It has been reported that apoptotic factor Bcl-2 is present more frequently in atrophic than in hyperplastic epithelium (Loyola et al. 2005) and that several factors indicative of apoptosis, such as p53, Bax, caspase-3, Fas, Fas-L and Ki-67, are present not only in the lining epithelium of periapical cysts, but also in residual apical cysts (Suzuki et al. 2005). Recently, Martin and colleagues (2011) found that Bcl-2 expression is inhibited by inflammation in radicular cysts and that Ki-67 expression is more prominent in the hyperplastic epithelium than in the atrophic one.

The previous results support the theory that regression of the lining epithelium in apical cysts and epithelial strands of periapical granulomas is a consequence of programmed cell death.

As far as true cysts are concerned, there have been suggested two possible theories in favor of complete healing. The first one rests on the assumption that regression of the cyst occurs concurrently with bone regeneration. Osteoblasts are activated to deposit bone around the lesion, while apoptosis is taking place in the lining epithelium, resulting in the progressive reduction in size of the lesion. The second theory postulates that part of the epithelial wall is disorganized due to apoptosis of local epithelial cells along with disintegration of the basal lamina by metalloproteinases. As a consequence, surrounding connective tissue may grow into the cystic lumen (Lin et al. 2009)

After the regression of the cystic epithelium, regeneration of periapical tissues takes place. Healing might be achieved by some degree of fibrous connective tissue replacement, which is known as fibrosis. The regeneration involves the alveolar bone, the cementum, and the periodontal ligament.

During periapical wound healing, damaged periodontal ligament cells and root cementum are removed by phagocytosis and cells from the adjacent viable periodontal ligament take their place and cover the area. Multipotent stem cells of the periodontal ligament can differentiate to cementoblasts under the influence of growth factors, such as fibroblast growth factors, insulin-like growth factor-1 IGF-1, transforming growth factor-β TGF-β, platelet-derived growth factor PDGF. These cells are only capable to differentiate into cementoblasts, therefore only repair of cement is feasible on the damaged root surface (Seo et al. 2004).

Bone would healing is mediated by the activated osteoblasts or mesenchymal stem cells of the endosteum, which differentiate to produce bone matrix. They are stimulated by numerous growth factors, such as TGF-β, IGFs, PDGE, BMPs, and cytokines, which are secreted by stromal cells, osteoblasts, platelets, and bone matrix after bone resorption (Al-Aql et al. 2008). These factors can stimulate osteoprogenitor cells of the inner cellular layer of the periosteum to differentiate into osteoblasts, in case one of cortical bone plates is resorbed (Linkhart et al. 1996).

There is evidence that if both cortical bone plates are resorbed as in large cyst-like periapical lesions, fibrous connective tissue will develop in place of mature bone due to the subsequent extensive periosteum destruction (Andreasen et al. 1972). The exact molecular mechanisms underlying scar tissue formation is still unclear. Nair (1999) proposed that periapical scar tissue probably develops due to

the ingrowth of fibroblasts from periosteum or submucosa into the defect before the responsible for the regeneration cells, which have the potential to restore various structural components of the apical periodontium. Scar tissue can be misdiagnosed as persistent apical periodontitis. Recent studies using histological examination of biopsies obtained during periapical surgery or tooth extraction found that the incidence of scar tissue formation is 1-3 % (Schultz et al. 2009, Wang et al. 2004). Although differential diagnosis is difficult, special characteristics have been proposed, such as: the radiolucent area is smaller than the preoperative but not completely gone and it appears to be separated from the root apex by a layer of bone. Furthermore, it has been proposed, after long-term radiographic evaluation of periapical healing, that in these cases the root apex is completely covered by normal periodontal ligament and alveolar bone (Molven et al. 1996)

2.3. Notch Signaling Network

2.3.1. Introduction

2.3.2. Components and structure of notch signaling pathway

v. Receptors

vi. Ligands

vii. Notch activation pathways

viii. Target genes

c. HES/E(spl) family

d. HERP family

2.3.3. Notch signaling regulation

2.3.1. INTRODUCTION

Cell to cell interactions are pivotal for the development of multicellular organisms. Such interactions are mediated by communication mechanisms among cells through signals targeting specific genes. These genes are associated with cell survival, proliferation, differentiation, patterning, adhesion, epithelial-mesenchymal transition, migration, angiogenesis and apoptosis. Thus, cell-cell signaling permits neighboring cells to influence each other's fate and behavior.

Notch pathway is a fundamental evolutionary conserved intercellular signaling cascade. It regulates morphogenesis, development and homeostasis during embryonic and adult life through local cell-to-cell interactions (Artavanis et al. 1999), dictating cell fate of epithelial, neural, muscle, blood, bone and endothelial cells. Since Notch seems to critically influence many fundamental processes in a wide range of tissues, it is not unexpected that disruption of its homeostatic control has been directly linked to multiple human disorders, such as ongogenesis/carcinogenesis and inherited congenital disorders, such as Alagille Syndrome, Tetralogy of Fallot, syndactyly Spondylocostal Dysostosis, (Gridley 1997, Garg et al. 2005) Celebral Autosomic Dominant Arteriopathy with subcortical infarcts and leukoencephalopathy (Louvi et al. 2006).

The gene encoding the Notch receptor was initially identified by Dexter in 1914 (Fiúza et al. 2007) in a mutant Drosophila melanogaster with "notches" in its wings, lending the name to the gene and demonstrating its importance in wing outgrowth. Based on this finding, new alleles were identified by Morgan and Bridges, leading Poulson in 1937 to describe an embryonic lethal phenotype due to complete lack of gene's function (Metz & Bridges 1917, Poulson 1939). Later, Bill Welshons in Iowa conducted cytologic and genetic analysis at the 3C7 region of the X chromosome, which harbors the Notch gene, shedding light on its complex allelic series and genetic interactions and producing a quite accurate genetic map. (Welshons et al. 1962)

The first evidence of its role in human development and disease came in 1991, when the first association of altered Notch function with human disease was reported, with the identification of rare chromosomal rearrangements in human T lymphoblastic leukemias/lymphomas (T-ALL) involving Notch 1 receptor (Ellisen et al. 1991)

Furthermore, there is evidence that Notch is an essential requirement in development, playing a critical role in at least three different types of processes, such as a) lateral inhibition, b) lineage decisions and c) boundaries formation (Bray 1998).

Notch belongs to the so called “neurogenic” genes since it controls the balance between those cells of the neurogenic region that delaminate as neuroblasts and those that stay behind as epidermal precursors. Thus, embryos lacking the function of any of the neurogenic genes show an increased number of delaminating neuroblasts, at the expense of epidermal precursors (Hartenstain 1992). Key components of Notch signaling were originally recognized genetically through mutant animals whose phenotypes resembled those of Notch mutants. Later analyses refined and extended Poulson’s observations, demonstrating conclusively that when Notch activity is lost, cells which under normal circumstances would give rise to epidermal precursors (dermoblasts), instead they switch fate and become neuroblasts. These excessive neuroblasts continue their normal differentiation to produce morphologically deranged, unviable embryo that displays hypertrophy of the nervous system at the expense of epidermal structures. Because of this neural hypertrophy, the phenotype was baptized later with the term “neurogenic”.

2.3.2. COMPONENTS AND STRUCTURE OF NOTCH PATHWAY

Notch proteins regulate tissue homeostasis through receptor-ligand interactions via signals requiring physical contact between cells (Wilson et al. 2006). The transmembrane nature of Notch ligands not only assists to specify signaling to local cell interactions, but also contributes to a signaling system for cells to communicate directly with their neighbors. Receptor's activation requires the interactions of two elements: a) the extracellular domain of the ligand, expressed on the surface of one cell and b) the extracellular domain of the receptor expressed on an opposing cell surface (Fig. 1).

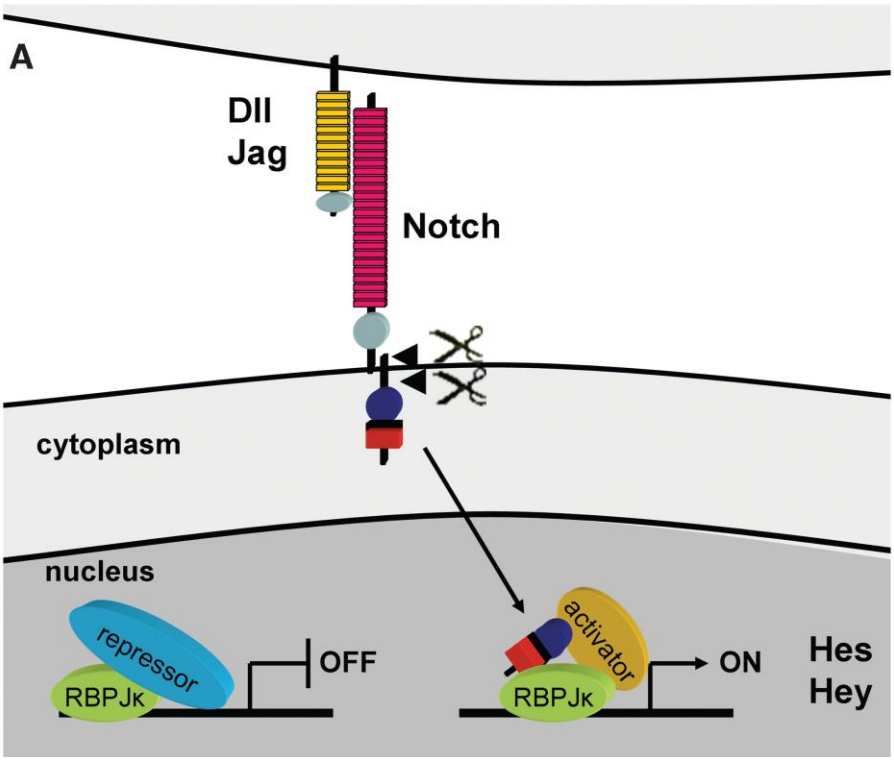


Figure 1: Notch Signaling Pathway. Ligands of the Delta (Dll) or Jagged (Jag) family induce intramembrane cleavage of the Notch receptor. The intracellular domain replaces transcriptional corepressors with activators enabling transcription of Hes and Hey genes by RBPJk. (Fischer et al. 2007)

i. RECEPTORS

Notch genes encode large single-pass Type 1 transmembrane glycoproteins. Fly Drosophila Melanogaster possesses only one Notch gene, worm Caenorhabditis elegans two (glp-1 and lin-12), while mammals posses four (Notch-1, Notch-2, Notch-3, Notch-4)

Most Notch proteins are cleaved in mammals by furin-like convertases at an external site close to the plasma membrane (the site 1 (S1)). This proteolytic event takes place in the trans-Golgi network transforms the Notch peptide into a heterodimer at the cell surface composed of two non-covalently associated subunits: the Notch Extracellular Domain (NECD) and the Notch Transmembrane Domain (NTMD), which contains a small portion of the extracellular region, the transmembrane region, and the intracellular domain (Kopan et al. 2009)

The N-terminal end of the extracellular domain of the receptor mediates interactions with the ligand and contains 36 tandem Epidermal Growth Factor EGF-like repeats (ELRs), which are responsible for ligand binding. Specifically, trans-interactions between receptor and ligand among neighboring cells require EGF-like repeats 11-12, while cis-inhibition with ligands presented in the same cell requires EGF-like repeats 24-29. Recently, it has been proposed that it is more possible the NECD to be in a compact than in a linear structure in a rod-like conformation (Xu et al. 2005, Cordle et al. 2008a)

Many EGF repeats contain calcium-binding sites which are pivotal for the perseverance of the structure and affinity of Notch in ligand binding (Cordle et al. 2008b) and can prevail/influence signaling effectiveness (Raya et al. 2004). They are followed by 3 cysteine-rich Lin12-Notch repeats (LNR), a hydrophobic heterodimerization region and both S1 and S2 cleavage sites. These units compose a negative regulatory region (NRR) (Gordon et al. 2009), which plays a crucial role/ is crucial for the prevention of the ligand-independent activation of the Notch receptor by concealing and protecting the S2 cleavage site from ADAM metalloproteases (Sanchez-Irizarry et al. 2004, Kopan et al. 2009).

The single transmembrane domain ends with a C-terminal “stop translocation” signal comprised of 3–4 arginine/lysine (Arg/Lys) residues. The S3 cleavage site lies within the transmembrane segment and is cleaved by the γ-secretase complex to liberate NICD.

The Notch Intracellular Domain (NICD) contains a RAM domain (composed of 12-20 amino acids) for Notch binding to the transcription factor

CSL/CBF1/Suppressor of Hairless/Lag-1, followed by seven ankyrin repeats (ANK domain) . Both the RAM domain and ANK repeats have been identified as regions participating in the interaction with CSL transcription factors. NICD also contains an evolutionary divergent transcription activation domain (TAD) and a conserved proline/glutamic acid/serine/threonine-rich (PEST) domain, which harbors degradation signals and whose mutation leads to increased receptor stability (Tamura et al. 1995). Mutations related to this region are associated with T-cell acute lymphoblastic leukemia lymphoma (T-ALL) highlight the pivotal functional role of regulated NICD degradation. The TAD region is found in Notch-1/-2, but it is not present in Notch-3/-4 in mammals.

ii. LIGANDS

The Notch receptor is activated by Delta-like and Serrate/Jagged ligand families and they are type 1 integral cell surface proteins. Delta and Serrate are identified in Drosophila, Delta and Jagged in vertebrates, while Lag-2 and Apx-1 in Caenorhabditis Elegans. More specifically, mammals express five canonical transmembrane ligands, 3 belonging to the Delta-like family (Delta-like-1, -3 and -4 and two to the Jagged family (Jagged-1and -2) which are homologous to Serrate (Lindsell et al. 1995, Shawber et al.1996, Callahan et al. 2001).

All ligands are presented with a extracellular domain-like architecture, starting from the N-terminal MNNL (Module of the N-terminus of Notch Domain) and followed by the DSL domain (Delta/Serrate/Lag-2) (Tax et al. 1994). The latter contains a binding-region for the Notch receptor interaction. The NT domain can be further subdivided into two distinct regions based on the presence or the absence of cystein: N1 is cysteine-rich while N2 is cysteine-free (Parks et al. 2006). Recently, a conserved glycosphingolipid (GSL)-binding motif (GBM) has been identified within the N2 region which may regulate ligand membrane association and endocytosis (Hamel et al. 2010)

The DSL motif is followed by 2 conserved atypical EGF-like repeats, named DOS domain (Delta and OSM-11-like proteins) (Komatsu et al. 2008), which is absent in mammals. All canonical ligands have additional EGF-like repeats before transmembrane segment (both calcium binding and noncalcium binding), ranging from 16 for Jagged, 5-9 for Delta and only 1 in DSL-1 in C.Elegans, and a C-terminal

cytoplasmic tail. The MNNL and the DSL domain and the DOS motif are required for canonical ligands to bind to Notch.

Jagged and Delta ligand are different in that the former have larger EFG-like repeat domains and an additional cysteine-rich domain (CRD) sharing partial homology with the Willebrand factor type C, which is not present in Delta-like. Special characteristic of the Jagged ligands is a tyrosine 255 at DOS domain, contrary to a small hydrophobic amino acid in delta like ligands. The Intracellular domain of the ligands is highly divergent among Notch ligands and contain multiple lysine residues (except for Delta-like 3) and most of them (Jagged-1,Delta-like-1, 4) a C-terminal PDZL (PSD-95/Dlg/ZO-1)-ligand domain which facilitate interactions with the actin cytoskeleton in promoting cell–cell adhesion, thus favoring epithelial cell assembly, and inhibiting cell motility and appears to be independent of Notch signaling (Mizahara et al. 2005). (Fig.2)

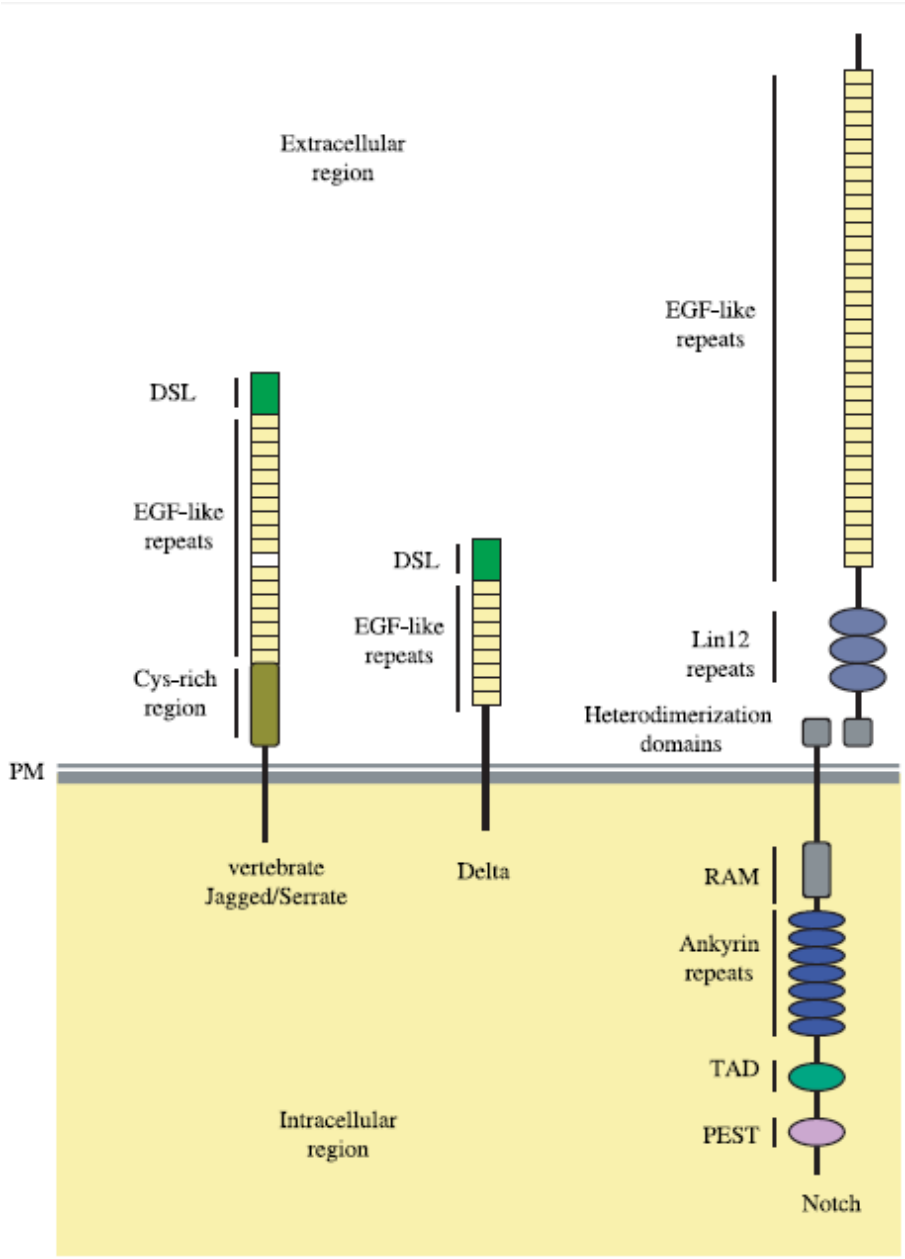


Figure 2: Structure of Notch and its ligands. Notch ligands, Delta and Jagged/Serrate, are composed of a DSL region responsible for the interaction with the Notch receptor and several EGF repeats. Jagged/Serrate also contains an extracellular cystein-rich region. Notch is composed by up to 36 EGF-like repeats. EGF repeats 11 and 12 are sufficient to mediate the interaction between Notch and its ligands. Notch also contains a cysteine-rich region known as Lin-12 repeats in close proximity with heterodimerization domains that bind noncovalently extracellular Notch with membrane-tethered intracellular Notch. In its intracellular part, Notch has a region called RAM (RBPjk Associate Molecule) followed by repeated structural motifs named Ankyrin repeats (mediate the interaction between Notch and CBF1/Su(H)), a transactivation domain (TAD) and a PEST domain. The PEST domain is involved in the degradation of Notch. (PM: plasma membrane) (Fiúza et al. 2007)

iii. NOTCH ACTIVATION PATHWAYS

Interaction between ligand and receptor initiates the activation of the Notch pathway, which is mediated by a sequence of proteolytic events and depends on the ability of the ligand to successfully induce receptor proteolysis, producing the release of an active Notch fragment. In brief, Notch signaling transmission depends on three fundamental events: (i) ligand recognition, (ii) conformational exposure of the ligand-dependent cleavage site, and (iii) assembly of nuclear transcriptional activation complexes. (Fig.3)

Upon ligand–receptor binding, Notch Extracellular domain separates from Notch Transmembrane Domain to be endocytosed with the ligand into the ligand-expressing cell. The sequence of proteolytic cleavages begins with cleavage at site 2 (S2) mediated by ADAM metalloproteases. The ADAM metalloproteases are encoded by Kuzbanian/kuz gene (van Tetering 2009) and essential for Notch activity in all phyla. In vitro Notch receptors seem to be mainly cleaved by ADAM17/TACE metalloprotease (Brou et al. 2000), while in vivo experiments using animal models suggest ADAM10/Kuzbanian metalloprotease for this essential function (Peschon et al. 1998, Hartmann et al. 2002, Lieber et al. 2002, Sotillos et al. 1997, Zhang et al. 2010).

ADAM proteases releases a short extracellular peptide and creates a short-lived membrane-tethered form , called Notch Extracellular Truncation (NEXT) fragment, which becomes a substratum for the aspartyl-protease presenilin(s), a component of the γ-secretase complex (De Strooper et al. 1999, Schroeter et al. 1998).This protease complex is a multicomponent member, including four core proteins, i.e. presenilin 1 or 2, Anterior Pharynx defective 1 (APH1), nicastrin, and Presenilin Enhancer 2 (PEN2) (Francis et al. 2008), of a growing family of intramembrane cleaving proteases (I-CLiPs). The NEXT fragment is cleaved progressively (S3) by the γ-secretase complex, most likely starting near the inner plasma membrane leaflet at site 3 (S3) and ending near the middle of the transmembrane domain at site 4 (S4) */[from site 3 (S3) to site 4 (S4)]* to release the Notch Intracellular Domain (NICD) and Nβ peptide. Then, NICD is free to translocate to the nucleus where it interacts with the DNA-binding protein CSL (Cp-binding factor 1 (CBF-1)/recombination signal sequence-binding protein Jk (RBP-Jk) in mammals, Su(H) (*Suppressor of Hairless)* in Drosophila, and LAG-1 in nematodes) through its RAM domain (Dou et al. 1994, Ghai et al. 2008, Morel et al. 2001). The ANK domain of NICD then interacts with CSL family, displacing co-repressors and recruiting

transcriptional co-activators such as Mastermind-like (MAML)/Lag-3 proteins, homologous to Drosophila Mastermind. Neither NICD nor CSL are able to bind to MAML1 alone, but can bind cooperatively to it in a complex. The produced tri-protein Notch–CSL–MAML complex in turn recruits multiple transcriptional regulators, such as MED8 mediator transcription activation complex and Co-R (co-repressor) proteins, thereby inducing up0regulation of downstream target genes to activate transcription (Hsien et al. 1999, Wu et al. 2000).

Target genes of Notch signaling include the Hairy/enhancer of split (Hes/E(spl), Hes-related (HERP, also known as Hey/HRT/HRT/CHF and gridlock) families, which belong to the helix-loop-helix transcription factors (bHLH) (Andersson et al 2011, Tanigaki et al. 2010, Borggrefe et al. 2009, Iso et al. 2001b, Iso et al. 2003), cyclin D1 (Ronchini et al. 2001) and c-Myc (Weng et al. 2005). The CSL-Notch complex also activate genes such as p21/Waf1, NFk B2, glial fibrillary acidic protein (GFAP), Nodal, GATA3, bcl-2 and CD25 (alpha chain of the IL-2 receptor), although their role as direct Notch targets has still not been conclusively determined (Hansson et al. 2004, Borggrefe et al. 2009).

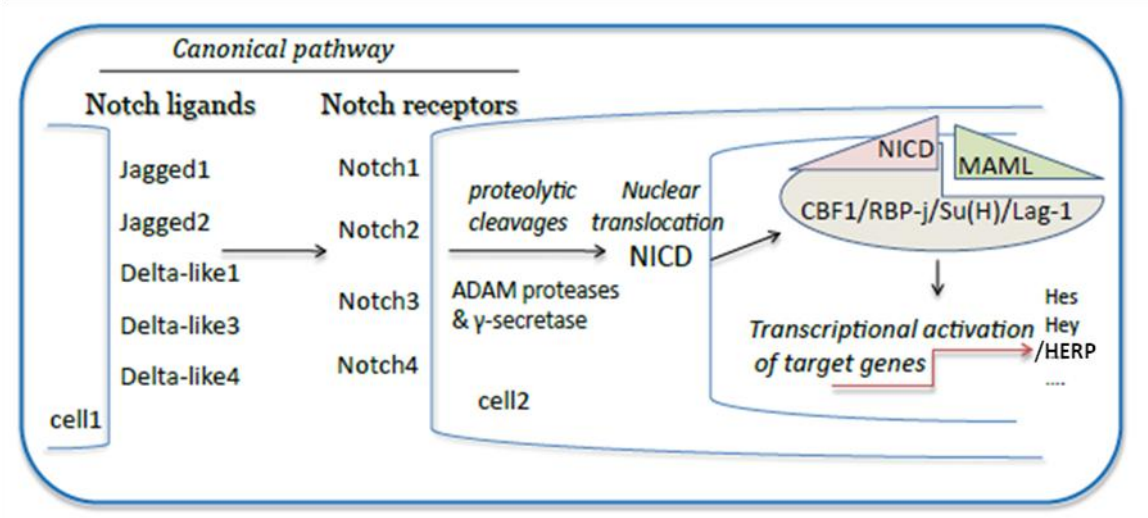


Figure.3: Schematic illustration of the canonical Notch signaling pathway (Quillard et al. 2013)

iv. TARGET GENES HES/HERP

The best-characterized Notch targets are the bHLH genes of the HES/ HEY families, depicted by the E(spl) genes in Drosophila and HES1 in mouse. HES/E(spl) had been the only known target of Notch signaling until a related but distinct bHLH protein family, termed HERP (HES-related repressor protein, also known as Hey/Hesr/HRT/CHF/gridlock), was isolated. They are characterized by their transient expression, reflecting the dynamic nature of Notch signaling. Additionally, there is evidence for autoregulation such that oscillations of HES expression have been observed and are thought to contribute to clocks that regulate somitogenesis, limb segmentation, and neural progenitor maintenance (Bray et al. 2010). All together HES/HEY have now been shown to function downstream of Notch in many critical processes and to contribute to oncogenesis. For instance, in tumor cells HES1 may take part in the regulatory circuitry sustaining cell growth by repressing expression of PTEN (Palomero et al. 2008).

a. HES/E(spl)

The HES/E(spl) family is a basic helix-loop-helix (bHLH) type transcriptional repressor which consists of seven members (HES1-7) and serve as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors (Bray et al. 2010)

b. HERP

This distinct family consists of three members (HERP1-3) (Iso et al. 2001a).

Both HES and HER genes can be expressed in the same cell. As a consequence, HES and HERP may act not only as homodimers but also as HES-HERP heterodimers in those cells on which HES and HERP are co-expressed. Although the genes of both families act as transcriptional repressors, HERP engages different repression mechanisms than does HES. Consequently, HERP could play a critical role in mediating Notch effects by regulating target genes, in both HES-expressing and non HES-expressing tissues, acting either as a hetero- or homo-dimer (Iso et al. 2001b)

There are several common features between the two families, as well as remarkable differences. First of all, they both contain a bHLH domain, and another

domain, the Orange (or helix3- helix4) in the corresponding regions carboxy-terminus to bHLH region. The amino acid sequences of these domains are highly conserved within the respective family, but less so among the two different families (Fig.4).

The most prominent characteristic which differentiate and distinguishes HES from HERP is a proline instead of a glycine residue at the basic region, respectively. This proline residue is strictly conserved from Drosophila to human among HES family members, from which they are also given the name proline bHLH proteins. On the contrary, the HERP family has a glycine at the corresponding position. This glycine residue is invariable trait among HERP family members across species from Drosophila to human, too. Thus, these prolines and glycines are hallmarks for the HES and HERP families, respectively.

Another distinction between the two families of target genes is a tetrapeptide C-terminal region. All HES members have in common the C-terminal tetrapeptide WRPW motif, whereas the HERP family has YRPW or its variants, as well as an additional conserved region carboxyl-terminal to the tetrapeptide motif, TE(V/I)GAF, which is not present in HES. Both HES and HERP serve as transcriptional repressors. A phylogenetic tree shows that they form a distinct subgroup in a large bHLH protein family. The above mentioned findings support the view that HERPs are closely related to the HES family belonging to class C protein, but they form a /different subgroup. Despite the similarities of their domains, HES and HERP seem to employ different repression mechanisms involving heterologous sets of co-repressor proteins-Groucho/TLE for HES and N-CoR/mSin3A/ HDAC for HERP (Iso et al. 2003)

The regulation by Notch of E(spl)/HES/HERP/Ref1 bHLH repressors appears to have a strictly conserved pattern and these proteins play a critical role in many Notch-dependent processes where they repress fundamental cell fate determinants and cell cycle regulators (Fischer et al. 2007).

Figure 4: Alignment of HES and HERP amino acid sequences. A schematic diagram. Conserved domains are marked by distinct colors: Blue for the basic domain, green for the helix-loop-helix domain, orange for the Orange domain, and pink for the tetrapeptide motif. Potential target genes of Notch are listed on the right.

2.3.3. NOTCH SIGNALING REGULATION

This core signal transduction pathway is used in most Notch-dependent processes and is known as the “canonical” pathway. However, depending on the cellular context, the amplitude and duration of Notch activity can be further regulated at various points in the pathway.

The relative affinity of Notch receptors for Delta and Jagged family ligands is controlled by receptor glycosylation and specifically by the addition of Fuc-GlcNac (fucose-N-acetylglucosamine) moieties by a fucosyltransferase and Fringe family N-acetyl-glucosaminidyl-transferases. Generally, cell-to-cell contact is essential for the activation of Notch signaling, usually resulting in coordinated modulation of genes which participate in cell fate determination, such as proliferation, survival, or differentiation (Espinoza et al. 2013).

Except for the HESR genes, rather few genes have been found to be regulated by Notch, both in vertebrates and invertebrates. This observation not only can be attributed to the fact that previous studies have not focused on the same processes, but also it may reflect species divergence in the outputs. However, various consistent findings have risen:

A. Notch has been reported to directly regulate genes involved in proliferation and apoptosis. (Table 1)

- Myc gene is a direct target of Notch in several types of cancer cells and in Drosophila cells (Klinakis et al. 2006, Palomero et al. 2006). The extent of proliferation was compromised when myc was knocked-down in these contexts, demonstrating that myc is a significant intermediate in the proliferative response to Notch activation.
- Other direct targets which take part in proliferation include CyclinD (Joshi et al. 2009), string/CDC25 and CDK5 (Palomero et al. 2006). Although Notch activates these proliferative genes in several contexts, in others it activates cell cycle inhibitors like p21 (Rangarajan et al. 2001) reflecting the differing consequences on proliferation (Koch et al. 2007).
- Hence reaper and Wrinkled/hid in Drosophila have been found as Notch direct targets, directly controlling apoptosis (Bray et al. 2010).

- Likewise, bcl2 in mammals has been found to respond rapidly to Notch activation consistent with being a direct target (Deftos et al., 1998), but direct CSL binding to its promoter has not been proved yet.

It is necessary, for the clarification of the contradictory and various roles that Notch plays in development and oncogenesis, to unveil what governs the selection of apoptotic and proliferative targets.

Notch Target Genes	Role
myc, CyclinD, string/CDC25, CDK5	Cell proliferation
p21	Cell circle inhibition
Hence reaper, wrinked/hid	Apoptosis
DELTEX, NRARP, Serrate, Su(H), neutralized, numb, Kuzbanian/Adam10	Notch auto-regulation
kinase phosphatase (MKP) lip-1	RASMAPK pathway regulation
ErbB2	Up-regulation of MAPK regulators in hematopoietic progenitors
Myc, IFL, String/CDC25	"realizator" genes

Table 1: Notch Target Genes/ Core components and modifiers of Notch signaling

B. Many components of the Notch pathway are themselves direct targets.

- DELTEX is a cytoplasmic protein acting as a Notch activator by directly binding to the intracellular domain of the receptor. DELTEX1 encodes a ubiquitin ligase that regulates Notch trafficking, was first shown to be positively regulated by Notch in C2C12 cells (Kishi et al. 2001) and has subsequently emerged as a target in multiple vertebrate tissues but not yet in invertebrates.
- NRARP is a Notch inhibitor and seems to be a target in a range of vertebrate cell types.
- Other components of this signaling pathway have so far only found as direct targets in invertebrates e.g., Serrate, Su(H), neutralized, numb, Kuzbanian/Adam10 (Krejci et al. 2009), although indirect evidence propose that some are also targets in mammalian processes. (Bray et al. 2010)

Furthermore, Notch autoregulates its own expression in some mammalian (Yashiro-Ohtani et al. 2009) and Drosophila cells (Krejci et al. 2009) as well as in C. elegans (Christensen et al. 1996), thus providing in this way a feedback mechanism that augments signaling (Christensen et al. 1996).

C. Common targets are components of other signaling pathways.

Multiple **Ras** pathway regulators were identified through bioinformatics and genetic screens in C. elegans, where the MAP kinase phosphatase (MKP) lip-1 is a direct target along with five other negative regulators of the RASMAPK pathway (Yoo et al. 2004). A similar elaborate cross talk with EGF receptor signaling network and with other signaling pathways is evident in Drosophila and direct Notch targets include positive as well as negative regulators (Krejci et al. 2009). Hints at similar cross talk in mammalian cells are seen with the identification of **ErbB2** as a direct target, with up-regulation of MAPK regulators in hematopoietic progenitors (Weerkamp et al. 2006) and with the oscillatory network related to Notch signaling in somitogenesis (although in this case there is as yet no proof that the cross talk involves direct regulation).
The exact nature of the Notch targets as well as the consequences for the cross-regulation of signaling pathways are likely to differ and they depend on the context of the cell.

D. Notch directly regulates expression of genes encoding proteins that actually implement cell functions ("realizator" genes).

For instance, in T-ALL cells many of the direct targets take part in metabolism (Palomero et al. 2006). And in several developmental contexts direct targets include cytoskeletal regulators such as cytoskeletal crosslinkers **Short stop and Gas2** and the genes encoding Ig cell adhesion receptors Roughest and Hibris (Krejci et al. 2009). Likewise, **Tenascin-C** is a target of Notch2 in glioblastoma cells, where it may contribute to invasiveness of the tumor cells (Sivasankaran et al. 2009).

Finally, several regulatory motifs are beginning to rise from systematic studies of Notch targets. These include positive feed-forward loops, exemplified by the role of Myc in T-ALL cells (Palomero et al. 2006), and incoherent **(IFL**), characteristic of the response in Drosophila myogenic precursors (Krejci et al. 2009). In this type of IFL, the stimulus (Notch) regulates both a gene and a repressor of the gene. Classic examples involve members of the HERP family. For example, PTEN, atonal and twist are all directly responsive to CSL/Notch, and in each case these genes can also be repressed by HESR proteins (Ligoxygakis et al. 1998, Palomero et al. 2008). Genome-wide studies revealed further targets that form IFL independent of HESR

members including String/CDC25-hindsight and myc-brat (Krejci et al. 2009). The overall output of IFL is difficult to predict since it is dependent on several criteria such as the rate of synthesis and the thresholds required for activation and repression, but in some conditions it has been shown to create pulse of target activities (Alon 2007) and it is proposed to render the response proportional to the fold change in the input signal.

Pleiotrophy

Pleiotrophy occurs when one gene influences multiple, seemingly unrelated phenotypic traits. Notch is not only pleiotropic in its action as judged by the near-universal array of tissues it affects throughout ontogeny, but also pleiotropic in terms of the fundamental developmental processes it is involved in. Numerous cellular processes, such as differentiation, proliferation and apoptosis, can be significantly affected by Notch-pathway activation, depending on the developmental context, with the latter being a fundamental parameter. It is widely known that Notch action in one tissue can induce cellular proliferation, while in another it can lead to apoptosis. Thus, it can be assumed is that cell fate changes can be triggered by the modulation of Notch activity, at least in cells that are not terminally differentiated. However, it is not possible to predict the nature of the resulting cell fate changes , a priori, as the fates affected will depend both on developmental context (spatial and temporal) and on the dosage of Notch activity.

2.4. Notch in Development

2.4.7. Central nervous system development and adult brain

2.4.8. T-Cells lineage

2.4.9. Somitogenesis

2.4.10. Cardiovascular development and homeostasis

2.4.11. Endocrine development

2.4.12. Maxilla morphogenesis

2.4. NOTCH IN DEVELOPMENT

2.4.1. Central Nervous System development & Adult Brain

Notch signaling is widely known as a major regulator of neural stem cells and neural development (Louvi et al. 2006). In vertebrates, Notch is required when the epidermal and neural lineages have already segregated and its inactivation leads to a “neurogenic phenotype” characterized by premature differentiation of neuronal progenitors. Based on the above findings, it can be proposed that Notch maintains a progenitor state and inhibits differentiation (Bolós et al. 2007).

Contrary to its inhibitory role in neuronal differentiation, Notch is found to have an opposite effect on gliogenesis by directly promoting the differentiation of many glial subtypes. Evidence of several *in vivo* studies performed in animal models (Furukawa et al. 2000, Sheer et al. 2001) is consistent with a supportive role of Notch signals in gliogenesis through bHLH targets. Activation of Notch signaling favors the generation of Muller glia cells at the expense of neurons, whereas reduced Notch signaling induces production of ganglion cells, causing a reduction in the number of Muller glia (Bolós et al. 2007).

Notch expression is eminent not only in the development of the central nervous system, also but throughout the adult brain and in differentiated cells in the central nervous system (Berezovscka et al. 1998). The pivotal role that Notch signaling plays in normal adult human brain function can be proved by its involvement in diseases as diverse as Alagille (Li et al. 1997, Oda et al. 1997), CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Joutel et al. 1996) and Hajdu–Cheney (Isidor et al. 2011, Simpson et al. 2011) syndromes, as well as Down’s syndrome (Fischer et al. 2005) and Alzheimer’s disease (Berezovscka et al. 1998, Veeraraghavalu et al. 2010). The former present functional mutations in key Notch pathway elements, while the latter are characterized by abnormal Notch expression levels. The neurogenic niches of the adult brain, which are the subventricular zone and the subgranular zone, are depicting a preservation of the embryonic germinal zones. These zones continue to generate cells throughout life to varying extents, depending on the species (Alvarez-Buylla et al. 2004). Although these niches are only a fraction of total adult brain tissue, Notch pathway components are expressed throughout the adult brain (Givorgi et al. 2006). This suggests that Notch might have roles beyond regulating stem cell maintenance and differentiation (Ables et al. 2011).

2.4.2. T-Cells lineage

Notch-1 receptor is required for the commitment of progenitors to T cell fate, as well as for the subsequent assembly of pre-T cell receptor complexes in immature thymocytes. Thus, it is evident that the human Notch-1 receptor is pivotal for the normal development of T cell progenitors (Koch et al. 2011).

2.4.3 Somitogenesis

Notch's role is crucial in the patterning process leading to somite boundary formation and the establishment of the anterior/posterior polarity of somites. Somitogenesis is the process by which somites form. Somites are paired blocks of paraxial mesoderm that form along the anterior-posterior axis of the developing embryo, at either side of neural tube. In vertebrates, somites give rise to skeleton and the associated muscles, tendons, cartilage, endothelial cells and skin (Ordahl et al. 1992). Notch involvement in somitogenesis was first suggested by the defects in somite morphology observed in mice with targeted mutations in the Notch-1. Mutants present abnormal somites with an anomalous positioning of segmental boundaries (Conlon et al. 1995). Additionally, Delta-1 targeted mutants present irregular somitogenesis with disturbed anterior/posterior polarity (Hrabe et al. 1997).

2.4.4. Cardiovascular development and homeostasis

There is evidence that Notch ligands and receptors (Shatter et al. 2000, Villa et al. 2001) as well as their downstream effectors and target genes (Nakagawa et al. 1999) are widely expressed in the vascular system. In vivo studies in animal models (Lawson et al. 2002) presented evidence that Notch is a crucial regulator of cardiovascular development and it is established that Notch signaling plays an especially crucial role in the development and pathophysiology of the cardiovascular system (Gridley et al. 2007). Furthermore, the finding that human CADASIL syndrome (cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy), which involves the Notch-3 gene, causes stroke and vascular dementia (Joutel et al. 1996) underlines the fundamental function of Notch in vascular development and homeostasis. In addition, Alagille syndrome results from

Jagged-1 haploinsufficiency, which among other features is characterized by vascular anomalies (Li et al. 1997, Oda et al. 1997)

2.4.5. Endocrine development

The role of Notch signaling in the development of endocrine system is well established.

PANCREAS: Specific Notch pathway elements and downstream effectors are expressed in the developing pancreas, suggesting a role for Notch in pancreatic development (Apelqvist et al. 1999). The available data indicate that Notch regulates the progressive recruitment of endocrine and exocrine cell types from a common precursor pool in developing pancreas.

GUT: Several evolutionary conserved pathways, such bone morphogenetic protein (BMP)/TGF, sonic hedgehog (Shh), wingless (Wnt) and Notch are regulating intestine homeostasis (Sancho et al. 2004).

BONE ENDOCRINE CELLS: Several in vitro studies have reported the potential role that Notch pathway plays in osteoclastogenesis and osteoblastogenesis. There is evidence indicating that Notch down-regulates osteoclastogenesis activation. Early findings suggest that Notch reduces the surface expression of macrophage colony-stimulating factor, CSF-1 (M-CSF), which is a homodimeric glycoprotein required for the lineage-specific growth of cells of the mononuclear phagocyte series. CSF-1 not only stimulates the proliferation of bone marrow-derived precursors of monocytes and macrophages, but also acts as a survival factor and primes mature macrophages to carry out differentiated functions (Sherr et al. 1988). Furthermore, it leads to the enhancement of the expression of osteoprotegerin in stromal cells, which subsequently results in the down-regulation of osteoclastogenesis (Yamada et al. 2003).

It has been found that Notch-1, Delta-1 and Jagged-1 are expressed in cultured osteoblast precursor cells as well as in differentiating osteoblasts during bone regeneration and that Notch-1 is activated in these cells. These results suggest that Notch signaling plays a significant role in the commitment of mesenchymal cells to the osteoblastic cell lineage (Nobta et al. 2005). Concomitant expression of Delta-1 and Jagged-1 during *in vivo* bone regeneration indicates that there is a functional redundancy between Delta-1 and Jagged-1 and that these ligands direct osteoprogenitor cells to the differentiated status through identical signaling pathways (Nobta et al. 2005). Thus, it can be proposed that Notch may demonstrate a

therapeutic potential in bone regeneration and osteoporosis (Bolos et al. 2007). Furthermore, it has been found that Jagged-1/Notch signaling modulates osteoblastic differentiation and Jagged-1 may play a significant role in mediating the effects of PTH on osteoblasts (Watanabe et al. 2003).

2.4.6 Maxilla morphogenesis

Humphreys and colleagues (2011) found that the deletion of Jagged-1 from cranial neural crest cells, but not Notch-1, resulted in a reduced maxilla, aberrant vascular branching and decreased proliferation and extracellular matrix formation during embryogenesis. The above mentioned findings indicate that Jagged-1 induces maxilla morphogenesis and that Notch signaling is pivotal for mid-face development in mice.

2.5. Notch in Disease

2.5.8. Alagille Syndrome

2.5.9. Cardiac Disease (non-syndromeic)

2.5.10. Celebrovascular Disease- CADASIL

2.5.11. Metabolic Bone Disease (Hajdu-Cheney Syndrome)

2.5.12. Buergers Disease

2.5.13. Notch in inflammation

2.5.14. Spondylocostal dysostosis (SDC) & Spondylothoracic Dysostosis (STD)

2.5. NOTCH IN DISEASE

2.5.1. Alagille Syndrome

Alagille syndrome is a hereditary pleiotropic disease which is linked with mutations and deletion of Jagged-1 and Notch-2. This multi-system, developmental disorder syndrome presents several clinical features including anomalies of the heart, vertebrae, eye or face, bile duct paucity, abnormal kidney and chronic liver disease. Additionally, numerous lower penetrance findings are frequently identified including renal anomalies, growth retardation, bone abnormalities, a high pitched voice, vascular malformations and delayed puberty (Alagille et al. 1987). Alagille patients have characteristic facial features consisting of a prominent forehead, deep-set widely spaced eyes, a straight nose with a flattened tip and a prominent pointed chin. These features give the impression of an inverted triangular face. The cardiac defects include right branch pulmonary artery stenosis, peripheral pulmonary stenosis, Tetralogy of Fallot (TOF), valvar pulmonic stenosis, ventricular septal defect, atrial septal defect, coarctation of the aorta and dextropositioning of the aorta. Penton et al. 2012)

2.5.2. Cardiac disease (Non-syndromic)

Cardiac defects have been also found, not only in association with Alagille syndrome, but also with mutations in Jagged-1 or Notch-1 genes in individuals exhibiting non-syndromic right-sided cardiac disease, similarly to the type that is seen in Alagille syndrome. Jagged-1 mutations, and in fewer cases Notch-1 mutations, have been identified in patients with isolated tetralogy of Fallot (Bauer et al.2010, MacGrogan et al. 2010). Jagged-1 sequence variants have been identified in 4% of patients presenting with pulmonic stenosis, peripheral pulmonic stenosis or pulmonary artery stenosis who did not meet the diagnostic criteria for Alagille syndrome (Bauer et al. 2010)

Notch-1 mutations are associated with structural abnormalities of the aortic valve, such as bicuspid aortic valve and have been found in individuals with more serious left ventricular outflow tract abnormalities, such as aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome (MacGrogan et al. 2011).

Furthermore, Notch receptors and ligands link cellular effectors and mechanisms associated with major cardiac disorders, such as myocardial infarction,

atherosclerosis and cardiac allograft vasculopathy (Quillard et al. 2013). Regarding myocardial infarction, it is known to be the most common and clinically crucial form of acute cardiac injury which leads to ischemic necrosis of a significant amount of cardiomyocytes. The subsequent necrosis elicits an inflammatory reaction which initially aids the removal of the necrotic tissue debris within the infarct and ultimately promotes healing and repair of the damaged tissue (Fragogiannis et al. 2008). Recent evidence supports that the Notch-1 receptor regulates the cardiac response to stress (Takeshita et al 2007, Kratsios et al. 2010). More specifically, the receptor Notch-1 and its ligand Jagged-1 are the predominant members of the Notch family expressed in the adult heart. The activation of the Notch pathway in the heart in response to stress is likely to be dependent on the expression of Jagged-1 ligand on the surface of cardiomyocytes (Li et al. 2009). There have been reported advanced levels of Notch ligands and receptors in damaged and regenerating tissues, including heart, as well as in vessels. An increase of Notch activation after myocardial infarction enhances survival rate, improves cardiac function and minimizes fibrosis, promoting anti-apoptotic and angiogenic mechanisms. As a consequence, the transient activation of endogenous Notch signaling has been observed following myocardial infarction, but is insufficient to launch an effective response to cardiac damage (Kratsios et al. 2010)

2.5.3. Celebrovascular disease-CADASIL

In 1996, Notch-3 mutations were found to cause Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), a disorder characterized by stroke and dementia (Joutel et al. 1996). The clinical presentation of the syndrome may vary and includes subcortical ischemic events, cognitive impairment and dementia, migraine with aura, mood disturbances and apathy (Chabriat et al. 2009). The arteriopathy affects mainly the small penetrating cerebral and leptomeningeal arteries and is characterized by thickening of the arterial wall and prominent morphological alterations on vascular smooth muscle cells and pericytes (Chabriat et al. 2009, Dziewulska et al. 2012)

2.5.4. Metabolic bone disease (Hajdu-Cheney Syndrome)

Hajdu-Cheney Syndrome is a rare, mostly sporadic, multisystem disorder of connective tissues characterized by severe and progressive focal bone destruction, osteoporosis, variable craniofacial abnormalities, renal cysts, cleft palate and cardiac defects. Its pathogenesis has been associated with mutations in Notch 2 (Isidor et al. 2011).

2.5.5. Buerger's disease

Buerger's disease [Thromboangiitis obliterans (TAO)] is an uncommon and an intractable disease characterized by a non-atherosclerotic, inflammatory endarteritis that causes a prothrombotic state and subsequent vaso-occlusive phenomena. The inflammatory process is initiated within the tunica intima and it involves small and medium-sized arteries and veins of the upper and lower extremities, while only rarely affecting visceral or cerebral vessels (Kobayashi et al. 1999)**.** Recently, Tamai et al. (2012) proposed that Notch signal activation may be involved in the inflammatory pathophysiology of Buerger's disease.

2.5.6. Notch in inflammation

Several studies demonstrate that Notch plays a significant role in the development of inflammatory processes as Notch is an important regulator of immune cell differentiation and activation. Recent studies provide evidence about its role in T CD4+, CD8+ lymphocyte, regulatory T-cell, B-cell, natural killer (NK) and dendritic cell homeostasis (Kassner et al. 2010). More specifically, Notch signaling regulates differentiation of macrophages, dendritic cells (DCs) and lymphocytes (Tanigaki et al. 2003) and is essential for T cell lineage commitment and early stages of thymocyte, as well as for Marginal Zone B cell (MZB) development (Feyerabend et al. 2009).

2.5.7. Spondylocostal Dysostosis (SCD) and Spondylothoracic Dysostosis (STD)

Mutations in several of the Notch signaling pathway genes cause severe vertebral and costal abnormalities in Spondylocostal Dysostosis (SCD) and Spondylothoracic Dysostosis (STD). Several genes in the Notch signaling pathway have been associated with autosomal recessive SCD and STD including the ligand Delta like 3, the glycosyltransferase Lunatic Fringe (LFNG) and the basic helix–loop–helix Notch target genes, Hairy-and-enhancer-of-split-7 (HES7)and Mesoderm Posterior 2 (MESP2) (Penton et al. 2012).

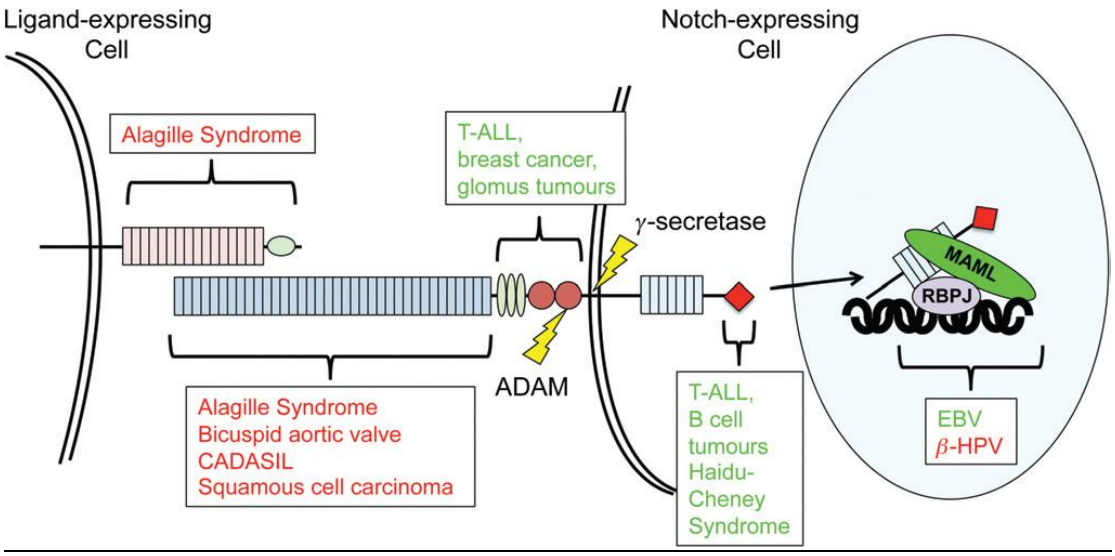


Figure 5.Normal and pathophysiological Notch signalling. Congenital disorders, cancers and viral infections are highlighted that are characterized by increased (green) or diminished (red) Notch signalling, due to aberrations affecting the function of Notch ligands, receptors or the nuclear Notch transcription complex. (Aster et al. 2014)

2.6. NOTCH IN CANCER

Notch pathway can dramatically affect so cell fates and, as a consequence, the balance between differentiation, apoptosis, and proliferation. Either aberrant increases or deficiencies of Notch signaling lead to human developmental anomalies and cancer development, underlying the significance of the accurate regulation of the intensity and duration of notch signals.

More specifically, Notch signaling pathway is a fundamental factor contributing in the maintenance of a pool of self-renewing hematopoietic stem cells (Duncan et al. 2005), therefore it is expected that its deregulation may lead to the development of hematological malignancies. In 1991, the first association of altered Notch function with human disease was reported, with the identification of rare chromosomal rearrangements in human T lymphoblastic leukemias/lymphomas **(T-ALL)** involving Notch 1 receptor (Ellisen et al. 1991). The critical role of Notch signaling in T-ALL cells is to drive a gene expression program maintaining growth, high metabolism, and survival. Thus, the Notch pathway became an attractive therapeutic target and multiple tools (e.g., γ-secretase inhibitors, neutralizing antibodies against Dll4 or NOTCH-1) that interfere with Notch signaling are currently areas of active research (Louvi et al. 2012). Furthermore, mutations in Notch-2 receptor, which are responsible for the partial or complete deletion of the PEST domain, have recently been discovered in a subset of diffuse large B-cell lymphomas, a subtype of mature B-cell lymphomas. Additionally, increased copied of the mutated Notch-2 allele have been found in some diffuse large B-cell lymphoma. (Lee et al. 2009)

Notch signaling has been implicated in human breast cancers, as the co-expression of high levels of Jagged-1 and Notch-1 has been shown to correlate with poor survival in this malignancy (Reedijk et al. 2005, Stylianou et al. 2006).
Additionally, activation of the pathway has been reported in several human cancers such as multiple myeloma (Jundt et al. 2004), pancreatic cancer (Mijamoto et al. 2003), prostate cancer (Santagata et al. 2005), cervical cancer (Zagouras et al. 1995), colon and gut (Kranenburg 2015) neoplasms and lung cancer (Konishi et al. 2007). Notch signaling pathway seems to be activated in two types of skin cancer (Proweller et al. 2006), basal-cell carcinoma (Thelu et al. 2002) and primary melanomas (Liu et al. 2006).

Moreover, there is evidence that Notch deregulation results in brain malignancies, such as gliomas, medulloblastomas and astrocytomas (Purow et al. 2005, Ntxiachristos et al. 2014, Somasundaram et al. 2005)

2.7. Notch in Dentistry

2.7.10. Odontogenesis

2.7.11. Pulp injury

2.7.12. Stem Cells

2.7.13. Caries & Cavity preparation

2.7.14. Periodontitis

2.7.15. Orthodontic movement induced root resorption

2.7.16. Palate formation

2.7.17. Taste

2.7.18. Notch in Head and Neck Cancer

2.7. NOTCH IN DENTISTRY

2.7.1. Odontogenesis

The tooth represents a powerful model, depicting the molecular mechanisms which participate in cell fate determination and differentiation of various cell lineages during embryonic development (Mitsiadis et al. 2009). The reciprocal inductive interactions between the oral epithelium and the underlying neural crest-derived mesenchyme finally result in the formation of the teeth (Thesleff et al. 1981, Cobourne et al. 2006).

There is strong evidence that the Notch signaling pathway plays a crucial role in the differentiation of the dental epithelium and mesenchyme, generation of cusp patterns and the teeth morphogenesis. Several studies have shown that components of the Notch signalling pathway are expressed also in developing mouse teeth. Expression of Notch-1, Notch-2 and Notch-3 receptors (Mitsiadis et al. 1995a), Delta-likel-1 (Mitsiadis et al. 1998a), Jagged-1 (Mitsiadis et al. 1997) and Jagged-2 ligands (Mitsiadis et al. 2005) in developing teeth prefigures the subdivision of the epithelium into ameloblastic (capable of enamel-matrix synthesis) and non-ameloblastic regions already at the initiation stage (Fig.1).

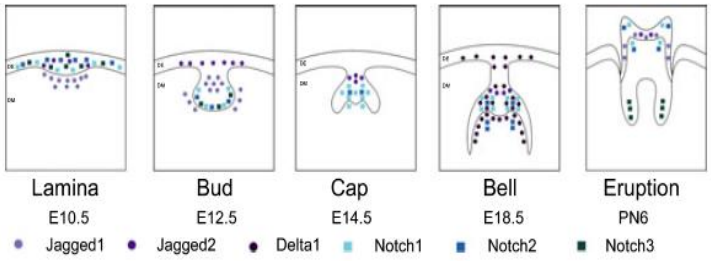


Figure 1. Expression pattern of Notch receptors. Expression patterns of Notch1, Notch2, Notch3, Jagged1, Jagged2 and Delta1 at different stages of developing tooth germ (DE, dental epithelium; DM, dental mesenchyma).

- **During the lamina stage, E11-E12:** During tooth initiation and morphogenesis, the receptors Notch-1, Notch-2 and Notch-3 are intensely expressed in the mesial side of the thickened dental epithelium, while Jagged-1 ligand is absent in dental epithelium, but intensely expressed throughout the condensed mesenchyme at E12. During the same stage, Jagged-2 ligand is expressed in the oral epithelium and in dental epithelium, while Delta-1 ligand is weakly expressed.

- **In the bud stage, E13**: Notch-1, -2 and -3 receptors are expressed in superficial dental epithelium, but they are absent from the condensed mesenchyme. Regarding Notch pathway ligands, Jagged 1 is expressed in the stellate reticulum and its expression region overlaps with that of Notch. Interestingly, at E13.5, Jagged-1 mRNAs are transiently expressed in the enamel knot, but are absent in epithelial cells. At the late bud stage, Jagged-1 is downregulated in the pulpal mesenchyme closest to the bud, but is maintained in cells of the peripheral dental mesenchyme forming the follicle that may contain the mesenchymal stem cells. Jagged-2 is weakly expressed in dental epithelium, including basal epithelial cells (Mustonen et al. 2002).
- **During the cap stage, E14-E15**: Receptors Notch-1 and Notch-2 are present in cells of the enamel organ. Except for its basal epithelium Notch-1 mRNAs are expressed throughout the dental epithelium, including the stellate reticulum and the epithelium overlapping the enamel knot, with the strongest signal in cells forming the stratum intermedium and overlying the basal layer (Mitsiadis et al. 1995a, Munccielli et al. 2000, Harada et al. 2006). Notch-2 mRNAs are found only in the oral half epithelium forming stellate reticulum, while their expression is weak or absent in the enamel knot and cervical loop compartments (Munccielli et al. 2000). Regarding pulp mesenchyme, Notch-1 and -2 receptors seem to be absent, but Notch-1 is present in dental papilla mesenchyme with a patchy pattern around the tooth germ. Notch-3 receptor is not expressed in the enamel organ but presumably present in perivascular structures (Mitsiadis 1995a). During this stage, Jagged-1 ligand is not present in dental papilla cells and the stellate reiculum (Mustonen et al. 2002), while Jagged-2 is present only at inner enamel epithelium (Mitsiadis et al. 2005).
- **During the bell stage, E16-E19**: During cytodifferentiation, all Notch receptors are found to be expressed in the enamel organ. More specifically, Notch-1 is mainly expressed in cells of the stratum intermedium and cervical loop area (Mitsiadis et al. 1998, Mitsiadis et al. 1995a), while Notch-2 mRNAs are most abundant in stellate reticulum but it is progressively expressed in the stratum intermedium, outer enamel epithelium and cervical loop area (Mitsiadis 1998, Harada et al. 2006). Interestingly, transcripts of all three Notch genes are absent in preameloblasts. Regarding dental papilla mesenchyme of the cusp region, weak signals of Notch-2 and -3 signals are observed, while Notch-1 and Notch-3 receptors are also correlated with the endothelial cells of blood vessels (Mitsiadis et al. 1998, Mitsiadis et al. 1995a). Late at this stage (E19), Notch-1 and -3

receptors are mainly found in SI, whereas Notch-2 mRNA is also present in the stellate reticulum and outer enamel epithelium. In the dental papilla and follicular mesenchyme the expression of Notch-2 and -3 is rather weak and absent from polarizing odontoblasts. Regarding ligands' presence during bell stage, Delta-1 expression seems to be upregulated in the epithelium-derived ameloblasts and mesenchyme- derived odontoblasts (Mitsiadis et al. 1998, Mustonen et al. 2002), while Jagged 2 ligand is mainly expressed in the inner enamel epithelium (Mitiadis 2005, Harada et al. 2006). At E18.5, a gradient of Delta-1 expression is detected in differentiating odontoblasts, with the strongest signal at the tips of the cusps and progressively lower levels of expression in the developmentally less advanced odontoblasts farther down. Weak expression of Delta-1 occurs in the subodontoblastic layer (Mitsiadis et al. 1998).

- **During the eruption stage (PN1–PN6):** With its terminal differentiation of mesenchymal cells and preameloblasts, Notch-1, -2 and -3 transcripts persist in the enamel organ. All Notch genes are transiently expressed in cells of the papilla mesenchyme of the cusp area just underlying differentiating odontoblasts, while Notch-3 transcripts are also found in the dental follicular mesenchyme. In differentiated odontoblasts or ameloblasts, no expression of any Notch gene has been detected (Mitsiadis et al. 1995a). Jagged-1 ligand has been detected in the inner enamel epithelium and ameloblasts (Mustonen et al. 2002, Harada et al. 2006), while Jagged-2 ligand has been found to be expressed only in the inner enamel epithelium (Mitsiadis et al. 2005, Harada et al. 2006).

Taking everything into consideration, the above mentioned findings provide strong evidence that Notch receptors and ligands control tooth morphogenesis and influence differentiation events.

The specific complementary expression appears to be established by a Notch-mediated feedback regulation mechanism between adjacent cells (Mitsiadis et al. 1998a, Mitsiadis et al. 1999). Similarly, the feedback regulation exerted by Delta-Notch signalling may be responsible for the asymmetries and spatial segregation of Notch-1, 2 and 3 and Delta-1 in different cell layers. This feedback includes positive regulation of Notch-1 and Notch-2 and negative regulation of Delta-1 expression (Lovschall et al. 2005). Moreover, Jagged-2 ligand seems to activate the Notch-1 receptor in mammalian cells (Mitsiadis et al. 2005). That feedback regulation may change the expression level of ligands and Notch receptors in opposing cells. On the epithelial side, the dental epithelial cells appear to constitute a developmentally

equivalent group, in which Delta-Notch signaling between preameloblasts / ameloblasts and the adjacent stratum intermedium may prevent immediately neighbouring cells from adopting an ameloblast fate through lateral inhibition (Mitsiadis et al. 2005). On the other hand, on the mesenchymal side, the expression of Delta-1 in newborn odontoblasts may direct adjacent cells towards an alternative fate (i.e. cells of the subodontoblastic layer), or, alternatively, it may inhibit the adjacent cells from exiting the cell cycle, thus providing a feedback mechanism to control the proportion of cells that will differentiate into odontoblasts (Lovschall et al. 2005).

In the tooth germ of vertebrates, little is known about the ligand-receptor pairs in the feedback regulation loops (Weinmaster 1997). The existing evidence suggests that Delta-1 can interact with any of the three Notch receptors, but the activation level differs among different receptors (Lovscall et al. 2005). More specifically, Jagged-1 is more effective than Delta-1 in activating Notch-2, while both Jagged-1 and Delta-1 can activate Notch-1 efficiently (Lindshell et al. 1995, Weinmaster 1997). Jagged-1 transcripts disappear from the dental mesenchyme at the early bell stage and persist in the epithelial components only in the stratum intermedium at the stage at which Delta-like 1 is upregulated in the dental epithelium (Lovschall et al. 2005).

Although Notch receptors and Jagged-1 ligand are expressed during early tooth morphogenesis in both the epithelium and the mesenchyme (Mitsiadis et al. 1997), it has been shown that Delta-1's expression is not affected by epithelio-mesenchymal interactions in dental explants. This finding suggests that signals intrinsic to both the epithelium and the mesenchyme are responsible for inducing Delta-1 in dental tissues. Intrinsic properties of progenitors have been postulated to control the generation time of different cell types in other systems as well (Lovschall et al. 2005)

2.7.2. Pulp Injury

Mitsiadis et al. 1999 examined the expression of the Notch pathway components in injured rat teeth. They found that Notch receptors were absent from normal adult dental tissues, whereas their expression was upregulated after injury. Both Notch receptors (Notch-1, Notch-2 and Notch-3) and ligand Delta-1 are expressed in injured pulp, but with different immunoreactivities. Notch staining is mainly restricted to mesenchymal cells adjacent to the lesion, while there is no

immunoreactivity in odontoblasts. Notch-2, the most prominent Notch receptor reactivated in injured pulp, is strongly expressed in mesenchymal cells of the pulp either close to the site of injury and at a distance from injured areas. This finding suggests that there may be a potential progenitor pool at the root, which differentiates into odontoblasts or pulp fibroblasts under the influence of growth factors effusing from a lesion (Mitsiadis et al. 2003) Notch-3 expression was mainly associated with vascular structures, while Notch-1 exhibited a rather faint expression which was restricted to few pulpal cells close to the lesion. None of the Notch receptors were expressed in odontoblasts. Expression of Delta-1 was upregulated in odontoblasts of the injured teeth, as well as in vascular structures.

More recent findings demonstrated that Notch-3 receptor has been detected in pericytes of injured rat molar, suggesting that pericytes may represent an alternative source for Dental Pulp Stem Cells (DPSCs) (Lovschall et al. 2007). Furthermore, ligand Delta--1 has been found in odontoblasts of injured molars (Lovschall et al. 2005). The activation of Notch signaling by either Jagged-1 or N1ICD inhibits odontoblast differentiation without affecting dental pulp cell proliferation (Zhang et al. 2008). In contrast, studies on human DPSCs have shown that activation of Notch by Delta-like1 ligand stimulates both cell proliferation and differentiation (He et al. 2009). Taken together, these results indicate that Notch signaling may act as either a negative (through Jagged-1 activation) or a positive (through Delta-like1 activation) regulator of odontoblast differentiation.

The above mentioned evidence not only highlight similarities between developmental and regenerative processes and add further weight to the hypothesis that activation of Notch is fundamental for tooth homeostasis (About et al. 2001), but also depict the context-dependent function of Notch signaling (i.e.: intact/physiological status vs. injury/pathological status, rodent vs. human tissues).

2.7.3. Stem Cells

As it has been already mentioned, Notch biological function depends on the developmental context, type, or state of the cell (Kopan et al. 2009, Artavanis-Tsakonas et al. 2010). However, Androutsellis-Theotokis *a*nd colleagues (2006) proposed that Notch activation promotes stem cell survival. Asymmetric stem cell division ensures stem cell renewal and a progeny of cells that will differentiate, thus ensuring repair and regeneration of tissues and organs. It has been reported that

during asymmetric stem cell division, Notch signaling is inhibited in one of the two daughter cells while it is activated in the other, enabling two different cell types to be generated (Artavanis-Tsakonas et al. 2010). Notch signaling plays a key role in cell fate determination and maintenance of stem cells in various tissues. In bone marrow, the hematopoietic stem cells (HSCs) are located in two different niches, endosteal and perivascular. Notch promotes HSCs self-renewal and inhibits cell differentiation, thus increasing the number of HSCs (Stier et al. 2002, Duncan et al. 2005). HSCs are regulated by signals that are derived from stromal fibroblasts and osteoblasts, which form the HSC niche and express Jagged-1 (Mitsiadis et al. 2007).
He et al. (2009) suggested that activation of Notch by Delta-1 ligand stimulates both cell proliferation and differentiation of human DPSCs.

2.7.4. Caries & Cavity Preparation

It has been shown that Notch-2 receptor is observed in adult carious teeth, while it is completely absent in adult intact teeth. In dental pulp, staining was observed in odontoblasts located beneath the carious front and in cells of the blood vessels.

Nine weeks after cavity preparation, odontoblasts facing the injury site produce either reactionary or reparative dentin. Reactionary dentin matrix is synthesized by odontoblast-like cells replacing the dying odontoblasts after the injury. Interestingly, Notch-2 expression was absent from the site of the reactionary dentin production, but was evident at a distance from the cavity preparation. Furthermore, Notch-2 staining was absent from odontoblast, but cells of the subodontoblastic layer exhibited a strong signal (Mitsiadis et al. 2003).

2.7.5. Periodontitis

Notch pathway not only is implicated in osteoblasts and osteoclast differentiation, but also it may be involved in pathological bone remodeling processes, such as rheumatoid arthritis and periodontitis (Duan et al. 2013). The latter is characterized by loss of connective tissue attachment and alveolar bone destruction, as a result of inflammation triggered by bacterial biofilms attached on the tooth surface. The pathogenesis of the disease mainly depends on a host immune

response to the bacterial infection, which is dominated by the presence of T cells (Yoshie et al. 1987). Recent findings based on a clinical study (Zhao et al. 2011) propose that Th17 cells may take part in the development of periodontitis by upregulating the expression of cytokines IL-17. Several factors are known to affect Th17 differentiation, including antigenic stimuli (Mukherjee et al. 2009, Peters et al. 2010), expression of particular transcription factors (Zhou et al. 2009) and epigenetic changes in the IL-17 gene locus (Akimzhanov *et al.* 2007).

Keerthivasan and colleagues (2011) in their recent study highlight the importance of Notch signaling in Th17 differentiation and suggest that selective targeted therapy against Notch may be an important tool to treat autoimmune disorders. They reported that Notch-1 is activated in both mouse and human *in vitro*-polarized Th17 cells. Blockade of Notch signaling significantly down-regulates the production of Th17-associated cytokines, suggesting an intrinsic requirement for Notch during Th17 differentiation in both mice and human species.

2.7.6. Orthodontic movement -induced Root Resorpton

Several studies have been conducted in order to determine the relationship between Notch signaling pathway and bone metabolism. Sethi and colleague (2011) proposed that Jagged-1 promotes tumor growth by stimulating IL-6 release from osteoblasts and directly activating osteoclast differentiation. Furthermore, Nakao et al. (2009) suggested that Jagged-1 promotes RANKL-induced osteoclastogenesis, while Fukushima et al. (2008) reported that RANKL induces the production of Notch-2 and Jagged-1 in bone marrow macrophages during osteoclastogenesis. Recently, Kikuta et al. (2015) investigated the expression of Jagged-1, Notch-2, RANKL, and IL-6 in areas of root resorption during experimental tooth movement in rats, in an attempt to shed light on the possible involvement of Notch Pathway in orthodontically induced inflammatory root resorption. They found the compression force increased the production of Jagged-1, IL- 6, and RANKL from the human Periodontal Ligament (hPDL) cells and their overall findings indicate that the Notch signaling response to excessive orthodontic forces stimulates the process of root resorption via RANKL and IL-6 production from hPDL cells.

Taking everything into consideration, the above mentioned findings suggest that the increased Jagged-1 expression observed in hPDL cells treated with excessive compression force activates osteoclastogenesis and odontoclastogenesis.

IL-17 may stimulate osteogenesis and odontoclastogenesis via Notch signaling, which may subsequently contribute to the inflammatory response associated with ensuing orthodontic induced inflammatory root resorption.

On the other hand, recent pathological and in vitro studies contradict the findings of the present study. For instance, Li and colleagues (2014) demonstrated that Notch signaling enhances the osteogenic differentiation of periodontal ligament stem cells (PDLSCs) in osteoporotic rats, while Ugarte and colleagues (2009) reported that Notch signaling enhances osteogenic differentiation in primary human bone marrow stromal cells.

The discrepancies between the above mentioned studies can be attributed to differences in the cell types used and the stimuli applied to the cells.

2.7.7. Palate Formation

Casey and colleagues (2006) reported, in their animal study, that ligand Jagged-2 is expressed throughout the oral epithelium and is required for receptors Notch-1 activation during oral epithelial differentiation. Notch-1 is normally highly activated in the differentiating oral periderm cells covering the developing tongue and the lateral oral surfaces of the mandibular and maxillary processes during palate development. Thus, the activation of Notch pathway seems to regulate and guide oral epithelium in order to prevent premature palatal shelf adhesion to other oral tissues and to facilitate normal adhesion between the elevated palatal shelves

2.7.8. Taste

Seta and colleagues (2003) found that Notch pathway components are expressed in embryonic taste epithelium by E14,5. It is possible that Notch signaling may be involved in the specification of taste bud progenitor cell types from among epithelial cells of the circumvallate papilla.

2.7.9. Notch in Head and Neck Cancer

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common malignancy in the world, with an annual world-wide incidence of over 600,000 cases

per year and 350,000 deaths per year (Ferlay et al. 2008). Investigators have uncovered several critical genes and pathways important to the tumorigenesis of HNSCC, including Notch. To gain a comprehensive view of the genetic alteration in HNSCC, Agrawal and colleagues (2011) and Stransky and colleagues (2011) used a high-throughput next-generation sequencing technique to analyze the HNSCC genome. Both groups sequenced the exons of all known human genes in tumor DNA and compared the sequence to that of the corresponding normal DNA from the identical patient. In total, the genomic landscapes of 32 and 74 tumors were examined, respectively. Both of them reported novel mutations in Notch-1. In both studies, inactivating mutations of Notch-1 were found in 10% to 15% of the HNSCC tumors, making Notch-1 the second most frequently mutated gene after TP53. Moreover, Sun and colleagues (2014) demonstrated the frequent occurrence of aberrant DNA copy number gains of Notch signaling pathway genes in HNSCC tumors compared with that in normal mucosa and they reported that 8 Notch pathway's genes exhibited increased DNA copy numbers in HNSCC tumors versus normal mucosa based on outlier analysis. The broad copy number alterations identified in the above mentioned study imply that in addition to Notch-1 mutations, copy number gains of Notch signaling pathway genes may contribute to HNSCC tumor development. Among these genes featuring copy number gains in HNSCC tumors, the identification of Notch ligand Jagged-1 is intriguing, as the expression array analysis demonstrated that Jagged-1 was significantly over-expressed (30%) in HNSCC tumors compared with that in normal mucosa. Taking everything into consideration, their study suggests that genomic amplification in part contributes to the over-expression of Jagged-1 in HNSCC. In a recent study, Izumchenko and colleagues (2015) investigated the prevalence of Notch-1 mutations in Chinese patients with oral leukoplakia and invasive oral cancers. They demonstrated that 54% of the primary squamous cell carcinomas and 60% of the oral potentially malignant lesions carried a Notch-1 mutation.

Regarding Notch signaling pathway in benign and malignant ameloblastic neoplasms, Nakaro and colleagues (2008), using immunohistochemical methods and in-situ hybridization, found Notch-1 intracellular domain (NICD) positive products in the cells at the peripheral layer of most proliferating epithelial tumor nests in ameloblastoma and in ameloblastic carcinoma. In particular, small numbers of mitoses were identified in the nuclear region with intense NICD positive reaction. The authors concluded that Notch signaling may take part in cytological differentiation or acquisition of tissue specific characteristics in neoplastic cells of odontogenic

neoplasms, including ameloblastoma and ameloblastic carcinoma and that Notch-1 may also contribute to cell cycle arrest induced by Notch-1 activation in ameloblastic carcinoma. Moreover, Kumamoto and colleagues (2008) examined ameloblastomas by reverse transcriptase polymerase chain reaction and by in situ hybridization to determine the expression of Notch-1, Notch-2, Notch-3, Delta-1, and Jagged-1. They reported that mRNA expression of Notch-1, Notch-2, Notch-3, Delta-1, and Jagged-1 was detected in all samples of normal and neoplastic odontogenic tissues. In tooth germs, Notch receptors were expressed in odontogenic epithelium (except for inner enamel epithelium), and expression of Notch ligands was lower in inner enamel epithelium than in other epithelial components. Ameloblastomas showed expression of Notch receptors and ligands in central polyhedral neoplastic cells, while Notch-2, Delta-1, and Jagged-1 were expressed in some neoplastic cells neighboring the basement membrane. The authors concluded that the expression of Notch pathway components in tooth germs and ameloblastomas suggest that Notch signaling might control cell differentiation and proliferation of normal and neoplastic odontogenic epithelium.

Notch signaling abnormity has been implicated as important molecular event in recent next generation sequencing studies of adenoid cystic carcinoma (ACC). Whole-genome sequence analysis indicates a 13% Notch-1 gene mutations, and exon sequencing in a series of 24 adenoid cystic carcinoma samples also identifies this finding (Ho et al. 2013, Stephen et al. 2013). Recently, Zhao and colleagues (2015) investigated the expression of Notch signaling pathway, and its relation with EMT program, in ACC by a custom-made Tissue Microarray in order to evaluate the immunoreactivity of Notch signaling and EMT program. They observed that Notch Intra-Cellular Domain (NICD) expression was almost negative in the normal salivary gland. On the other hand, NICD's expression was increased in pleiomorphic adenoma and highest in ACCs, and it was mainly found located in the nucleus and cytoplasm at the glandular structure of neoplasm tissues. The authors suggest that Notch signaling pathway may play significant roles in adenoid cystic progression through their relationship with EMT progress.

3. AIM

The aim of the present study was to gain a better understanding of the role of the Notch Signaling Pathway periapical cysts. To our knowledge, currently, there are no reports about the presence of ligand Jagged-1 and HERP transcription factor in periapical cysts.

Thus, in this study, the immunohistochemical expression of Notch-1 and Notch-2 receptors, Jagged-1 ligand and HERP1 transcription factor in periapical cysts was evaluated and correlated with the presence of inflammation.

4. MATERIALS AND METHODS

Material

This is a retrospective study on archived biopsy specimens from the Department of Oral Pathology and Medicine, Dental School, University of Athens, between 1990-2011. The protocol was approved by the Committee on the Ethics of the Dental School, University of Athens (P. No.: 262). All lesions were fixed in 10% (v/v) neutral buffered formalin solution (24-48 hours), dehydrated in by graded alcohol 70%, 80%, 90% and 100%, then immersed in xylene and embedded in paraffin wax (Paraplast) at 59 ^{o}C and prepared for Immunohistochemistry.

Thirty lesions were selected according to the following criteria:

- Histological diagnosis of periapical cysts (PC): Diagnosis was established in conventional hematoxylin and eosin stained tissue sections. The criterion used to diagnose the lesions as periapical cyst was a well-delimited cavity lined by stratified squamous epithelium, as defined by Nair et al. 1998.

- Adequate amount of tissue.

- Adequate clinical and demographic data related to the sample.

The cysts were grouped according to the presence of inflammatory cell in the underlying connective tissue and the inflammatory infiltrate was classified as **scarce** or **intense** according to the presence of inflammatory cells. Lesions characterized by <20% inflammatory infiltration were classified as **non-inflamed** periapical cysts (Fig. 7), while lesions characterizes by >50% inflammatory infiltrate were classified as **inflammed** periapical cysts (Orstavic & Mjor 1988) (Fig.7). (Table 2, 3)

	N	%
INFLAMMATION		
Non-inflamed PCs	15	50
Inflamed PCs	15	50
Total	100	100

Table 2: Sample classification

A/A	#	DIAGNOSIS	A/A	#	DIAGNOSIS
1	**98-215**	Inflamed PC	1	**90-666**	Non-inflamed PC
2	**02-488**	Inflamed PC	2	**91-240**	Non-inflamed PC
3	**02-478**	Inflamed PC	3	**91-278**	Non-inflamed PC
4	**04-731**	Inflamed PC	4	**91-314**	Non-inflamed PC
5	**05-796**	Inflamed PC	5	**91-413**	Non-inflamed PC
6	**05-873**	Inflamed PC	6	**91-490**	Non-inflamed PC
7	**06-127**	Inflamed PC	7	**92-187**	Non-inflamed PC
8	**07-670**	Inflamed PC	8	**92-230**	Non-inflamed PC
9	**07-805**	Inflamed PC	9	**92-268**	Non-inflamed PC
10	**07-872**	Inflamed PC	10	**92-381**	Non-inflamed PC
11	**08-1047**	Inflamed PC	11	**92-394**	Non-inflamed PC
12	**10-450**	Inflamed PC	12	**94-569**	Non-inflamed PC
13	**10-799**	Inflamed PC	13	**95-325**	Non-inflamed PC
14	**10-1127**	Inflamed PC	14	**95-377**	Non-inflamed PC
15	**11-120**	Inflamed PC	15	**95-616**	Non-inflamed PC

Table 3: Sample Distribution

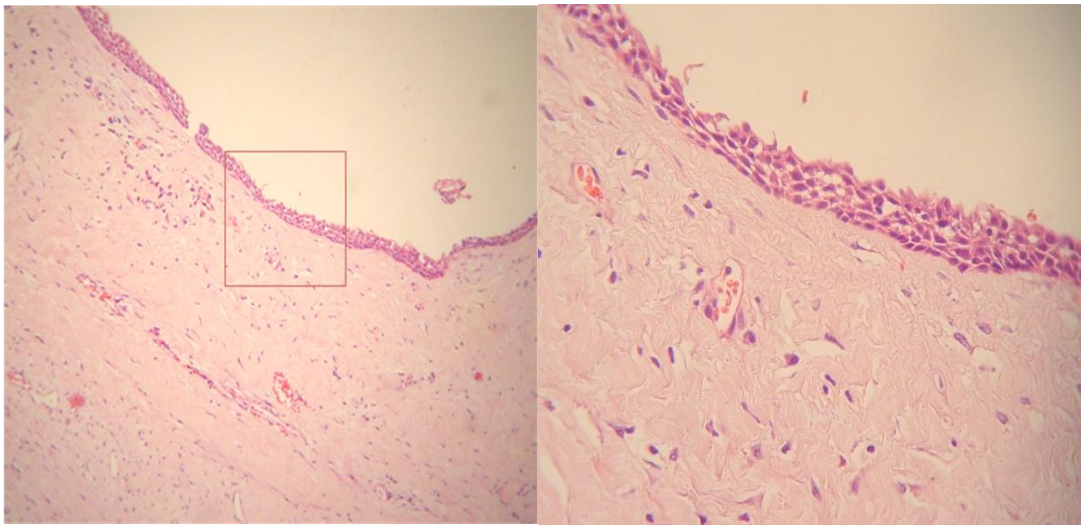


Fig.6: Representative section of a **non-inflamed** periapical cyst (90-666). Notice the scarce (<20%) inflammatory infiltration of the underlining connective tissue. (Hematoxylin and eosin stained, magnification x100 & x400, respectively)

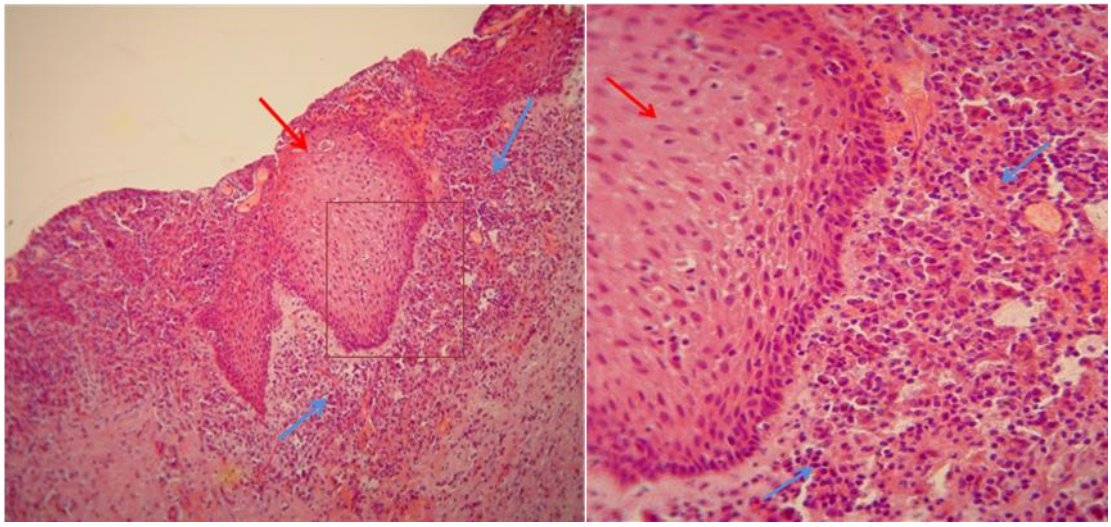


Fig.7: Representative section of a periapical **inflamed** cyst (07-872). Notice the epithelium (red arrows) and the **intense** inflammatory infiltration (blue arrows) of the sub-epithelial connective tissue. (Hematoxylin and eosin stained, magnification x100 & x400, respectively)

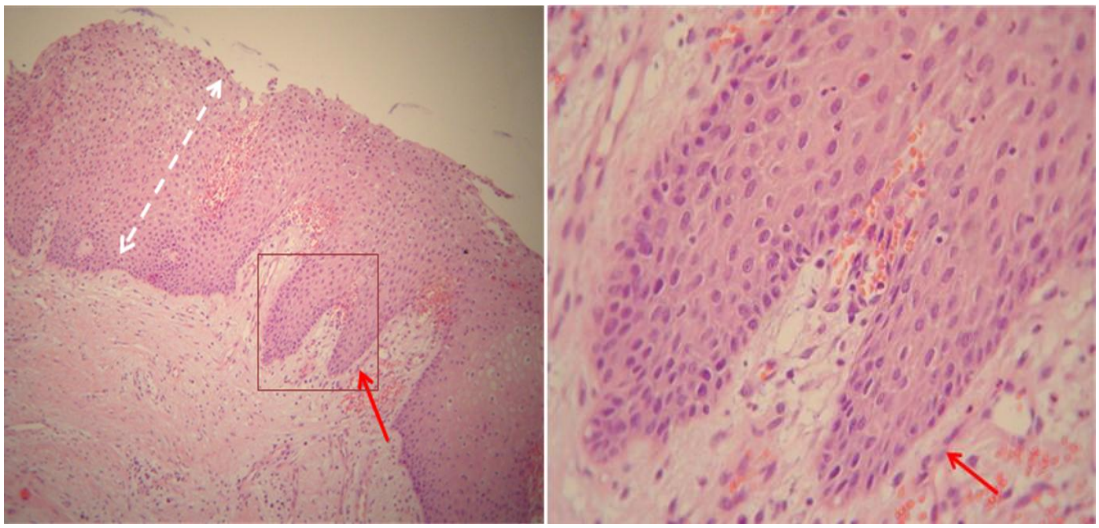


Fig.8: Representative section of a **non-inflamed** periapical cyst (92-187). Notice the epithelium (white arrow) with long rete pegs (red arrows), and **scarce** inflammatory infiltration of the underlining connective tissue. (Hematoxylin and eosin stained, magnification x100 & x400, respectively)

IMMUNOHISTOCHEMISTRY

The paraffin-embedded tissue blocks were cut at 5μm thickness on a microtome (Leica, RM2145) and floated in a 59°C water bath containing distilled water (Kunz instruments, HIR-3). The sections were transferred onto glass poly-L-lysine slides suitable for immunohistochemistry (#P8920, Sigma USA). The slides were dried in 60°C oven overnight and they were stored at room temperature until ready for use.

Immunohistochemistry was performed with 4 different antibodies: (Table 4)

1. Mouse Monoclonal Notch-1 Antibody, catalog No: NBP1-48289, Novus Biologicals, Inc., Littleton, USA

2. Rabbit Polyclonal Notch-2 Antibody, catalog No: AP07611SU-N, Acris Antibodies GmbH, Germany

3. Rabbit Polyclonal Jagged-1 Antibody, catalog No: NBP1-90208, Novus Biologicals, Inc., Littleton, USA

4. Rabbit Polyclonal HEY1/HERP Antibody, catalog No: ab22614, Abcam, Cambridge, UK

	Notch-1	**Notch-2**	**Jagged-1**	**HERP**
Clonality	Monoclonal	Polyclonal	Polyclonal	Polyclonal
Dilution	1:100	1:10	1:15	1:20
Epitope Retrieval Solution	CC1 (EDTA)	ER1 (citric)	ER1 (citric)	CC1 (EDTA)
Source	Novus Biologicals	Acris Antibodies	Novus Biologicals	Abcam

Table 4: Selected Antibodies

Immunohistochemistry Protocols

- The immunohistochemical staining of Notch-1 and HEY1 antibodies was performed with an automated Immunostaining Ventana Benchmark®XT XT system (Ventana Medical Systems, Inc., USA).

For the immunohistochemical staining of Notch-1 antibody the following protocol was applied:

1. Pretreatment with Cell Conditioning 1 (CC1) (EDTA pH 9) (catalog N. 950-124, Ventana Medical Systems, Inc.) for 60 min at 90°C for the epitope retrieval.
2. Incubation with Protease 1 (catalog No 760-2018) at 37°C for 4min, in order to allow the antibody to recognize and bind epitopes
3. Incubation with the primary antibody (dilution 1:100) at 37 °C for 28min.
4. Staining with iView DAB Detection Kit (catalog No 760-091, Ventana Medical Systems, Inc.) which is an indirect biotin streptavidin system.

For the immunohistochemical staining of HEY1 antibody the following protocol was applied:

1. Pretreatment with Cell Conditioning 1 (CC1) (EDTA pH 9) (catalog N. 950-124, Ventana Medical Systems, Inc.) for 60 min at 90°C for the epitope retrieval.
2. Incubation with the primary antibody (dilution 1:20) at 37 °C for 28min.
3. Staining with iView DAB Detection Kit (catalog No 760-091, Ventana Medical Systems, Inc.)

- The immunohistochemical staining of Notch-2 and Jagged-1 antibodies was performed in Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol:

1. Deparaffinization and pre-treatment with Epitope Retrieval Solution 1 (ER1) (citrate based pH 6.0) at 90°C for 20 min.
2. Peroxidase blocking was carried out for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH).
3. Incubation with the primary antibodies for 20 min at room temperature (Notch-2 dilution 1:10, Jagged-1 dilution 1:15)

4. Incubation with polymer for 10 min and development with DAB-Chromogen for 10 min.

Controls

Breast cancer tissues were used as positive control for all antibodies, with the above mentioned protocols.
For negative control, substitution of the primary antibodies with non-immune serum of the same specificity was utilized.

Evaluation of Immunostaining

Immunostained slides were evaluated on a consensus basis by two examiners using a double-headed light microscope.

The staining positivity, intensity and extent of immunohistological staining were evaluated using modification of the semi-quantative method described by Loreto et al (2013).

The intensity of the staining was graded using a three-step scale, (0: faint, 1: moderate, 2: strong positive staining) and the **extent** was graded using a three-step scale, (0: >5%, 1: 5-50%, 2: >50%) of the target cells.

For the final evaluation score, the product of intensity and extent was calculated: intensity * extent = score
Thus, a final 4-step score was used for the positivity of staining *(0: no positive staining, 1: mild, 2: moderate, 4: strong positive staining).*

Statistical analysis was performed regarding the presence of immunohistochemical staining in correlation with the degree of inflammation in periapical cysts using Chi-square Test, Fisher Exact Test with the Freeman-Halton extension.

5. Results

Patient Demographic Data

The samples were obtained by 16 males and 14 females patients and their age ranged between 22 and 73 years old (mean age 41 years).

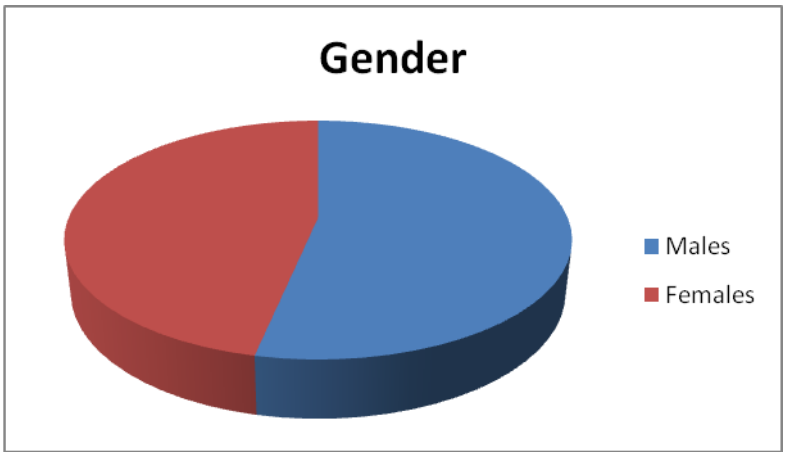
Thirteen patients (43.3%) reported the presence of symptoms, while seventeen (56.7%) were asymptomatic. Information on patient demographics, specific anatomical location and presence of symptoms are analyzed in the following table. (Table 5)

	N	**%**
GENDER		
Male	16	53.3
Female	14	46.6
Total	30	100
ANATOMICAL DISTRUBUTION (ARCH)		
Maxilla	19	63.3
Mandible	11	36.6
Total	30	100
SYMPTOMS		
Presence	13	43.3
Absence	17	56.7
Total	30	100
	MEAN	
AGE	41	

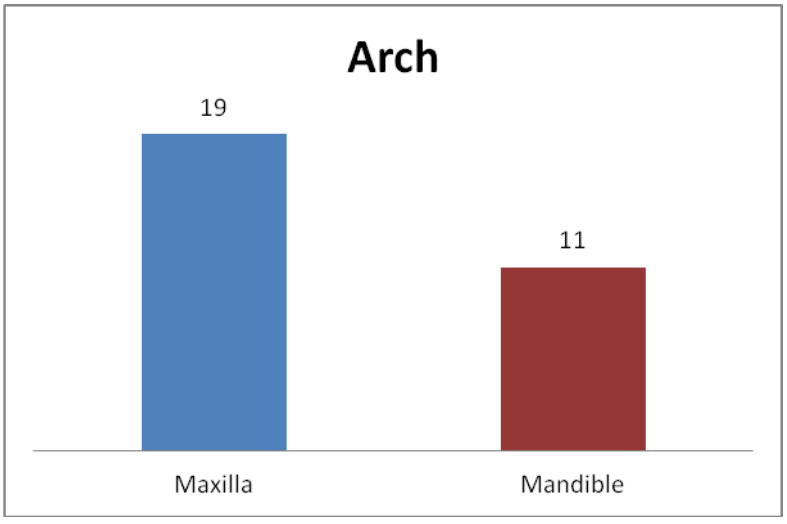
Table 5: Sample Demographic Characteristics

Most of the lesions included in the sample were surgically removed from the maxillary region (n=19), while the rest (n=11) were obtained from the mandible. Sixteen patients were males and fourteen were females (Histograms 1, 2).

All specimens (100%) presented lining epithelium, as it was one of the inclusion criteria, which presented various degrees of thickness.



Histogram 1: Gender distribution



Histogram 2: Anatomical Distribution

Regarding control specimens (breast tumor), cytoplasmatic immunostaining reaction of Notch-1, Notch-2, Jagged-1 and HERP was observed in all human breast tumor cells but not in the stromal cells (Fig.9-12).

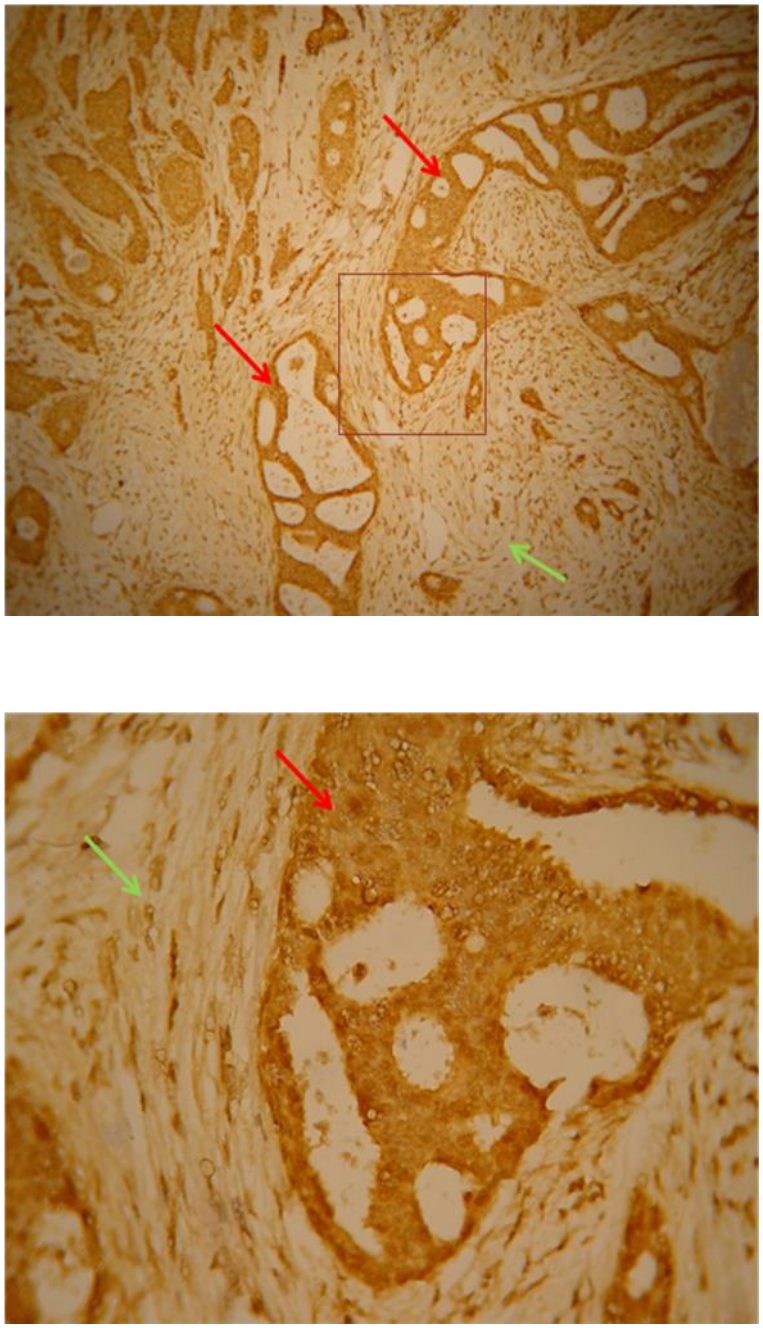


Fig.9: Immunohistochemical localization of Notch-1 in breast cancer biopsy. Notch-1 positive staining is distributed in the human breast tumor cells (red arrows), while no immunoreactivity was observed in the stromal cells (green arrows) (chromogen DAB, magnification x100 & x400, respectively)

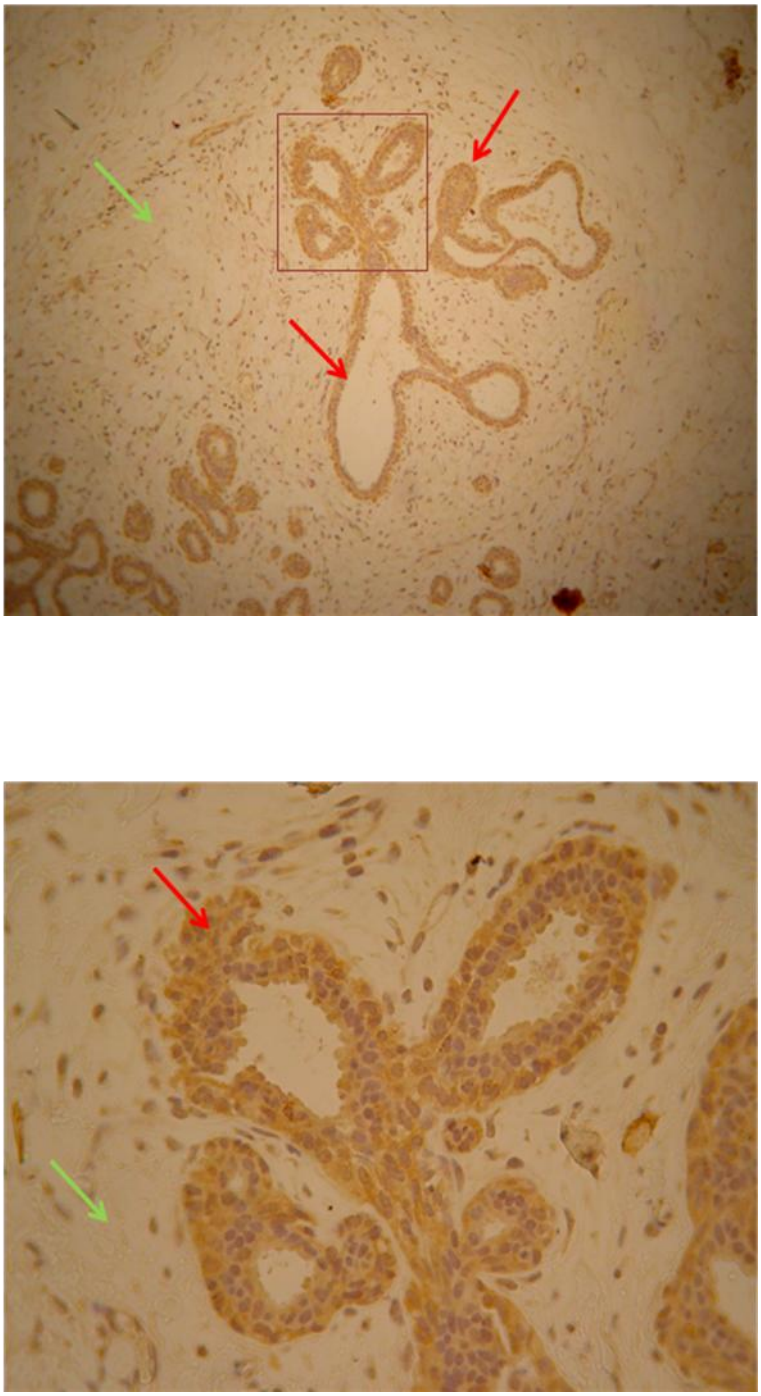


Fig.10: Immunohistochemical localization of Notch-2 in breast cancer specimen. Notch-2 positive staining is observed in the human breast tumor cells (red arrows), while no immunoreactivity was observed in the stromal cells (green arrows). (chromogen DAB, magnification x100 & x400, respectively).

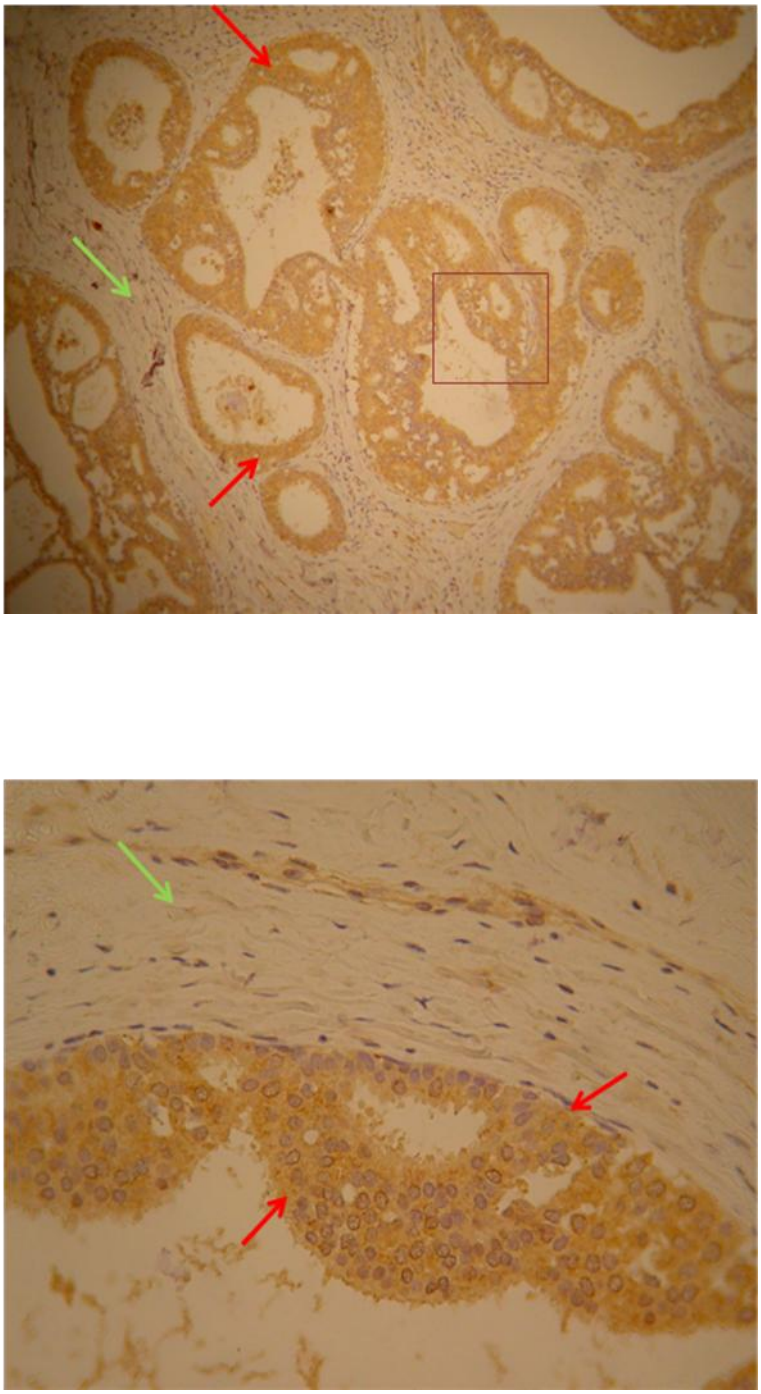


Fig.11: Immunohistochemical localization of Jagged-1 in breast cancer biopsy. Jagged-1 positive staining is distributed in the human breast tumor cells (red arrows), while no immunoreactivity was observed in the stromal cells (green arrows). (chromogen DAB, magnification x100 & x400, respectively)

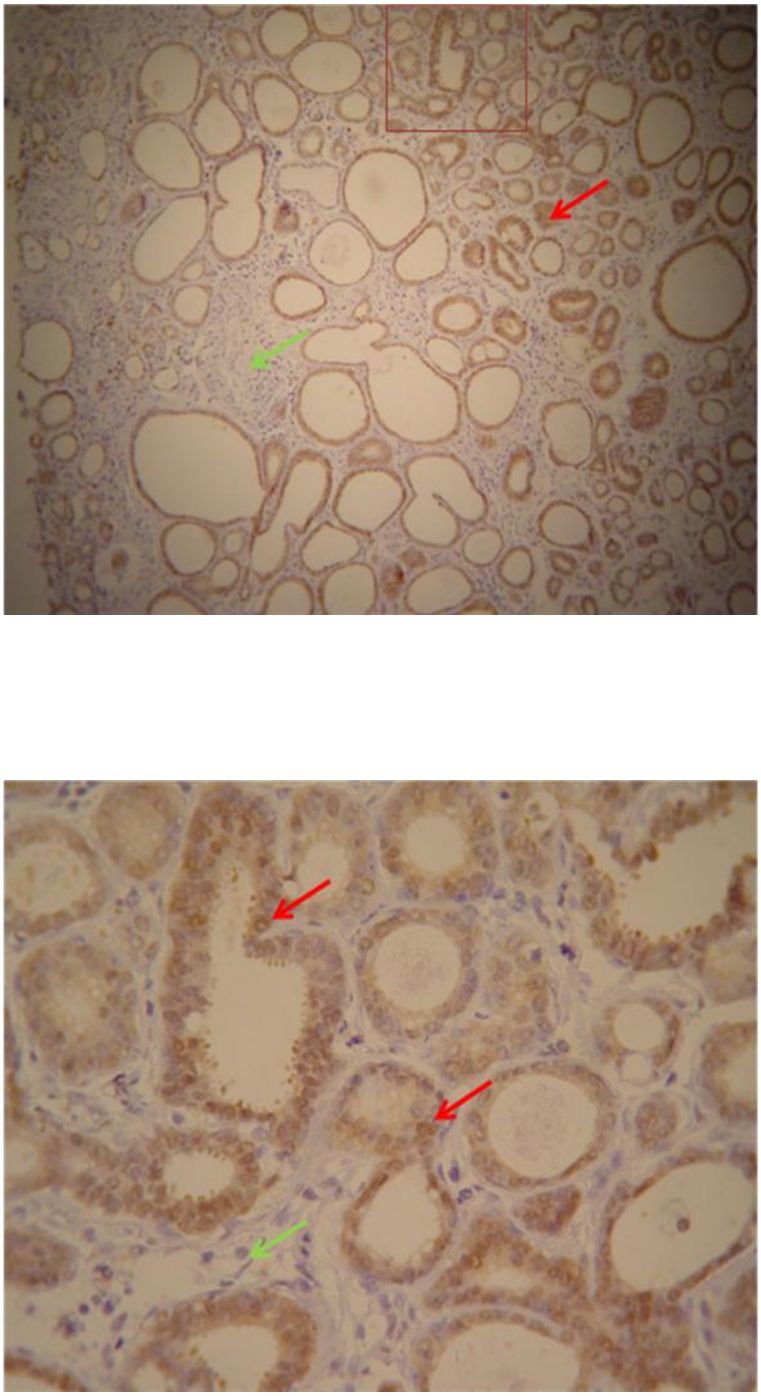


Fig.12**:** Immunohistochemical localization of HERP in breast cancer biopsy. HERP positive immunostaining is observed in human breast tumor cells (red arrows), while no immunoreactivity was observed in the stromal cells (green arrows). (chromogen DAB, magnification x100, x400 respectively)

A positive immunoreactivity was detected for Notch-1, Notch-2 and Jagged-1 antibodies in the epithelial cells of periapical cysts , while HERP was not detected. No immunoexpression could be detected in the underlying connective tissue, with the exception of their expression in some subepithelial inflammatory cells.

Notch-1

Immunohistochemical reactivity for Notch-1 was detected in the cytoplasm of the basal and suprabasal epithelial cells of all periapical cysts (100%), in some subepithelial inflammatory cells and endothelial cells. Only one specimen (3.33%) presented mild staining for Notch-1, while fourteen (46.67%) had moderate expression and fifteen (50%) strong expression. (Fig.13- 17).

Presence of inflammation does not affect the expression of Notch-1 in a statistically significant manner (Fishers Exact Test =1)

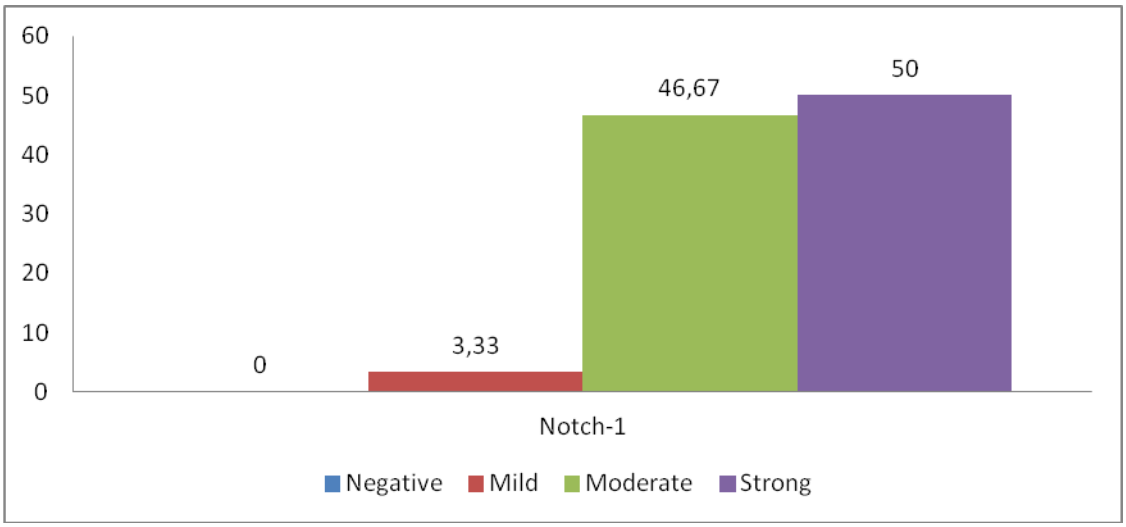
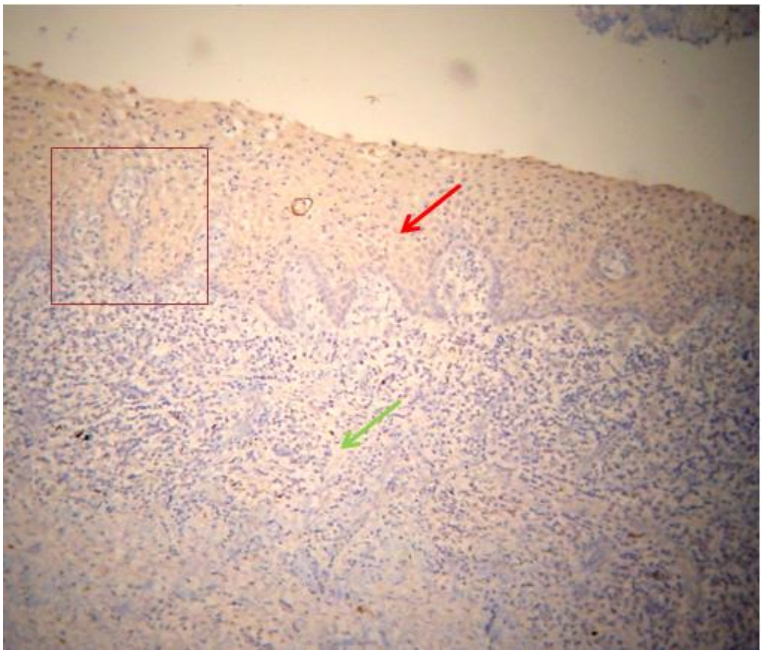


Figure 13: Histogram of Notch-1 expression distribution



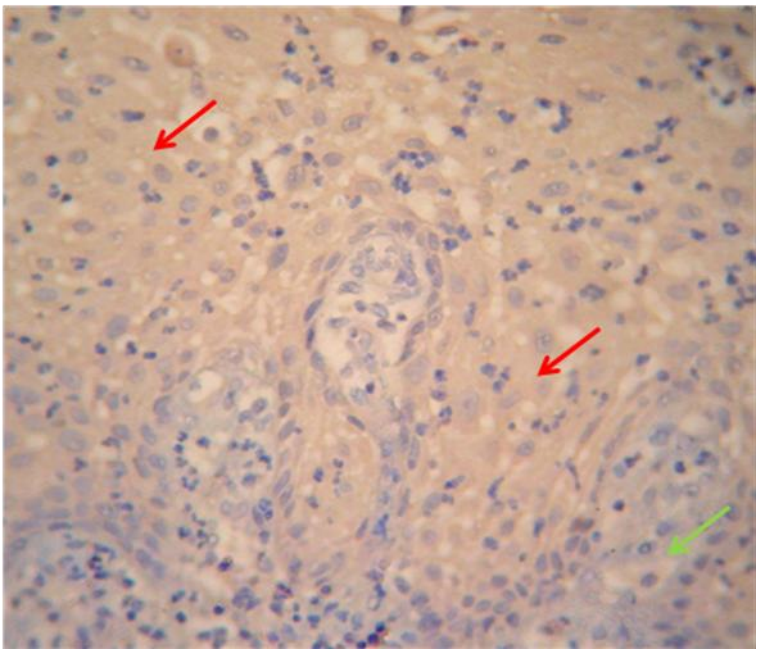
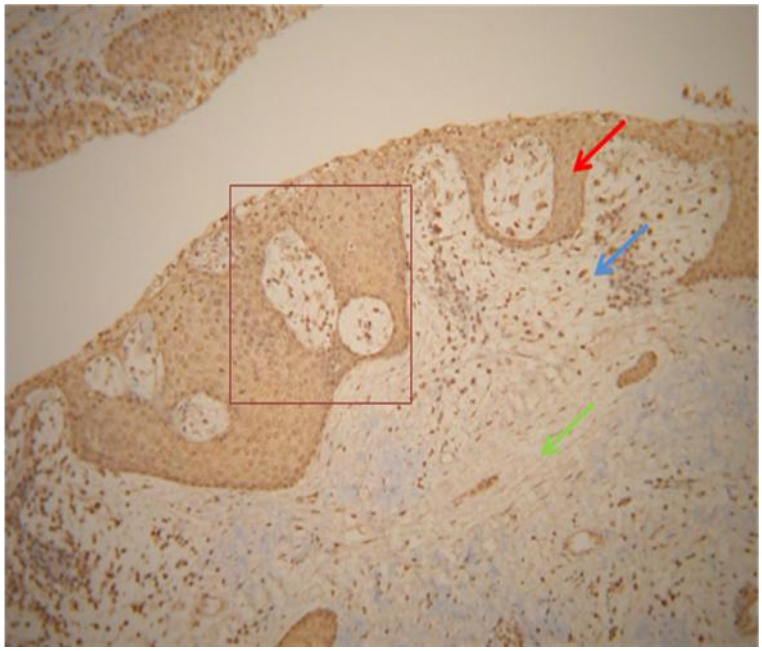


Fig. 14a, b: Notch-1 localization in a periapical **inflamed** cyst (04-731). **Moderate** staining is observed in the cytoplasm of epithelial cells (red arrows). The underlining connective tissue remains negative for Notch-1 (green arrow). (chromogen DAB, magnification x100 & x400, respectively)



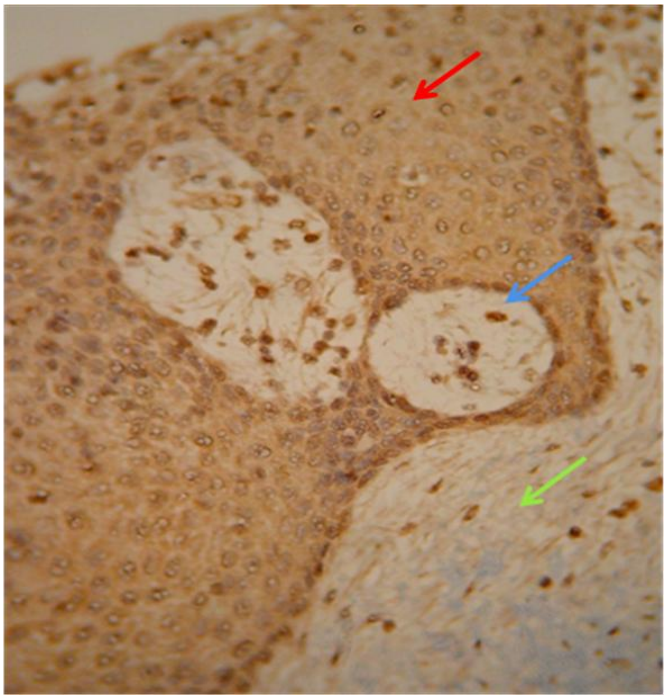
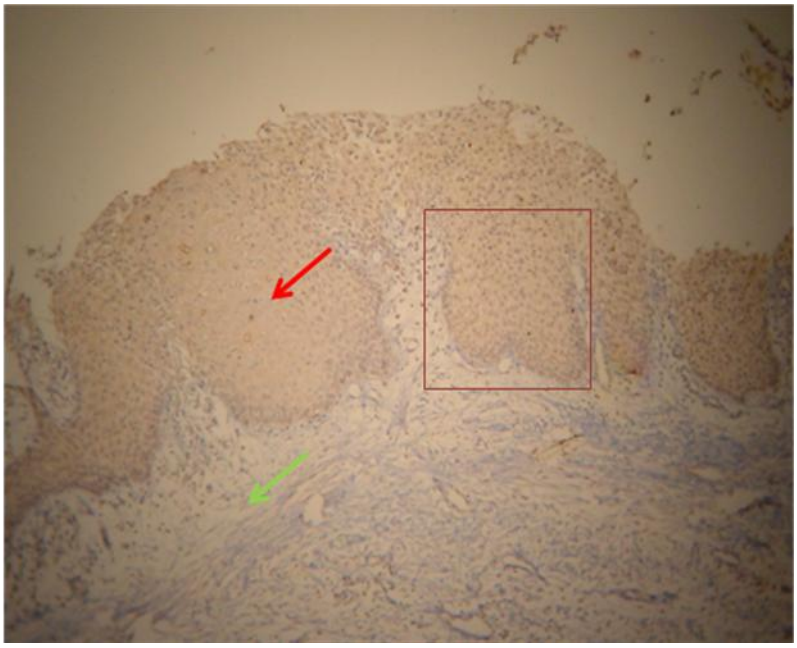


Fig. 15a,b: Notch-1 localization in a periapical **inflamed** cyst (07-805). **Strong** staining is observed in the epithelial cells (red arrow) and some sub-epithelial inflammatory cells (blue arrow). The underlining connective tissue remains negative for Notch-1 (green arrow). (chromogen DAB, magnification x100 & x400, respectively)



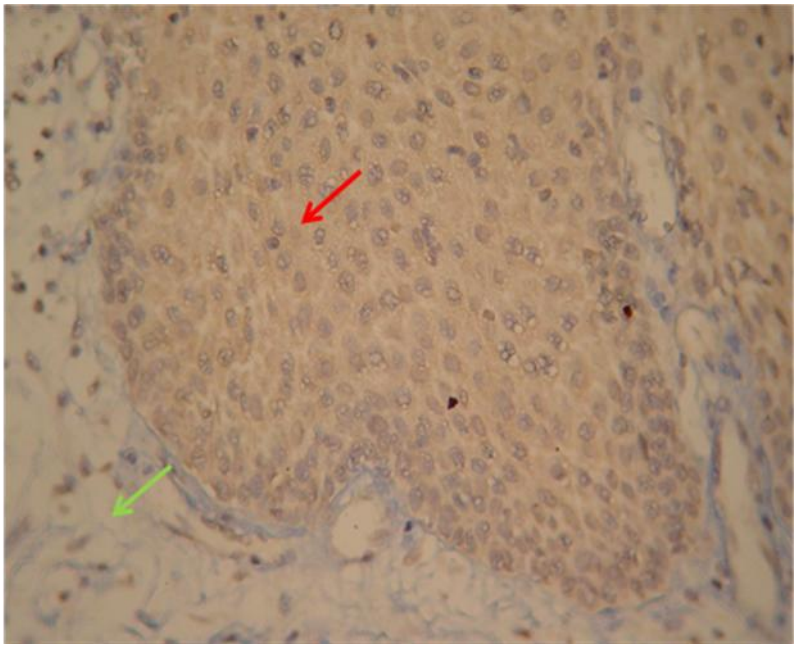


Fig. 16a, b: Notch-1 localization in a **non-inflamed** cyst (91-314). **Moderate** staining is observed in the epithelial cells (red arrow). The underlining connective tissue remains negative for Notch-1 (green arrow). (chromogen DAB, magnification x100 & x400, respectively)

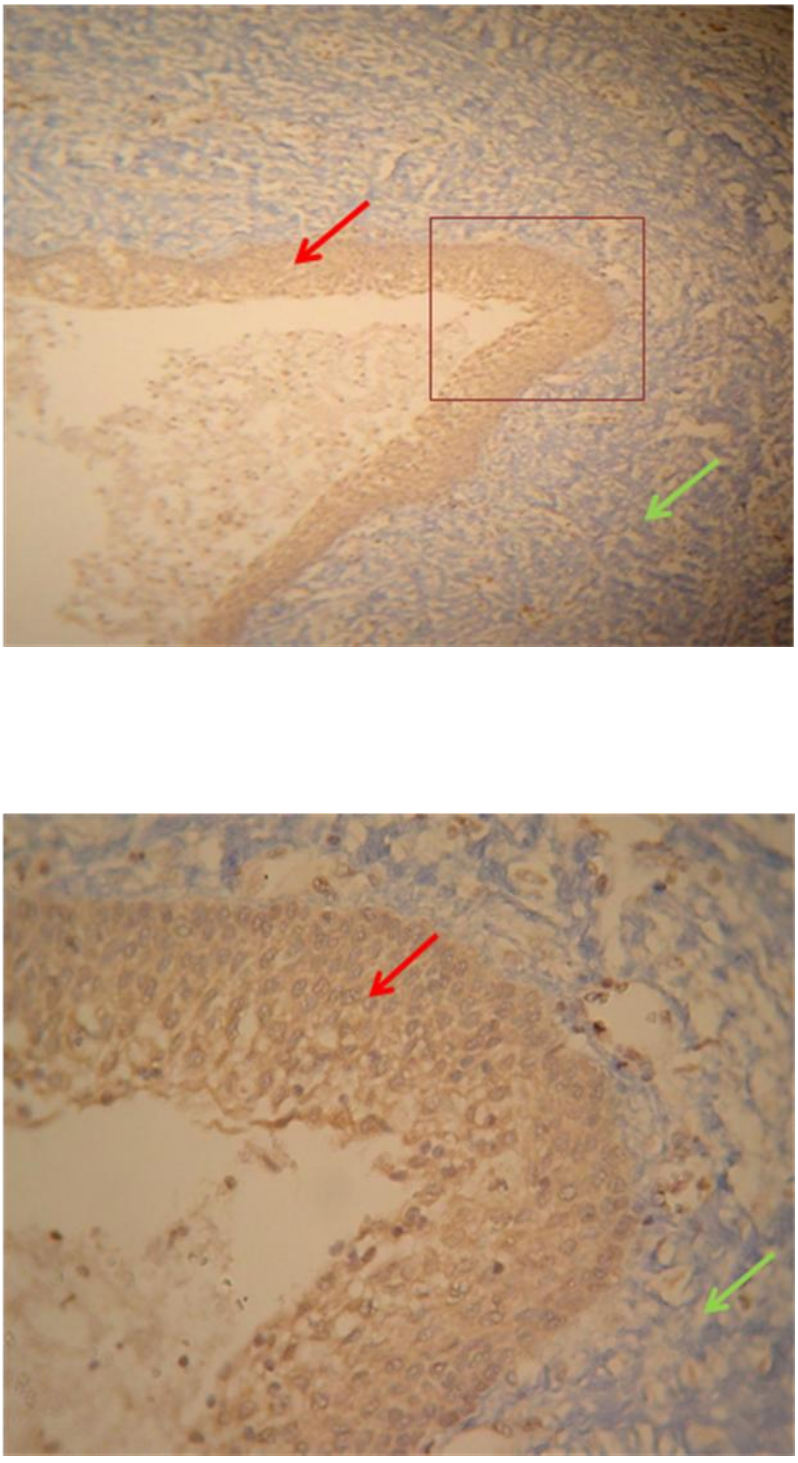


Fig. 17a, b: Notch-1 localization in a **non-inflamed** cyst (92-230). **Strong** staining is observed in the epithelial cells (red arrow), while the subepithelial connective tissue remains negative for Notch-1 (green arrow). (chromogen DAB, magnification x100 & x400, respectively)

Notch-2

Expression of Notch-2 was detected in the cytoplasm of the basal and suprabasal epithelial cells of nineteen periapical cysts (79.1%) while in five specimens (20.83%) no expression of Notch-2 could be detected. Positive expression was also observed in some subepithelial inflammatory and endothelial cells**.** (Fig.19-24)

From the negative specimens, two belonged to the group of inflammatory cysts and three in the group of non-Inflammatory cysts.

From the positive specimens, one demonstrated mild expression (5.26%), nine (47.36) moderate expression and nine (47.36%) strong expression for this antibody. The remaining six specimens were lost during the immunohistochemistry process. (Figure 18)

Presence of inflammation does not affect the expression of Notch-2 in a statistically significant manner (x^2=0.086, p=0.768) (Fisher exact test=1).

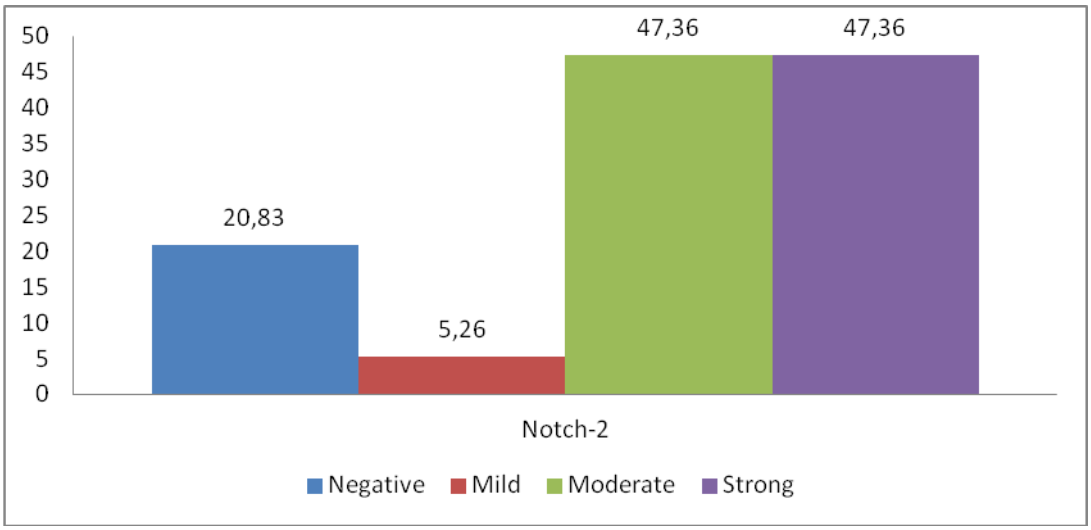


Figure 18: Histogram of Notch-2 expression distribution

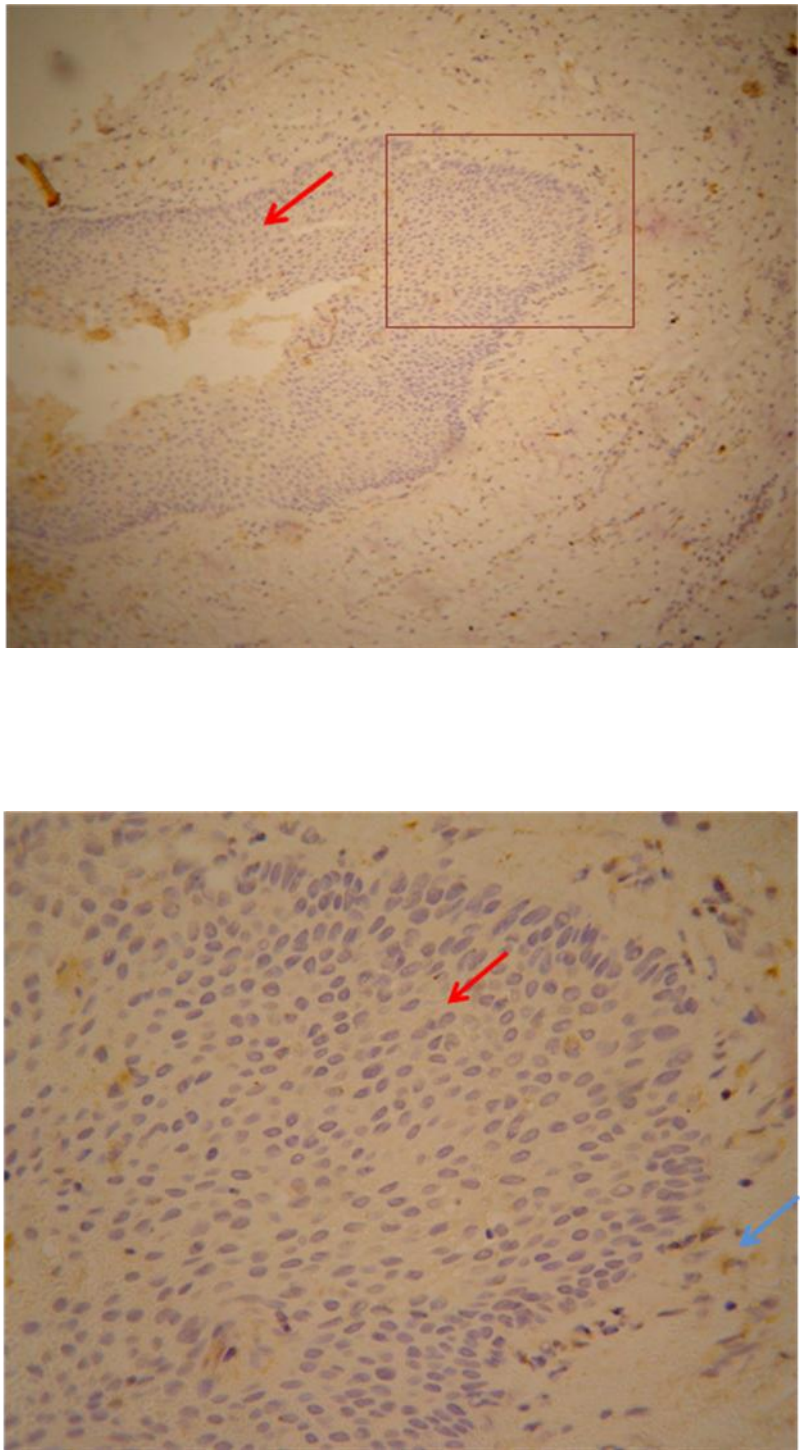
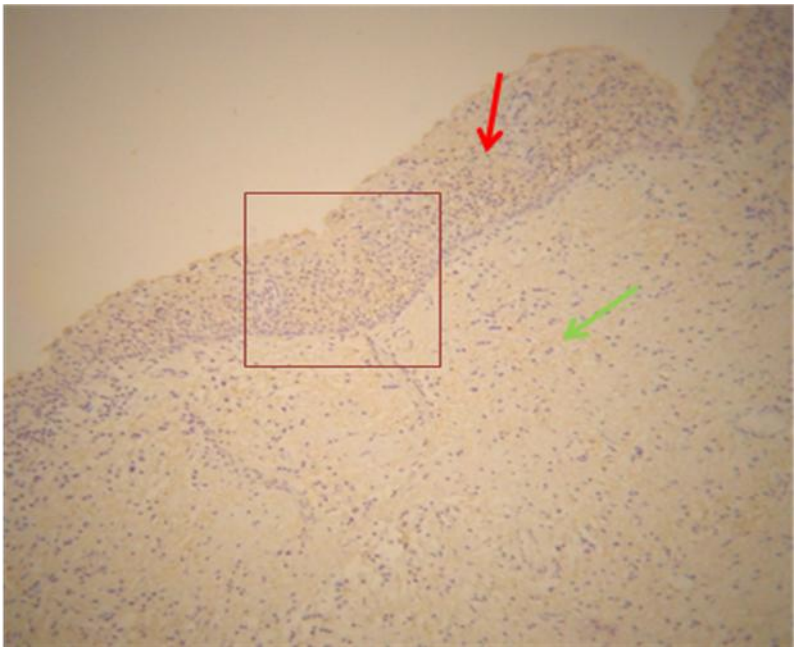


Fig. 19a, b: Specimen of a periapical **inflamed** cyst (10-1127). **Negative** immunoreactivity of Notch-2 is observed in the epithelial cells (red arrows) as well as the subepithelial connective tissue. Positive immunostaining can be observed in some sub-epithelial inflammatory cells (blue arrows). (chromogen DAB, magnification x100 & x400, respectively



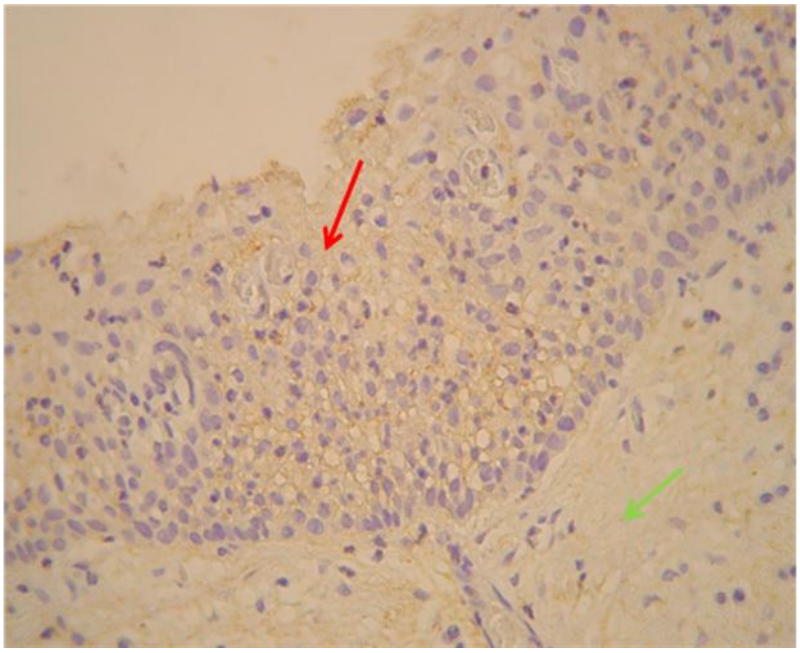
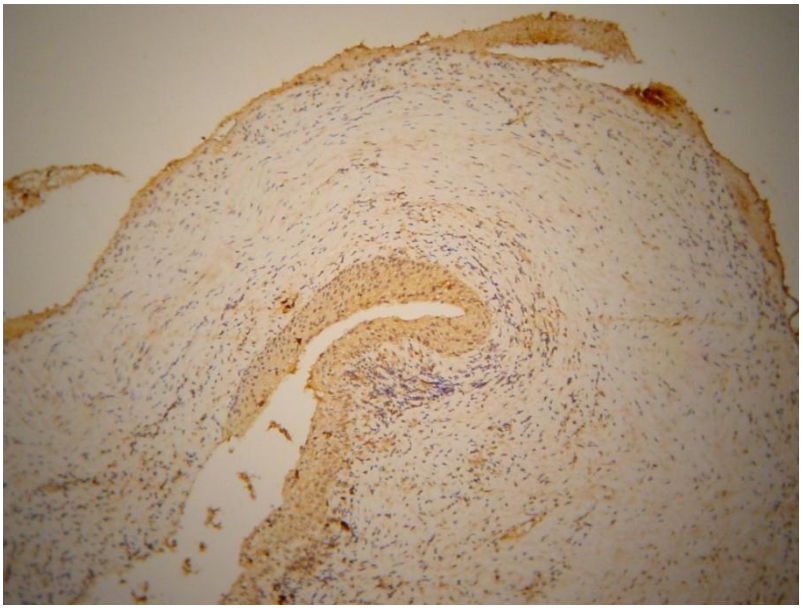


Fig. 20a, b: Notch-2 localization in a periapical **inflamed** cyst (02-478), where **moderate** immunoreactivity is observed in the epithelial cells (red arrows) while the subepithelial connective tissue remains negative for Notch-2. (chromogen DAB, magnification x100 & x400, respectively)



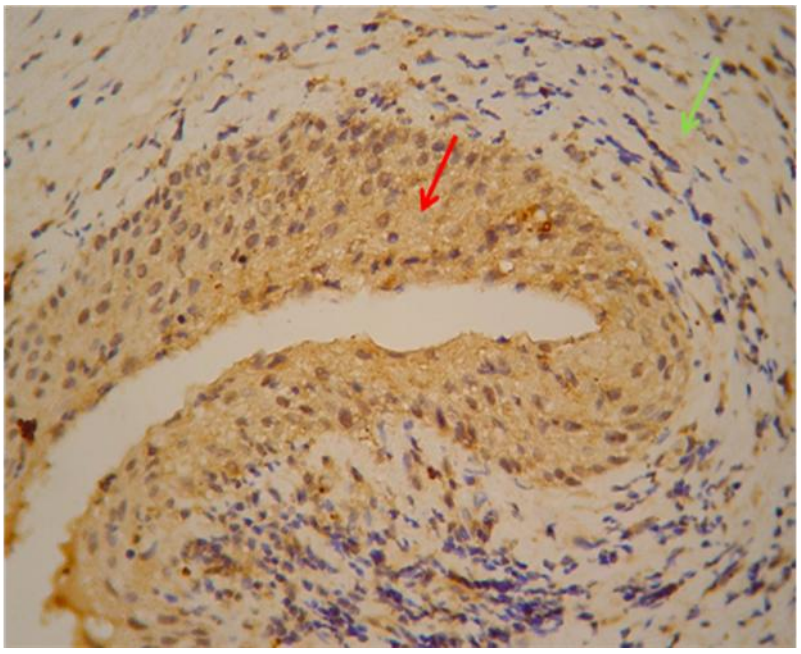
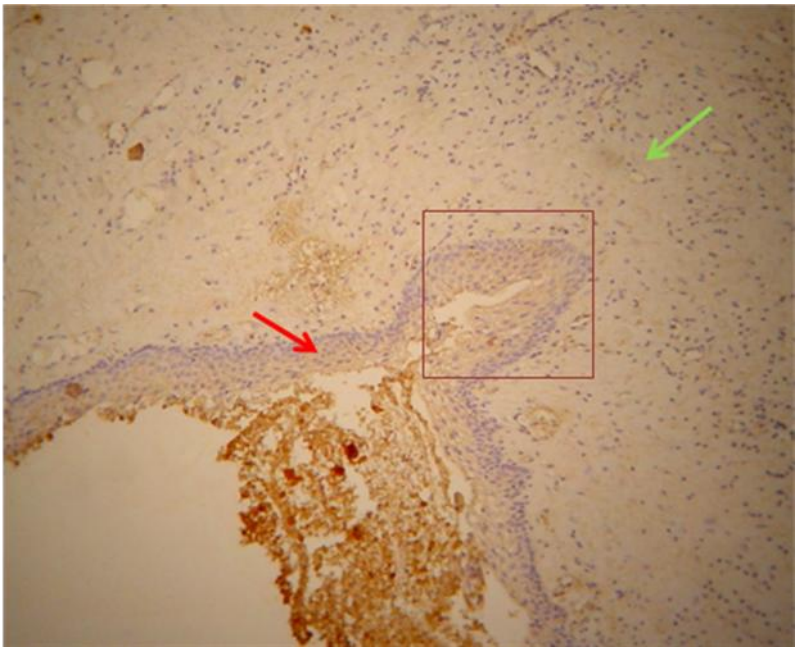


Fig. 21a, b: Notch-2 immunoreactivity in a periapical **inflamed** cyst (07-670), where **strong staining** is observed in the cytoplasm of epithelial cells (red arrows). The sub-epithelial connective tissue remains negative for Notch-2 (green arrows). (chromogen DAB, magnification x100 & x400, respectively)



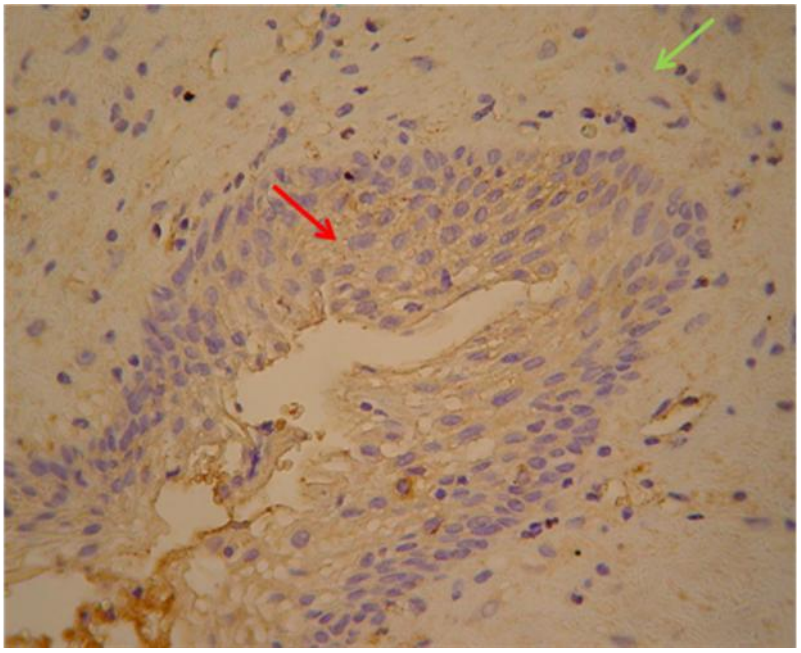


Fig.22a, b: Immunohistochemical localization of Notch-2 in epithelial cells of human **non-inflamed** periapical cyst (91-240). **Moderate** cytoplasmic staining can be observed in the epithelial cells (red arrows), while the underlining connective tissue remains negative (green arrow). (chromogen DAB, magnification x100 & x400, respectively)

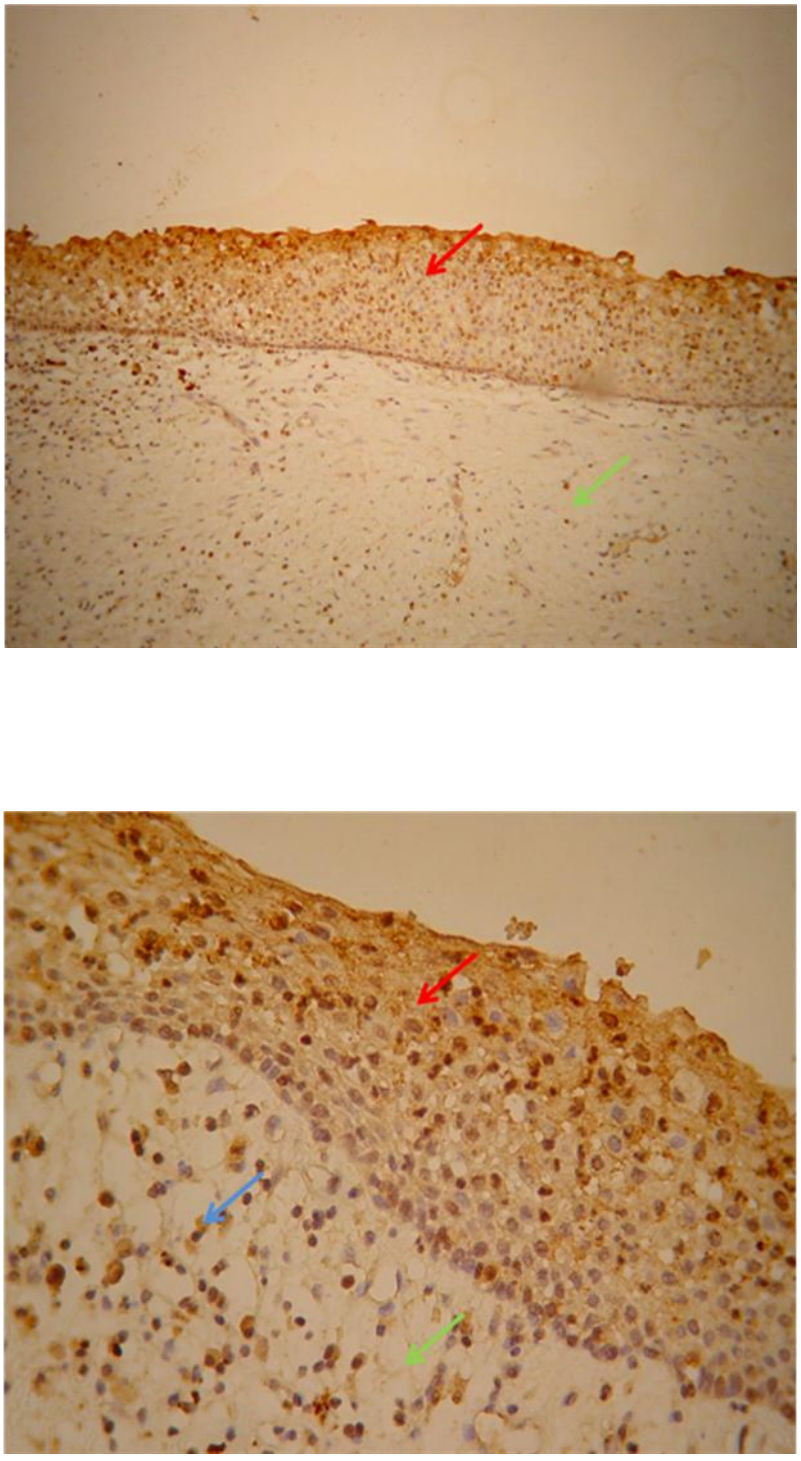


Fig.23a, b: Immunohistochemical localization of Notch-2 in epithelial cells of human **non-inflamed** periapical cyst (91-278). **Strong** immunoreactivity can be observed in the epithelial cells (red arrows), as well as some sub-epithelial inflammatory cells (blue arrow), while the underlining connective tissue remains negative for Notch-2 (green arrow). (chromogen DAB, magnification x100 & x400, respectively)

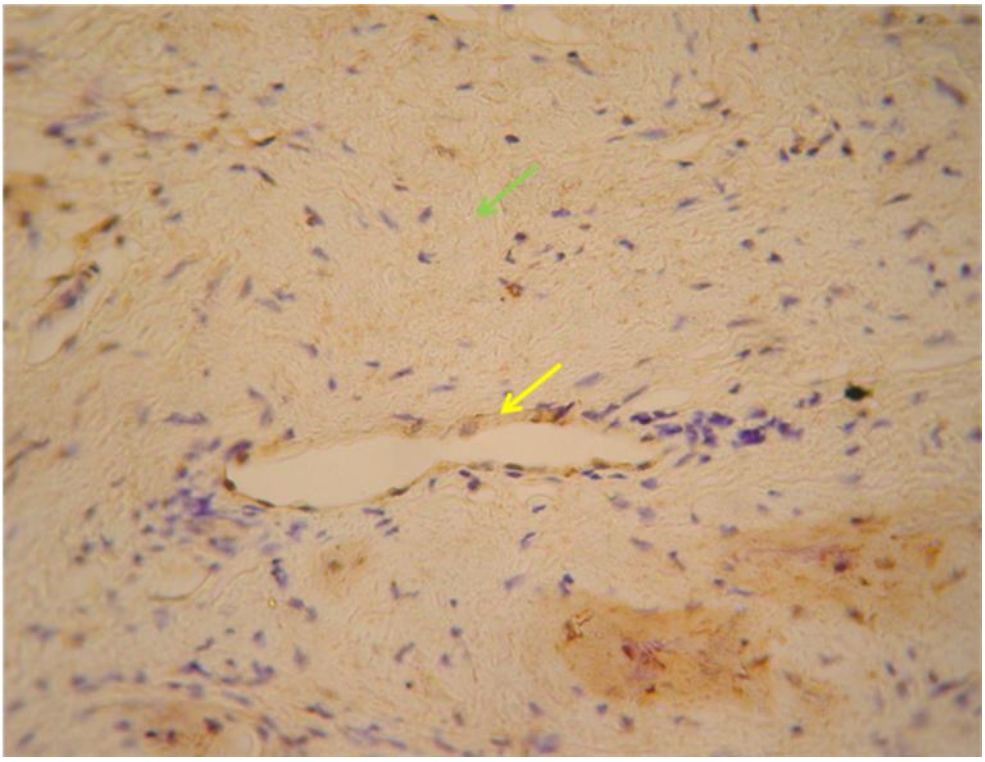


Fig.24: Notch-2 positive immunoreactivity of endothelial cells (yellow arrow) of a human periapical cyst (91-314). Notice that the surrounding connective tissue remains negative for Notch-2. (chromogen DAB, magnification x400)

Jagged-1

Positive immunostaining for Jagged-1 was detected in the basal and suprabasal epithelial cells of 27 periapical cysts (90%) and three (10%) presented negative immunoreactivity. The three negative specimens belong to the group of non-inflammatory cysts (25%).

From the periapical cysts which were positive for the expression of this antibody, eight (29.62%) demonstrated mild expression, sixteen (59.25%) demonstrated moderate expression and only three (8.10%) specimens presented strong expression. (Figures 25-30)

No apparent correlation was recognized between Jagged-1 expression and the inflammatory cell infiltration of periapical cysts. (x^2=3.33, p=0.067, Fisher Exact test= 0.22)

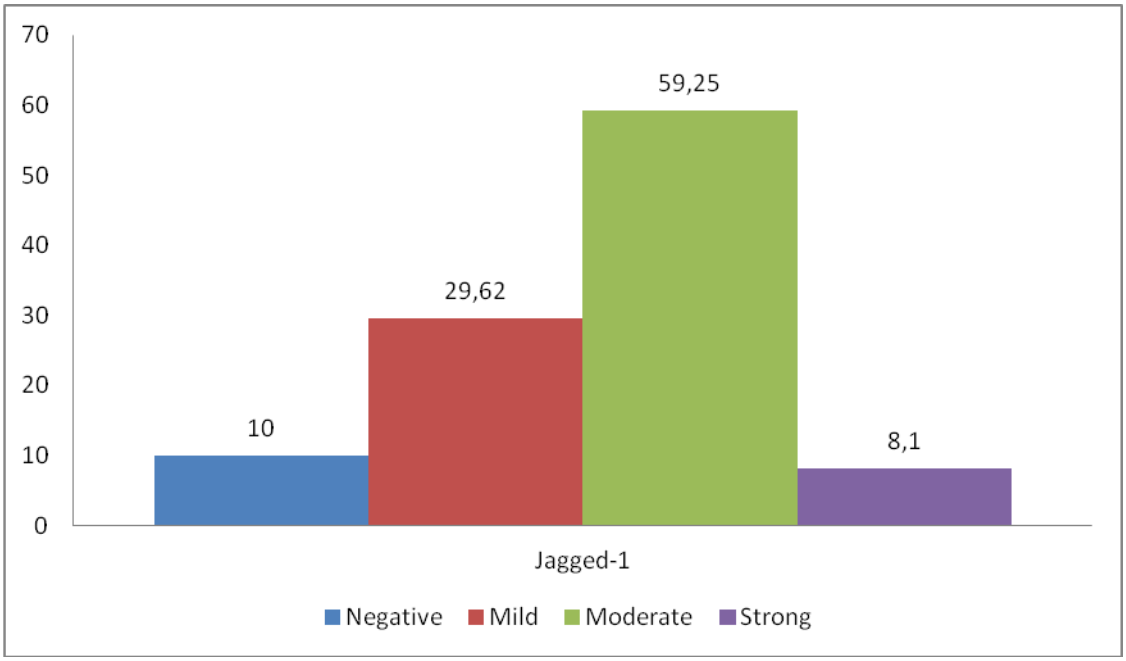
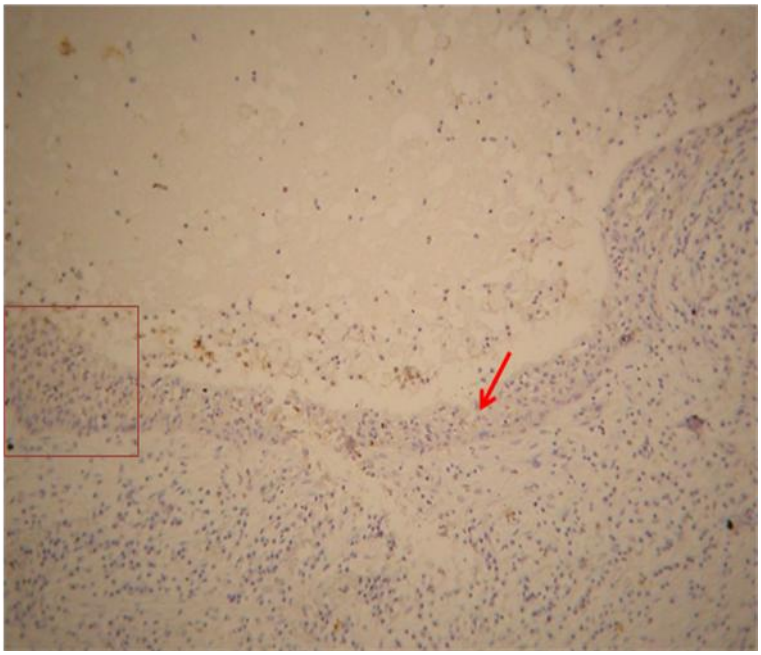


Figure 25: Histogram of Jagged-1 expression distribution



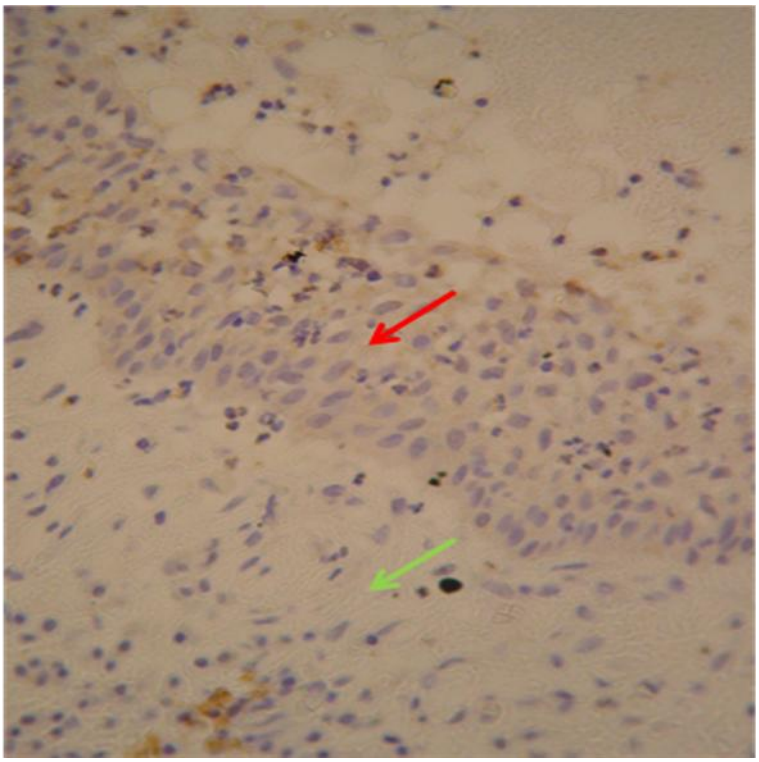


Fig.26a, b: Periapical **inflamed** (02-488) with negative immunoreactivity of Jagged-1, both in epithelial (red arrows) and stromal cells (green cell). (chromogen DAB, magnification x100 & x400, respectively)

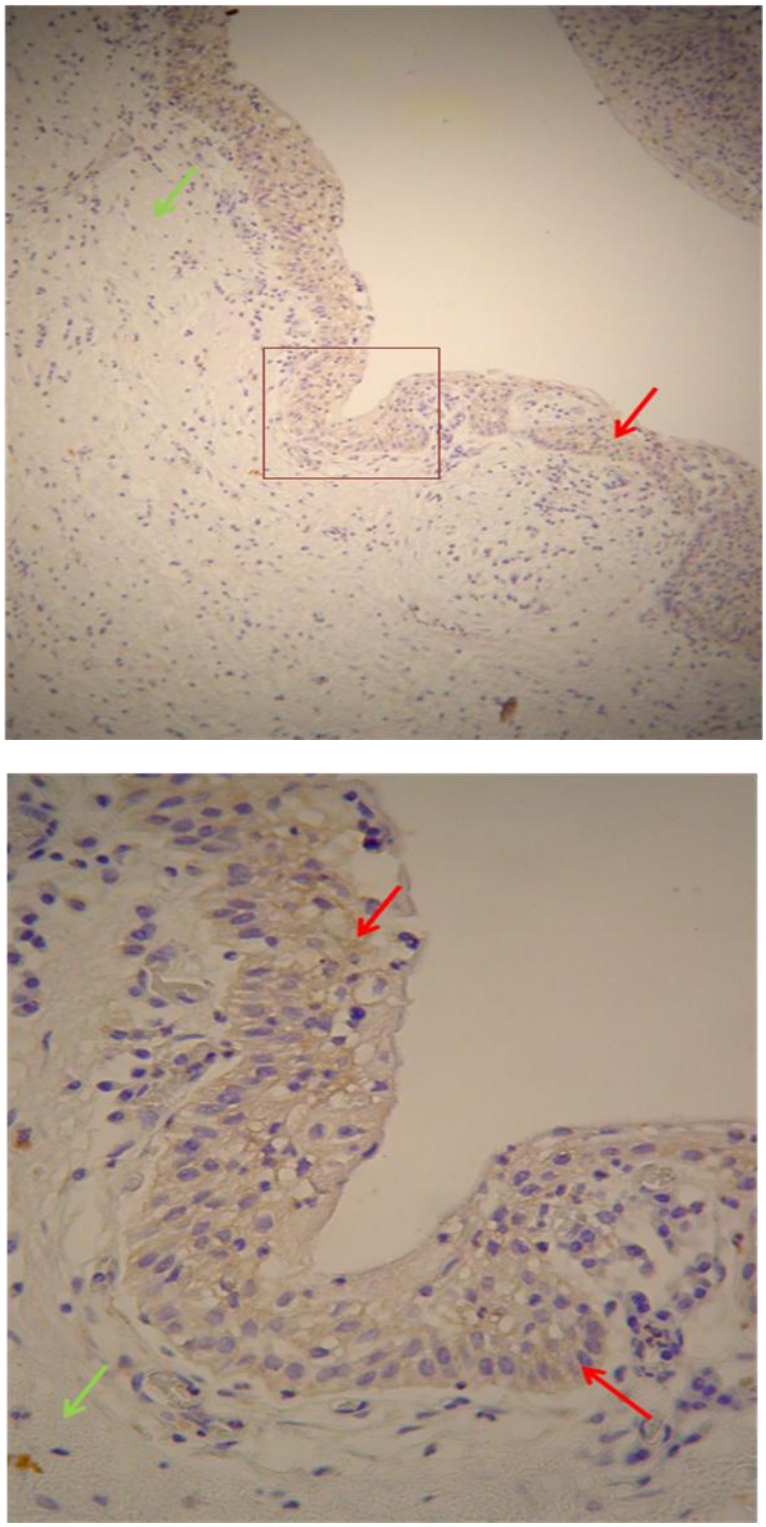


Fig.27a, b: Jagged-1 localization in a periapical **inflamed** cyst (02-478), where **moderate** immunoreactivity is observed in the epithelial cells (red arrows) while the subepithelial connective tissue remains negative for Jagged-1. (chromogen DAB, magnification x100 & x400, respectively)

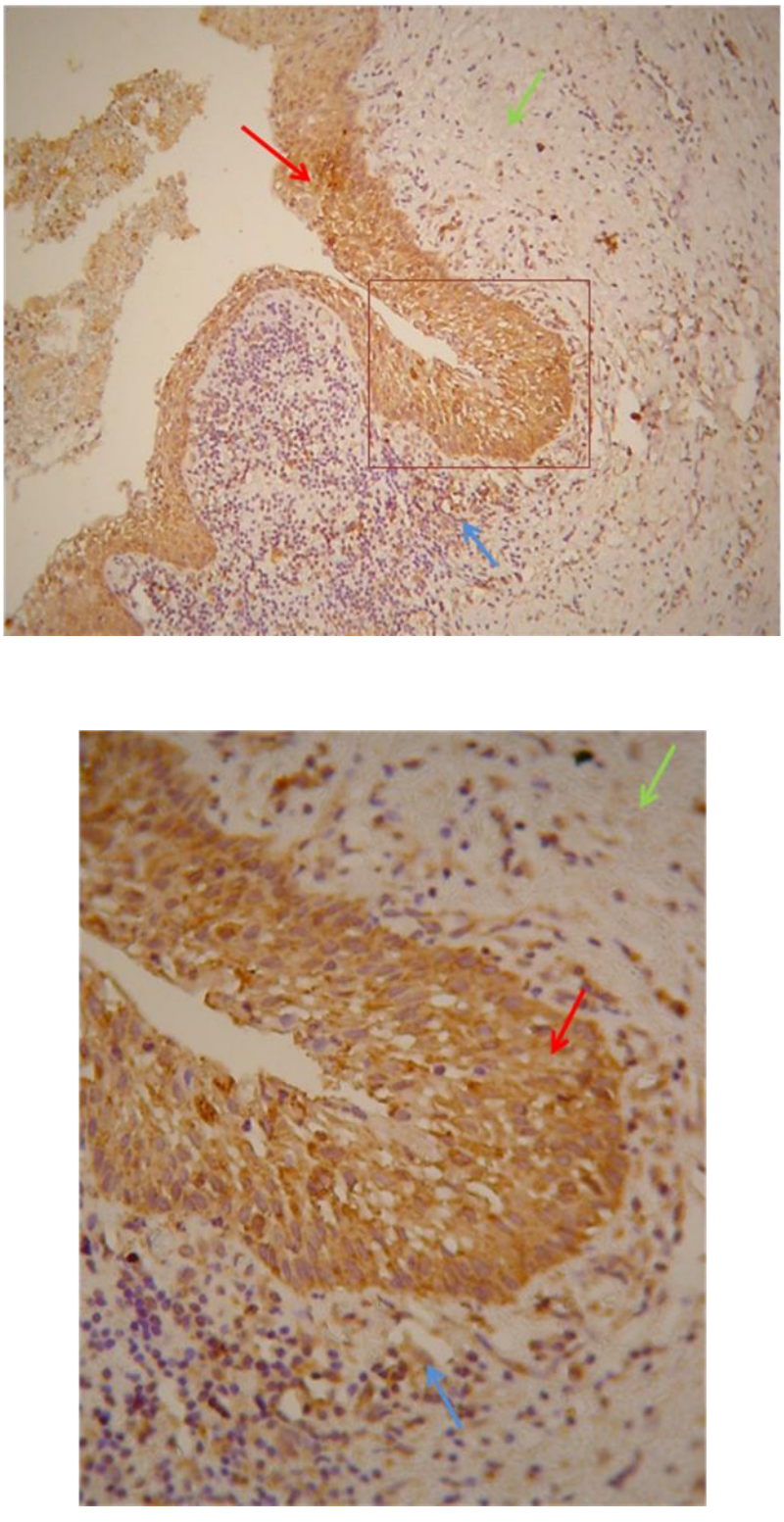
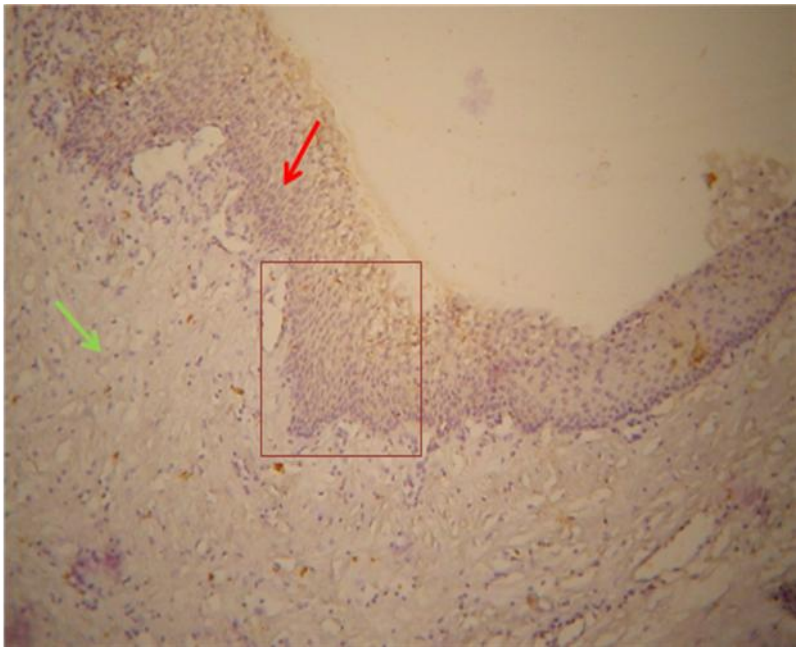


Fig. 28a, b: Jagged-1 localization in a periapical **inflamed** cyst (07-670). **Strong staining** is observed in the cytoplasm of epithelial cells (red arrows) and some sub-epithelial inflammatory cells (blue arrows). The underlining connective tissue remains negative for Jagged-1 (green arrows). (chromogen DAB, magnification x100 & x400, respectively)



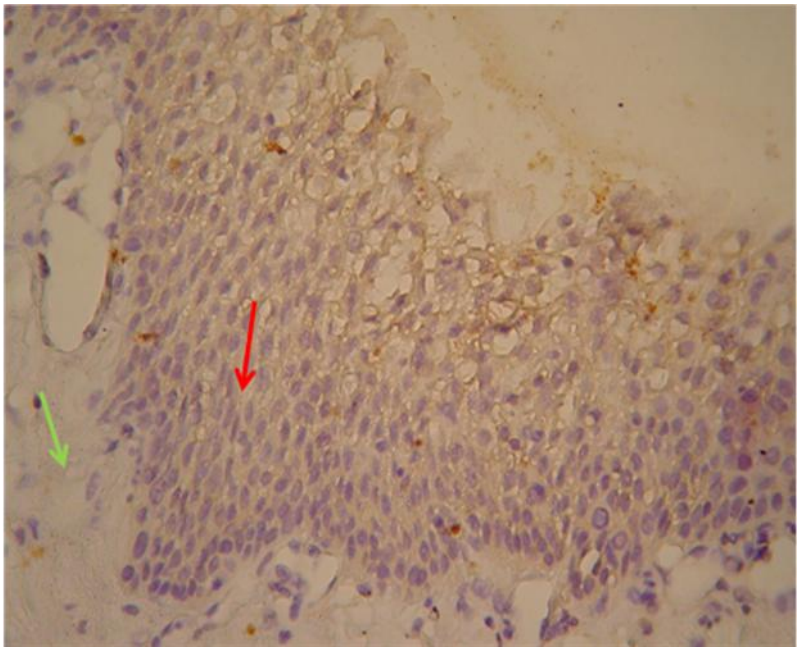
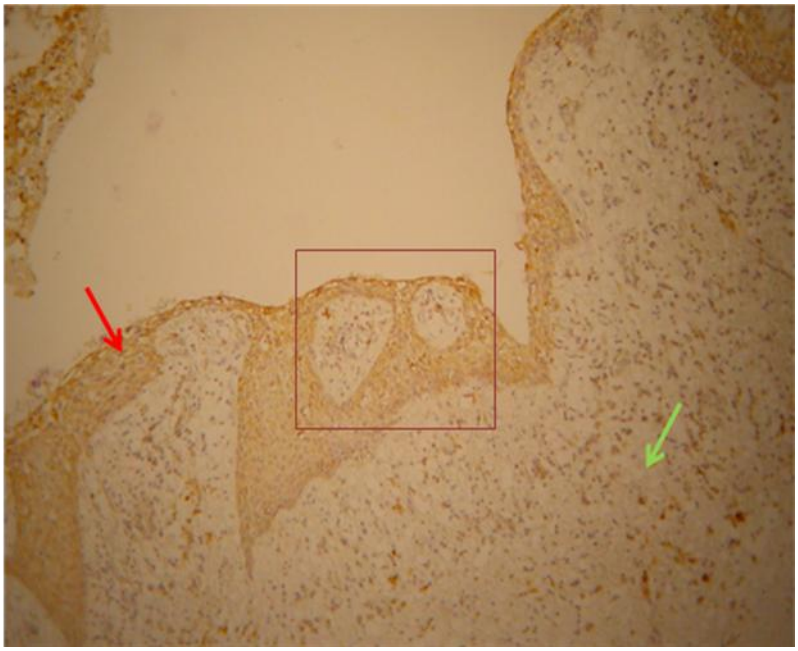


Fig.29a, b: Immunohistochemical localization of Jagged-1 in epithelial cells of human **non-inflamed** periapical cyst (92-230). **Moderate** cytoplasmic staining can be observed in the epithelial cells (red arrows), while the underlining connective tissue remains negative (green arrow). (chromogen DAB, magnification x100 & x400, respectively)



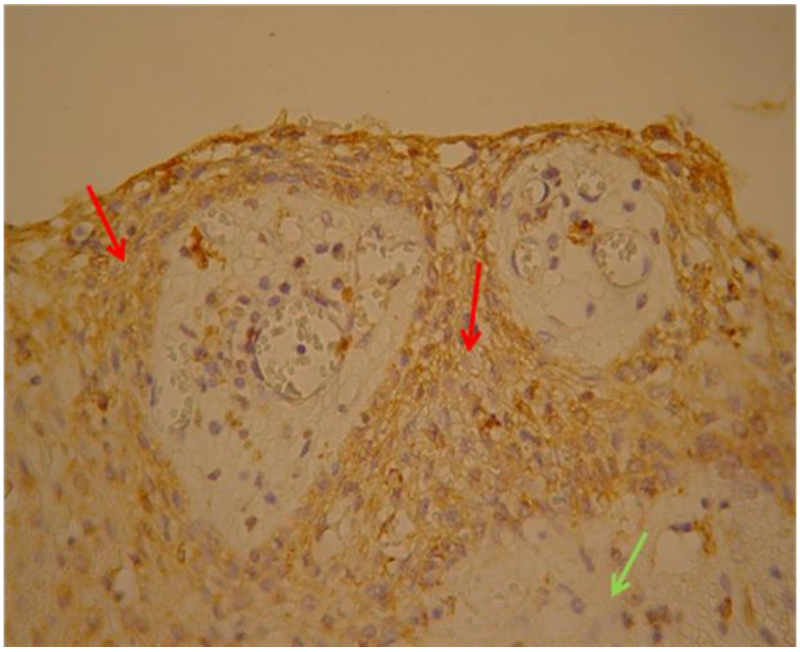


Fig. 30a, b: Jagged-1 localization in a periapical **non-inflamed** cyst (91-413). **Strong** staining is observed in the cytoplasm of epithelial cells (red arrows), while the underlining connective tissue remains negative for Jagged-1 (green arrows). (chromogen DAB, magnification x100 & x400, respectively)

HERP

No expression of HERP antibodies could be detected in the lining epithelium of periapical cysts, with the exception of their expression in some subepithelial inflammatory cells. (Fig.31, 32)

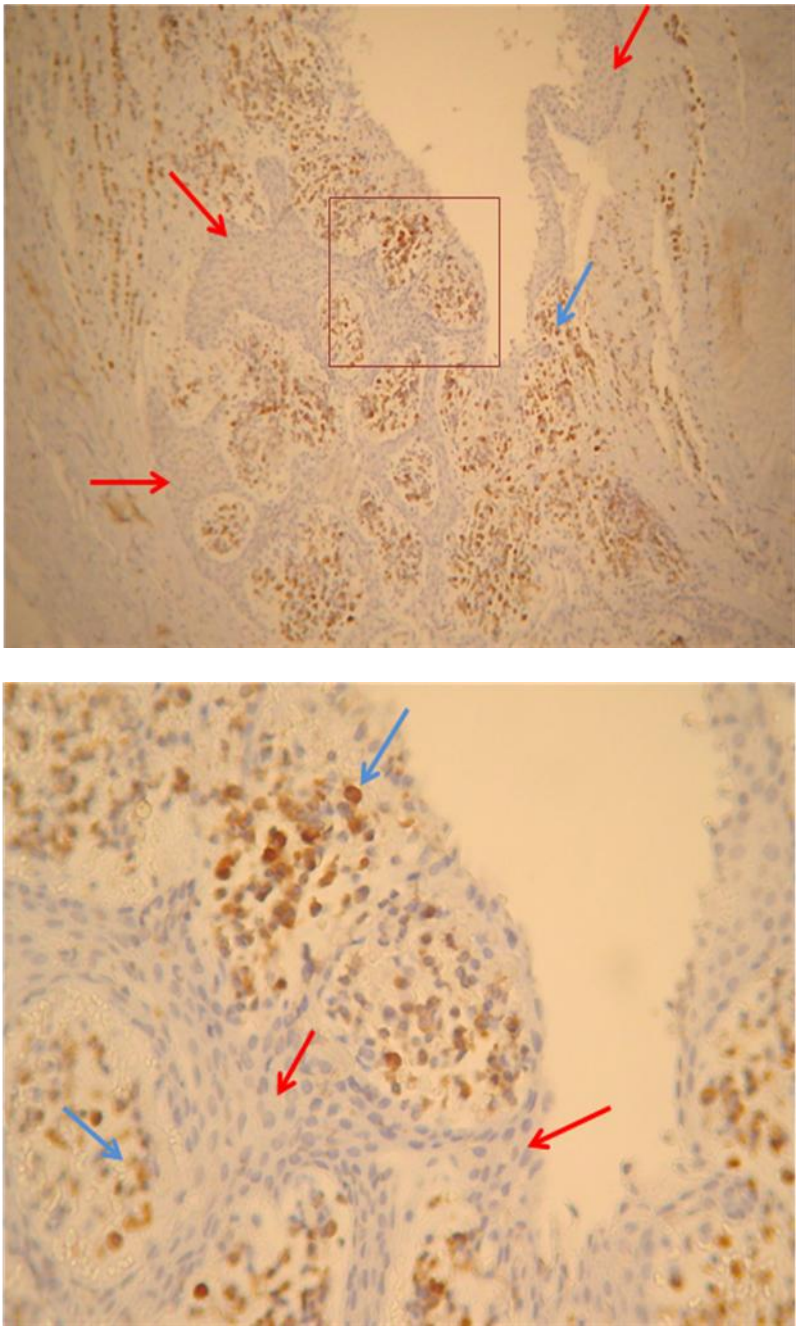
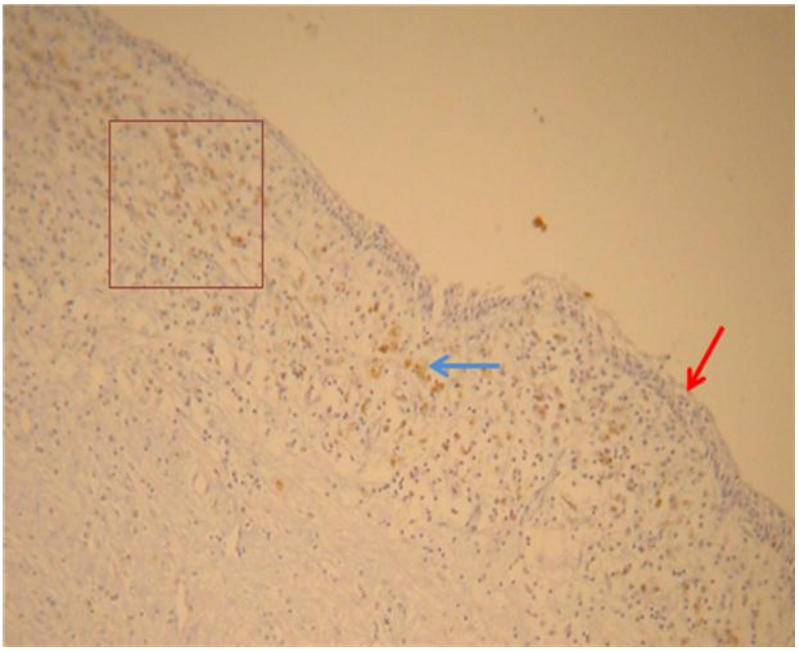


Fig.31a, b: Immunohistochemical localization of HERP in a periapical inflammatory cyst (07-670). Notice that epithelial cells present negative immunoreactivity for HERP (red arrows), while some sub-epithelial inflammatory cells (blue arrows) are positively stained. (chromogen DAB, magnification x100 & x400, respectively)



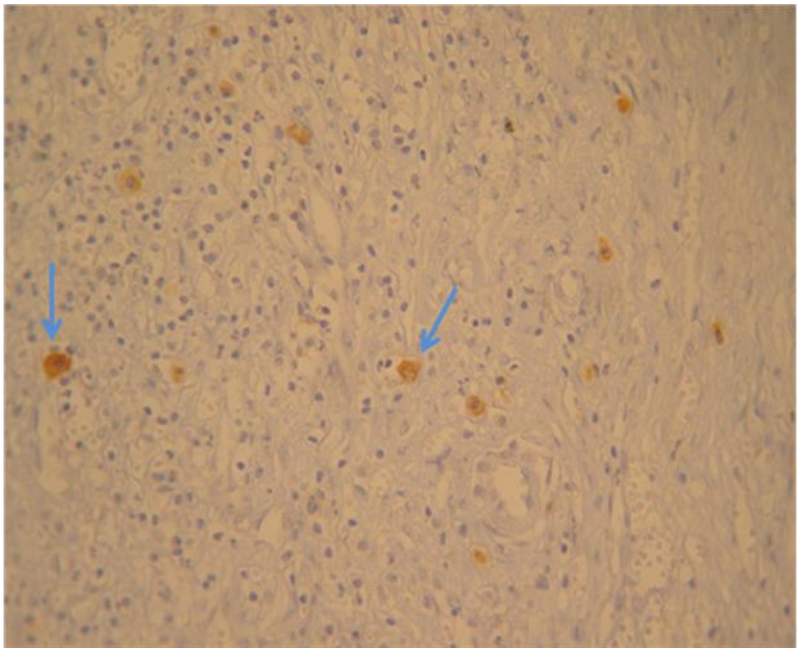


Fig.32a, b: Immunohistochemical localization of HERP in a periapical cyst with limited inflammatory infiltration (91-240). Epithelial cells demonstrate negative immunoreactivity for HERP (red arrow), while some sub-epithelial inflammatory cells, presumably histiocytes (blue arrows) are positively stained. (chromogen DAB, magnification x100 & x400, respectively)

Expression (Score)	Notch-1	Notch-2	Jagged-1	HERP
Mild (1)	3,33%	5,26%	29,62%	0%
Moderate (2)	46,67%	47,3%	59,25%	0%
Strong (4)	50%	47,3%	8,10%	0%

Table 6: Results of semi-quantative scoring of immunohistochemical stain

6. DISCUSSION

In the present study we investigated the immunohistochemical expression of Notch-1, Notch-2 receptors, Jagged-1 ligand and HERP1 transcription factor in inflamed and non-inflammed periapical cysts in an attempt to evaluate and correlate their expression to the presence of inflammation.

The samples of our study were obtained from archival biopsies submitted to the Department of Oral Pathology and Medicine, Dental School, University of Athens. Due to the fact that all archival lesions were fixed in formalin solution and embedded in paraffin wax, the selected method for the detection of the studied molecules was immunohistochemistry.

Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label, thus it makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue (Polak et al. 1983). The antibodies used are glycoproteins which are produced by plasma cells and used by the immune system to identify and neutralize foreign objects, bacteria and viruses. They are able to recognize and bind to a specific antigenic site, which is called epitope, e.g. proteins, glycoprotein, polysaccharides, which poses a complementary determining region. Immunohistochemical studies use two major types of antibodies: polyclonal or monoclonal, regarding their specificity for a multiple or a single epitope, respectively. Thus, major components in a complete immunohistochemistry method are: 1) Primary antibody binds to specific antigen, 2) the antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated, antibody, 3) with presence of substrate and chromogen, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding. In this study the applied chromogen was 3,3'-Diaminobenzidine (DAB), which is widely used for immunohistochemical staining and immunoblotting. When in the presence of peroxidase enzyme, DAB produces a brown precipitate that is insoluble in alcohol and xylene. The resulting color of the chromogen solution can vary from colorless to pale brown (Hsu et al. 1981).

Depending on the method of fixation and tissue preservation, the sample may require additional steps to make the epitopes available for antibody binding, including deparaffinization and antigen retrieval. For formalin-fixed paraffin-embedded tissues,

antigen-retrieval is often necessary and involves pre-treating the sections with heat or protease. These steps may make the difference between the target antigens staining or not staining.

Depending on the tissue type and the method of antigen detection, endogenous biotin or enzymes may need to be blocked or quenched, respectively, prior to antibody staining. Although antibodies show preferential avidity for specific epitopes, they may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen. A great amount of non-specific binding causes high background staining which will mask the detection of the target antigen. To reduce background staining, samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind.

Given the high quality of the positive controls used for each antibody, we may conclude that the immunohistochemistry protocols used in this study were successful. In our study, IHC automated system was selected (Fig.33). Nowadays, most advanced IHC staining instruments are fully automated, handling all steps from baking to counterstaining. Manual IHC is regarded highly perplexed and technique sensitive. On the other hand, IHC automation overcome these difficulties and allocates pivotal key advantages (Prichard 2014), such as:

1. **Standardization**, which ensures that staining is repeatedly performed in the same way and does not depend on differences in personal skills.

2. **High reproducibility,** by ensuring that any given step of the staining procedure is continuously performed as specified by any given protocol.

3. **Optimized use of reagents.**

4. **Improved error control via process control** (e.g. control of slides to be stained and correct selection of reagents via barcode tracking) and **process monitoring** (e.g. reporting of correct liquid level or process temperature), plus alarm notification if action is needed.

5. **Reduced hands-on time,** which may compensate for increased staining volume.

Fig. 33: Fully-automated Immunostaining Ventana Benchmark®XT XT system (Ventana Medical Systems, Inc., USA).

Notch pathway is a fundamental evolutionary conserved intercellular signaling cascade that participates in a variety of cellular processes, such as morphogenesis, development and homeostasis during embryonic and adult life through local cell-to-cell interactions (Artavanis et al. 1999), dictating cell fate of epithelial, neural, muscle, blood, bone and endothelial cells (Miele et al. 1999). Since Notch seems to critically influence many fundamental processes in a wide range of tissues, aberrant Notch function has been associated with a wide range of developmental disorders and neoplasms (Bolos et al. 2007, Louvi et al. 2012). Disruption of its homeostatic control has been directly linked to multiple human disorders, such as ongogenesis/carcinogenesis and inherited congenital disorders, such as Alagille Syndrome, Tetralogy of Fallot, syndactyly Spondylocostal Dysostosis, (Gridley 1997, Garg et al. 2005) Celebral Autosomic Dominant Arteriopathy with subcortical infarcts and leukoencephalopathy (Louvi et al. 2006)

Expression of Notch receptors and ligands in tooth germs and odontogenic tumors suggests that Notch signaling might control the cell differentiation and proliferation of normal and neoplastic odontogenic epithelium (Kumamoto et al. 2008). However, Notch expression in cystic odontogenic lesions is scarcely studied. There are few studies investigating the expression of Notch pathway's components in

periapical cysts. First of all, Gonçalves et al. (2010) reported the presence of Notch-1 in the lining epithelium of radicular, dentigerous and keratocystic odontogenic cysts, and, along with the presence of EGF and p53, they suggested that these molecules participate in the development, maintenance and integrity of cystic odontogenic epithelial lining, favoring lesion persistence. However, in order to confirm that the Notch pathway is active, the final product of it should be examined. For this reason, Meliou et al. (2011) demonstrated that Notch pathway is activated downstream in the lining epithelium of non-inflamed periapical cysts, as they studied not only receptors and ligand, but also the transcriptor factors HES1 and HES5, which are the final products of the Notch Pathway. Thus, they proposed a possible involvement of this pathway in the proliferation of epithelial cells and hence to periapical cyst growth and expansion.

Our results confirm the previous reported findings by Meliou et al. (2011), as Notch-1 and Notch-2 receptors detected in the epithelial cells of periapical cysts and no expression of the antibodies could be detected in the underlying connective tissue, with the exception of their expression in some subepithelial inflammatory cells, which served as our internal positive control (Osborne & Miele 1999). However, the receptor's presence alone does not indicate that the signaling pathway is active. Therefore, the co-expression of other components should be examined, such as ligands and transcription factors, (in order the activation of the pathway to be confirmed). Meliou and her colleagues (2011) reported intense immunostaining of Notch-1 and -2 and transcription factors HES-1 and HES-5, but only moderate staining for Delta-1 ligand. The authors attribute this observation to the fact that the receptor might interact with more than one ligand. It is known that the Notch pathway is activated when the ligand Delta or the ligand Jagged of one cell interacts with the Notch receptor of its neighboring cell (trans-interactions), releasing the Notch Intracellular Domain (NICD) that activates many downstream target genes (Kopan et al. 2009). The existing evidence, from studies in injured pulps in rats, suggests that Delta-1 can interact with any of the three Notch receptors, but the activation level differs among different receptors (Løvschall et al. 2005). It is known that Jagged-1 is more effective than Delta-1 in activating Notch-2, while both Jagged-1 and Delta-1 can activate Notch-1 efficiently (Lindshell et al. 1995, Weinmaster 1997).

Recently, bHLH protein family, termed **HERP** (HES-related repressor protein, also known as **Hey**/Hesr/HRT/CHF/gridlock), has been isolated. The members of this distinct family are characterized by their transient expression, reflecting the dynamic nature of Notch signaling (Iso et al. 2003). For this reason, except for Noch-1 and -2,

we also investigated the expression of ligand Jagged-1 and HERP1 transcription factor in these lesions. To our knowledge, currently, there are no reports about the presence of ligand Jagged-1 and HERP transcription factor in periapical cysts.

NOTCH-1

In our study, immunohistochemical reactivity for Notch-1 was detected in the cytoplasm of the basal and suprabasal epithelial cells of all periapical cysts (100%), in some subepithelial inflammatory cells and in stromal endothelial cells, with the majority of the specimens presenting strong expression.

Notch-1 has been found to be normally highly activated in the differentiating oral epidermal cells covering the developing tongue and the lateral oral surfaces of the mandibular and maxillary processes during palate development (Casey et al. 2006). Furthermore, Notch-1 mutations, along with Jagged-1, have been associated with cardiac defects (MacGogan et al. 2010) and human T lymphoblastic leukemias/lymphomas (T-ALL (Ellisen et al. 1991). The critical role of Notch signaling in T-ALL cells is to drive a gene expression program maintaining growth, high metabolism, and survival.

NOTCH-2

Regarding Notch-2, it was detected in the cytoplasm of the basal and suprabasal epithelial cells of nineteen periapical cysts (79.1%), in some sub-epithelial inflammatory cells and in stromal endothelial cells. In five specimens (20.83%) no expression of Notch-2 could be detected. The majority of the specimens demonstrated moderate (47.36%) and strong (47.36%) immunostaining for this antibody.

Except for lining epithelium of periapical cysts (Meliou et al. 2011) Notch-2 has been detected in various stages of T cell development and differentiation (Fiorini et al. 2009). On the other hand, Notch 2 is the most prominent Notch receptor reactivated in injured pulp and it is strongly expressed in mesenchymal cells of the pulp either close to the site of injury and at a distance from injured areas, suggesting that there may be a potential progenitor pool at the root, which differentiates into odontoblasts or pulp fibroblasts under the influence of growth factors effusing from a lesion (Mitsiadis et al. 2003)

Furthermore, it has been found that Notch ligand's expression in the endothelium activates Notch pathway in neighboring cells, and that this function is critical for smooth muscle cell differentiation (High et al. 2008). More specifically, Jagged-1 expression by endothelial cells induces pericytes in the microvasculature or smooth muscle cells in larger vessels to express Notch-3 and Jagged-1, which subsequently promotes and maintains the differentiation phenotype of these cells (Liu et al. 2009). In tumor cells, over-expression of Notch signaling pathway has been shown to enhance neovascularization and tumor growth (Zeng et al. 2005), while it also appears to promote vascular sprouting by regulating VEGFR3 expression in endothelial tip cells. Sprouting angiogenesis is a dynamic process in which endothelial cells collectively migrate, shape new lumenized tubes and make new connections.

JAGGED

Furthermore, positive immunostaining for Jagged-1 was detected in the basal and suprabasal epithelial cells of 27 periapical cysts (90%), in some subepithelial inflammatory cells, presenting moderate immunoreactivity in the majority of the specimens (59.25%).

Co-expression of high levels of Jagged-1 and Notch-1 has been detected in breast cancer and it seems to correlate with poor survival in this malignancy. Jagged-1, expressed either in differentiated basal tumor cells, in other differentiated tumor cells, or in the tumor stem/progenitor cells themselves can activate Notch signaling to promote self-renewal of the tumor-initiating population (Reedijk et al. 2005). In our study, control samples obtained from breast cancer biopsies demonstrated the same expression pattern.

HERP

HERP transcription factor was not found to be expressed in these lesions, with the exception of their expression in some subepithelial inflammatory cells.

HES and HERP are individually expressed in different cells during development (Iso et al. 2003). It has been found that expression of HERP1 is not induced by ligand stimulation in several cell types initially tested, such C2C12 muscle cells, 10T1/2 fibroblasts, 293T, and P19 teratocarcinoma cells (Iso et al. 2001a). On the other hand, the observation that zebrafish gridlock/HERP-1 plays a central role in the

development of the aorta (Zhong et al. 2000) suggested a **cell type specific** role for HERP-1 in vascular tissue. Consistent with this, when an aortic smooth muscle cell line was used in co-culture studies, expression of endogenous HERP1 (as well as HERP-2) mRNA was induced by Notch in the absence of de novo protein synthesis (Iso et al. 2002). Since HERP-1 mRNA expression is detected in multiple tissues in mice embryos, its role may not be limited to vascular cells. However, the absence of HERP-1 mRNA induction in several different kinds of cells, as described above, proposes that HERP-1 might have a cell-type-restricted role (i.e., vascular tissue). The differential expression of HES and HERP in different tissues suggests that HES and HERP may work separately as the respective homodimers, explaining the negative immunoreactivity in the present study. Furthermore, it has been reported that HERP-1 negatively regulates its own gene expression (Nakagawa et al. 2000).

In an attempt to interpret the differences observed between the studies, one can assume that **the wide variety of Notch components**— ligands, receptors, and effectors—can raise complexities to Notch signaling. For instance, each ligand isoforms may demonstrate special affinity and bind to a specific isoforms of receptors which in turn may be linked to particular effectors' isoforms. Such a link among specific isoforms of ligand, receptor, and effector might create cell-type specific sub-pathway of Notch signaling, and contribute to generation of distinct cell fates, provided that different effector isoforms regulate distinct sets of target genes.

This hypothesis is supported partly by **the tissue specific distribution** of different isoforms of Notch components. For instance, HERP-1 appears to be important particularly in the development of vascular tissue, and HERP-1might be regulated by vascular-specific isoforms of ligands and receptors such as Delta-4 and Notch-4/int-3 that are predominantly expressed in vascular endothelial cells (Shutter et al. 2000). Distinct functions of each isoform in animals are clearly demonstrated at least for Notch receptors and ligands, by the gene disruption studies for three receptors (Notch1, Notch2, and Notch4) and four ligands (Delta-1, Delta-3, Jagged-1 and Jagged-2). Mice with a mutation of one of these genes show different phenotypic changes, indicating distinct roles of the isoforms (Conlon et al. 1995, Hrabe de Angelis et al. 1997, Hamada et al. 1999, Dunwoodie et al. 2002).

Furthermore, taking into account the **cross-talk between Notch and other signaling pathways** (Axelrod et al. 1996, Price et al. 1997, Oswald et al. 1998, Carmena et al. 2002), we can postulate that signals other than Notch might be involved in the regulation of HES1 and HERPs expression (Iso et al. 2003).

Regarding the **pathogenesis** of periapical cysts several theories has been proposed over the years, such as nutritional deficiency theory, the abscess theory and, recently, the theory of the merging epithelial strands. However, none of these seem to be widely and unanimously accepted:

- The nutritional deficiency theory (Ten Cate 1972, Shear 1992, Summers 1974) postulates that the central cells of the continuously growing epithelial mass are removed from their source of nutrients and undergo necrosis and liquefaction degeneration. Given the fact that oral epithelium may reach a thickness of 500μm (Markiewicz et al. 2010) and that the cells in the outer layers rely on the diffusion of nutrition from the basement membrane, it is rather unlikely that proliferating epithelial cells will continue to form a ball mass such that the inner cells cannot obtain nutrition (Huang 2010). Furthermore, Lin and colleagues (2007) proposed that the entrapment of the connective tissue inside the ball mass is unlikely to occur as this is not a natural relationship between connective tissue and epithelium and because the epithelial strands in apical granulomas are frequently infiltrated by polymorphonuclear leukocytes, but cell necrosis is not often seen in the center of epithelial strands.
- The abscess theory (Nair et al. 2008, Oehlers 1970, Summers 1974) assumes that the proliferating epithelium surrounds the pre-existing abscess cavity, based on the innate tendency of any epithelium to cover exposed connective tissue surfaces. Recently, supporter of this theory seems to be Nair and colleagues (2008) who investigated the role of abscess in the formation of apical cysts and proposed that abscess appears to be a factor causing cyst formation. Lin and colleagues (2007) do not support this view as epithelial proliferation is more prominent in chronic apical periodontitis than apical abscesses and they propose that apical cyst formation might be a genetically programmed event.
- Lin and colleagues (2007) proposed the third theory which postulates that merging of epithelial cell strands may occur to reach the formation of a ball mass and when the connective tissue trapped inside the ball mass degenerates, thus creating a cyst. However, the periapical inflamed connective tissue (apart from abscess or necrotic areas) is well vascularized (Nair et al. 2008) and it is quite unlikely that an inflamed vital area of periapical connective tissue would be strangulated by the proliferating epithelium.

Regarding the **expansion** of periapical cysts, several attempts have been made in order to unveil the possible mechanisms:

- It has been suggested (Toller 1970, Shear 1983) that radicular cysts may expand as a result of the osmotic and hydrostatic pressure. Increased osmosis, owing to the degradation of epithelial and inflammatory cell in the lumen, leads to an inward movement of exudate from the surrounding tissue into the cyst cavity. However, this theory does not take into account the cellular aspects of the cyst and the complex array of immunologic mechanisms which take part in such pathologic responses. Recently, Ward and colleagues (2004), in an attempt to clarify the dynamics of cyst enlargement and role of osmotic pressure forces throughout its growth, proposed a novel mathematical model. The authors concluded that, although osmotic pressure differences play a significant role during the early and intermediate stages of cystic growth, in very large cysts this role becomes inconsequential and that cell birth in the lining dictates expansion.
- Moreover, it has been suggested that activation of neutrophils, fibloblasts, mononuclear leukocytes and macrophages leads to secretion of matrix metalloproteinases and, as a consequence, to degradation of fibrous connective tissue capsule (Teronen et al. 1995). Additionally, epithelial cell rests of Malassez are found to secrete several proteins, prostaglandins (PGs) E and F, IL-1 and IL-6 (Bando1993), thus participating in bone resorption. (Brunette 1979, Birek 1982) Harris (1973) had also reported the existence of PGs in the cystic wall.

The current evidence highlights the complexity of the mechanisms underlying the immunopathogenesis, the maintenance and the concurrent slow expansion of periapical cystic lesions.

Furthermore, it is still unclear as *why* and *how* the stratified epithelium is formed. Several reports suggest that it originated from the Epithelial Rest cells of Malassez (ERM) (Ten Cate 1972). Recent reports discuss about the difficulty in culturing and isolation of the primary ERMs from the surrounding tissues, as well as the fact that they cannot indefinitely expand in vitro in order to be studied (Nam 2011, 2014). Taking this into consideration, skepticism may rise about the in vitro histologic and histochemical studies conducted decades ago, which initially proposed that the ERMs can proliferate and give rise to the lining epithelium of periapical cysts.

Interesting is the fact that the epithelial cell rests of Malassez may represent unique **stem-cell** populations that are capable of undergoing Epithelial-Mesenchymal Transition **(EMT)** (Xjong 2012), as they are found to express epithelial, embryonic (Nam et al. 2011) and bone marrow mesenchymal/stromal stem cell markers (Xiong

2012). Nam et al. 2011 proposed that ERM contain a primitive stem cell population that might be more primitive than epithelial stem cells.

Investigating the presence of **stem cell markers in periapical inflamed tissues**, Patel and colleagues (2010) found that mesenchymal stem cells (MSCs) are present in the granulation tissue that developed in response to foreign bodies in an animal model. Liao and colleagues (2011) demonstrated that mesenchymal progenitor cells are present in inflamed periapical tissue, which expressed markers of MSCs, are highly osteogenic, weakly adipogenic in vitro, and capable of forming mineralized tissue in vivo. More recently, Marrelli (2013) demonstrated that cells isolated from human periapical inflammatory cysts display MSC-like properties as well as self-renewal capability and osteogenic and adipogenic potential. The authors also proposed that this tissue could also be considered as a source of cells with MSC-like properties. However, the cell-surface marker of adult epithelial stem cells LGR5 was found to be expressed in the odontogenic epithelium of dental follicles, but, interestingly, LGR5 was not found to be expressed in the lining epithelium of periapical cysts (Nikolis 2013).

Taking into consideration the different expression patterns of stem cell markers in ERM and periapical cysts, we may assume that the epithelial cells which participate in the formation of periapical cysts may acquire mesenchymal phenotypes through EMT. In support of the first assumption, Nam and colleagues (2014) found that immortalized HERS/ERM cells could respond to TGF-β1 and acquire mesenchymal phenotypes through EMT.

Proliferation of epithelial cells in periapical lesions is likely to be related not only to the inflammatory mediators, growth factors and proinflammatory cytokines released by host cells during periapical inflammation (Torabinejad 1980, Tani-Ishii 1995, Meghji 1996), but also to microbial cytokines and endotoxins present in apical periodontitis (Meghi 1996). It has been suggested that PGE_2 induces proliferation of epithelial cell rests by raising the level of intracellular cAMP (Brunette 1984). Furthermore, IL-1, IL-6 and KGF have been shown to play an important role in up-regulating the epithelial cell proliferation (Saunder 1989, Grossman et al. 1989, Chelid et al. 1994). It has been reported that these factors, as well as PGE_2, TNF and TNF-a (Irwin et al. 1991, Lin et al. 1996, Chang et al. 1996), may up-regulate EGF receptors gene expression by influencing transcriptional factors. It has been scientifically proven that epidermal growth factor - EGF receptors are expressed by epithelial cells in normal periodontal tissues and odontogenic cysts, such as odontogenic keratocysts/dentigerous cysts (Thesleff 1897, Li et al. 1993), and are

up-regulated during inflammatory processes (Irwin et al. 1991, Lin et al. 1996). Several other growth factors has been proposed as stimulators for the proliferation of epithelial cell rests, such as insulin-like growth factor (Götz et al. 2003), fibroblast growth factor 2- FGF2 (So 2001).

At this point, it would be helpful to discuss about epithelial-mesenchymal transition process (EMT), as it has been recently found that the epithelial cell rests of Malassez may represent unique stem-cell populations that are capable of undergoing Epithelial-Mesenchymal Transition (EMT) (Xiong et al. 2012). Polarized epithelial cell tissues depend on the formation of intercellular tight and adherent junctions. This specific structure of the tissue can be rearranged by epithelial–mesenchymal transition (EMT): the epithelial cells lose both polarity and cell-to-cell contacts, dismantle their junctional structures, start expressing mesenchymal cell proteins, remodel their extracellular matrix and become migratory (Hay et al. 2005). Therefore, EMT can be described as a differentiation or morphogenetic process in which new tissue types are generated during embryogenesis and which is partly responsible for the pathogenesis of several diseases, such as metastatic cancer and tissue fibrosis (Huber et al. 2005, Radisky et al. 2005, Thiery et al. 2006. The reverse process has also been reported and it is known as mesenchymal–epithelial transition (MET) (Thiery et al. 2006). This process describes how transitory mesenchymal cells generate polarized epithelia after migration into new sites of tissue formation. MET has been described in the context of embryonic development and is also perturbed pathologically in fibrotic disorders (Zeusberg et al. 2008). Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) have been observed during multiple cell fate conversions including embryonic development (Thiery et al. 2009), tumor progression and somatic cell reprogramming. In addition, MET and sequential EMT-MET during the generation of induced pluripotent stem cells (iPSC) from fibroblasts have been reported recently. Such observation is consistent with multiple rounds of sequential EMT-MET during embryonic development which could be considered as a reversed process of reprogramming, at least partially (Li et al. 2014).

Such morphogenetic processes, as EMT or MET, **are regulated by** many signal transduction pathways (Fig.34), including the **Notch pathway**, usually initiated by secreted polypeptide factors, which aim at regulating a new set of transcriptional and post-translational events, leading to the generation of new cellular phenotypes. (Moustakas et al. 2007)

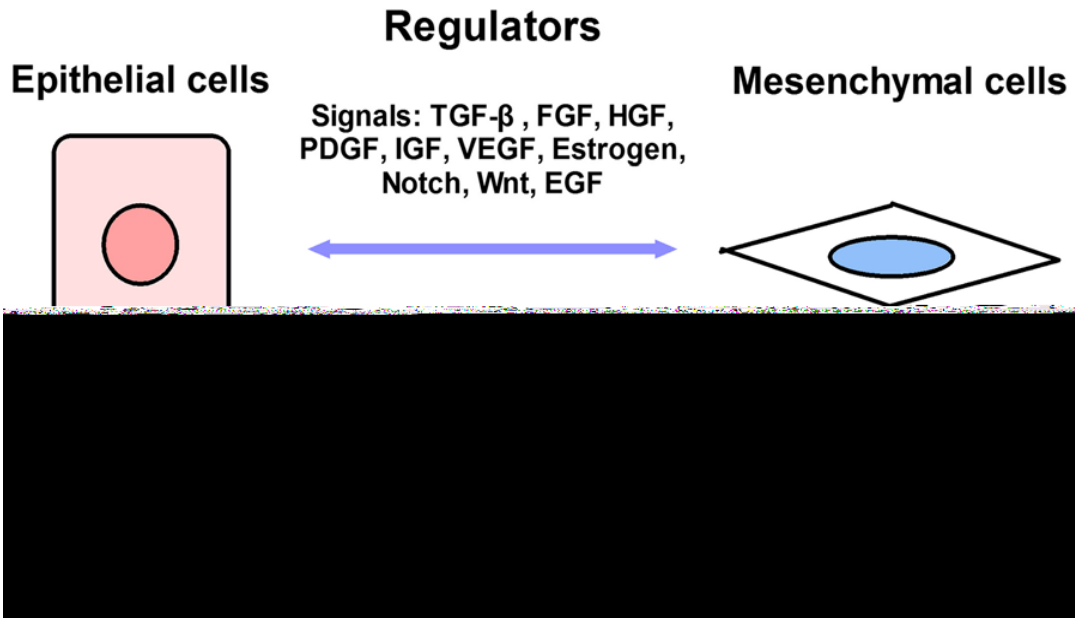


Fig.34: Regulatory network in EMT and MET. EMT can be regulated by many signaling pathways, transcription factors, and post-transcriptional mechanisms. (Guo et al. 2014)

To sum up the key points of **EMT-MET**:

- ✓ EMT and MET have key roles in embryogenesis and cancer metastasis (Chang et al 2013)
- ✓ EMT is an integral part of tissue remodeling that occurs during embryogenesis (Thiery 2006)
- ✓ In adults, it can be activated to promote wound healing after tissue injury (Lamoille et al. 2013)
- ✓ MET contributes to embryonic development (Wu et al. 2012)
- ✓ EMT induction allows cancer cells to disseminate from the primary tumor, invade surrounding tissues, and eventually generate metastases by colonizing remote sites via blood or lymphatic routes. Metastatic cells can then revert back via MET to re-acquire epithelial characteristics similar to those of cells in the primary tumor (Lamouille et al. 2013).
- ✓ EMT and MET are essential to the regulation of stem cell pluripotency. (Lamouille 2013)

To summarize our findings:

- Stromal cells and connective did not express any of the examined antibodies, with the exception of their expression in some subepithelial inflammatory cells.
- Notch-1, Notch-2 and Jagged-1 are expressed in the lining epithelium of periapical cysts.
- HERP transcription factor was not detected in these lesions.
- Expression of Notch-1, Notch-2 and Jagged-1 was not correlated with the presence or absence of inflammation.
- These findings further support the potential role of Notch signaling pathway in the expansion and maintenance of the lining epithelium of periapical cysts.

7. Clinical Implications

As it has already been mentioned, aberrant Notch signaling has been implicated in numerous human diseases, including a broad spectrum of cancers. For this reason, therapeutic approaches involving the modulation of the Notch pathway function have been proposed. Such therapeutic strategies include chemical and immunological targeting of Notch, its ligands, the ADAM and γ-secretase proteases, and the downstream transcriptional factor Mastermind in tumors (Groth et al. 2012) Therapeutic inhibition of Notch signaling is likely to be widely applicable, either alone or in combination with other chemotherapeutic approaches. Nowadays, in vitro, animal and human clinical trials are currently underway to evaluate the effectiveness of such techniques and strategies for treating a variety of different cancers, such as breast cancer (Nwabo Kamdje et al. 2014), gastric cancer (Kim et al. 2015) as well as brain and central nervous system tumors (Fouladi et al. 2011).

Furthermore, it should be noted that Notch-targeting therapies not only are relevant for cancer, but also they potentially apply to developmental, vascular, cardiac and other diseases associated with Notch pathway malfunction.

Taking everything into consideration, one may assume that inhibition of Notch signaling pathway might be an alternative, non-invasive, therapeutic strategy for the regression of true periapical cysts, combined with conservative root canal treatment.

8. CONCLUSIONS

In the light of our results and taking into consideration:

- The involvement of Notch signaling pathway in postdevelopmental **stem cell systems**, such as hair follicles (Vauclair et al. 2005), hematopoietic and immune systems (Suzuki et al. 2005) and in intestinal epithelial stem/progenitor cells (Sander et al. 2004),
- Notch's **regulatory role in EMT-MET** processes,
- **The unique and divergent properties** that characterize the epithelial rest **cells of Malassez** and the **lining epithelium** of periapical cyst,

it may be proposed that Notch signaling, in general, participates in the cell fate decision, differentiation and maintenance of the cells that are activated in order to form the lining epithelium of periapical cysts. Furthermore, our findings may suggest the potential role of Notch signaling pathway, in the expansion and maintenance of periapical cysts by enhancing neoangiogenesis, thus providing the pivotal vascular supply to the cyst. However, further support/research is needed for the determination of the ERMs actual role in the immunopathogenesis of periapical cysts and the potential role of EMT-MET in this process.

9. SUMMARY

Cell to cell interactions are pivotal for the development of multicellular organisms. Such interactions are mediated by communication mechanisms among cells through signals targeting specific genes. These genes are associated with cell survival, proliferation, differentiation, patterning, adhesion, epithelial-mesenchymal transition, migration, angiogenesis and apoptosis. Thus, cell-cell signaling permits neighboring cells to influence each other's fate and behavior. Notch pathway is a fundamental evolutionary conserved intercellular signaling cascade. It regulates morphogenesis, development and homeostasis during embryonic and adult life through local cell-to-cell interactions, dictating cell fate of epithelial, neural, muscle, blood, bone and endothelial cells. The aim of the present study was to gain a better understanding of the role of the Notch Signaling Pathway periapical cysts. To our knowledge, currently, there are no reports about the presence of ligand Jagged-1 and HERP1 transcription factor in periapical cysts. Thus, in this study, the immunohistochemical expression of Notch-1 and Notch-2 receptors, Jagged-1 ligand and HERP1 transcription factor in periapical cysts was evaluated and correlated with the presence of inflammation.

Materials and Methods: Thirty formalin-fixed, paraffin-embedded human periapical cysts were obtained and selected from archival biopsies submitted to the Department of Oral Pathology and Medicine, Dental School, University of Athens, between1990-2011. The obtained sample was grouped according to the presence of inflammatory cell in the underlying connective tissue infiltration were classified as non-inflammatory periapical cysts (cat.1 Orstavic % Mjor 1988) or inflammatory periapical cysts (>50% inflammatory infiltration). Immunohistochemistry was performed with 4 different antibodies: mouse monoclonal Notch-1 antibody (catalog No: NBP1-48289, Novus Biologicals, Inc., Littleton, USA), rabbit polyclonal Notch-2 antibody (catalog No: AP07611SU-N, Acris Antibodies GmbH, Germany), rabbit polyclonal Jagged-1 antibody (catalog No: NBP1-90208, Novus Biologicals, Inc., Littleton, USA), rabbit polyclonal HEY1/HERP antibody (catalog No: ab22614, Abcam, Cambridge, UK). The immunohistochemical staining of Notch-1 and HERP antibodies was performed with an automated Immunostaining Ventana Benchmark®XT XT system (Ventana Medical Systems, Inc., USA), while the immunohistochemical staining of Notch-2 and Jagged-1 antibodies was performed in Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany). Immunoreactivity was visualized by development with

3,3′-diaminobenzidine. Statistical analysis was performed regarding the presence of immunohistochemical staining in correlation with the degree of inflammation in periapical cysts using Chi-square Test, Fisher Exact Test with the Freeman-Halton extension; $P < 0.05$ was considered significant.

Results: Notch-1 was detected in the cytoplasm of the basal and suprabasal epithelial cells of all periapical cysts (100%), in some subepithelial inflammatory cells and endothelial cells. Only one specimen (3.33%) presented mild staining for Notch-1, while fourteen (46.67%) found to have moderate expression and fifteen (50%) strong expression. Presence of inflammation does not affect the expression of Notch-1 in a statistically significant manner (Fishers Exact Test =1)**.** Expression of **Notch-2** was detected in the cytoplasm of the basal and suprabasal epithelial cells of nineteen periapical cysts (79.1%) while in five specimens (20.83%) no expression of Notch-2 could be detected. Positive expression was also observed in some subepithelial inflammatory and endothelial cells**.** From the negative specimens, two were belonged in the group of inflammatory cysts and three in the group of non-Inflammatory cysts. From the positive specimens, one demonstrated mild expression (5.26%), nine (47.36) moderate expression and nine (47.36%) presented strong immunoreactivity for this antibody. Presence of inflammation does not affect the expression of Notch-2 in a statistically significant manner (x^2=0.086, p=0.768) (Fisher exact test=1). Positive immunostaining for **Jagged-1** was detected in the basal and suprabasal epithelial cells of 27 periapical cysts (90%) and three (10%) presented negative immunoreactivity. The latter three negative specimens belong to the group of non-inflammatory cysts (25%). From the periapical cysts which were positive for the expression of this antibody, eight (29.62%) demonstrated mild expression, sixteen (59.25%) demonstrated moderate expression and only three (8.10%) specimens presented strong staining. No apparent correlation was recognized between Jagged-1 expression and the inflammatory cell infiltration of lining epithelium in periapical cysts (x^2=3.33, p=0.067, Fisher Exact test= 0.22). No expression of **HERP** antibody could be detected in the lining epithelium of periapical cysts, with the exception of its expression in some subepithelial inflammatory cells

Conclusions: Notch pathway is an evolutionary conserved signaling mechanism which regulated cell fates decisions during development and postnatal life. The present observations further support the potential role of Notch signaling pathway in the expansion and maintenance of the lining epithelium of periapical cysts.

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