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VEIKO VENGERFELDT

Apical periodontitis: prevalence and etiopathogenetic aspects

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UNIVERSITY OF TARTU
Press

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Dissertation has been accepted for the commencement of the degree of Doctor of Philosophy in Medicine on August 29, 2018 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

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Commencement: November 15, 2018

Publication of this dissertation is granted by the University of Tartu.

This study was supported by the Estonian Research Council (grants No. IUT20-42 and IUT34-19), the European Union through the European Regional Development Fund (Project No. 2014-2020.4.01.15-0012), and Enterprise Estonia (grant No. EU48695).

ISSN 1024-395X
ISBN 978-9949-77-850-8 (print)
ISBN 978-9949-77-851-5 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I **Vengerfeldt V**, Mändar R, Nguyen MS, Saukas S, Saag M. Apical periodontitis in southern Estonian population: prevalence and associations with quality of root canal fillings and coronal restorations. BMC Oral Health. 2017 Dec 12;17(1):147. doi:10.1186/s12903-017-0429-7. PMID: 29233146

II **Vengerfeldt V**, Špilka K, Saag M, Preem JK, Oopkaup K, Truu J, Mändar R. Highly diverse microbiota in dental root canals in cases of apical periodontitis (data of illumina sequencing). J Endod. 2014 Nov;40(11): 1778–83. doi:10.1016/j.joen.2014.06.017. PMID: 25227214

III **Vengerfeldt V**, Mändar R, Saag M, Piir A, Kullisaar T. Oxidative stress in patients with endodontic pathologies. J Pain Res. 2017 Aug 24;10:2031–2040. doi:10.2147/JPR.S141366. PMID: 28894386

Contribution of Veiko Vengerfeldt to original publications:

Paper I: Study design, radiological evaluation, data analysis, writing the paper

Paper II: Study design, clinical evaluation and treatment of patients, collecting specimen, laboratory investigation, writing the paper

Paper III: Study design, clinical evaluation and treatment of patients, collecting specimen, laboratory investigation, writing the paper

LIST OF ABBREVIATIONS

8-EPI	8-isoprostanes
AAE	American Association of Endodontists
AChE	8-EPI-acetylcholinesterase
AIDS	Acquired Immune Deficiency Syndrome
AP	Apical periodontitis
CBCT	Cone beam-computed tomography
CEJ	Cemento-enamel junction
CI	Confidence intervals
DEJ	Dentinoenamel junction
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extra-cellular polymeric substances
EPT	Electric pulp test
FDI	Fédération Dentaire Internationale (World Dental Federation)
GI	Glass ionomer
HIV	Human immunodeficiency virus
HOMD	Human Oral Microbiome Database
hRCT	Healthy RCT tooth
IgG	Immunoglobulin G
IO	Intraoral
LDF	Laser Doppler flowmetry
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
MTA	Mineral Trioxide Aggregate
NaOCl	Sodium hypochlorite
NiTi	Nickel-Titanium
NS	Not significant
OR	Odds Ratio
OSI	Oxidative Stress Index
OTU	Operational taxonomic unit
OxS	Oxidative stress
PA	Periapical
PAI	Periapical index
pAP	Primary apical periodontitis
PDL	Periodontal ligament
PGF2α	Prostaglandin F2α
RC	Root canal
RCF	Root canal filling
RCT	Root canal treatment
RDP	Ribosomal Database Project

RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	ribosomal RNA
RS	Reactive species
sAP	Secondary apical periodontitis
SLOB	Same Lingual Opposite Buccal
TAC	Total antioxidant capacity
TMB	Tetramethylbenzidine
TPX	Total peroxide concentrations
ZOE	Zinc oxide eugenol

INTRODUCTION

A tooth is divided into two major parts; the crown and the root. Alveolar ridges contain the roots of the teeth. The tooth is situated in the alveolar bone of the jaws. Each tooth has a pulp cavity that contains the pulp-dentin complex. There is pulp cavity, which contains pulp-dentin complex inside of the tooth. The pulp cavity extends down through the root of the tooth as the root canal(s), which opens into the periodontium though the apical foramen. The blood vessels and nerves of dental pulp enter and leave the tooth through this foramen. This sets up a form of communication between the pulp and surrounding periodontium. Apical periodontitis (AP) is an inflammatory disease around the apex of a tooth root that is caused by infection in root canal system (Kakehashi *et al.,* 1965; Siqueira & Rocas, 2007). AP is a sequela of dental caries, irreversible pulpitis and pulpal necrosis, visible on the radiographs as a radiolucent lesion in the periradicular area.

AP is a widespread condition ranging from 27% in Finland (Huumonen *et al.,* 2017) up to 83% in Jordan (Al-Omari., *et al.,* 2011) if a patient as a person is a subject under investigation. When considering teeth as investigation subjects the presence of AP ranges from 1.4–8.0% of all teeth (Eriksen *&* Bjertness, 1991). Prevalence of AP has been shown to be positively correlated with age, gender, level of previous dental treatment, quality of dental treatment and socioeconomic status of the region (Kirkevang *et al* 2001). Knowing the distribution and prevalence of AP within a certain population helps plan the treatment need and evaluate the success of endodontic interventions (Eriksen *et al.,* 2002; Huumonen *&* Ørstavik, 2002). Prevalence of AP in Estonia was unknown before the present study.

AP is an inflammatory disease of periradicular tissues that is accompanied by destruction of periapical tissues (Nair, 2004). Its microbial etiology has been explored by culture and later also by molecular methods. The latter have expanded the list of putative endodontic pathogens by inclusion of some fastidious bacterial species or even uncultivated bacteria that had never been previously found in endodontic infections (Siqueira & Rocas, 2013). Most microorganisms in the root canal system congregate in the form of biofilms (Jhajharia *et al.,* 2015) thus creating additional difficulties during the treatment procedures.

AP usually results in the formation of an osteolytic apical lesion caused primarily by the immune response. Reactive oxygen species (ROS) produced by phagocytic cells in response to bacterial challenge represents an important host defense mechanism, but disturbed redox balance and high-grade oxidative stress (OxS) result in tissue injury and bone resorption (Hernández-Ríos *et al,* 2017; Dezerega *et al.,* 2012; Gomes *et al.,* 2018).

Very few studies have been carried out concerning the associations between OxS and AP and there are no studies comparing the role of excessive OxS and the salivary antioxidant system in the pathogenesis of AP.

In the first part of this study we collected data about the prevalence of AP for the first time in Estonia from panoramic radiographic images and compared the outcomes with the outcomes of other countries.

In the second part of the study we specified the determining factors of AP and found a correlation between different dental diseases/previous dentistry and signs of AP in panoramic radiographic images.

In the third part of the study we identified dental microbial communities as the main etiologic factor for endodontic pathologies using next generation sequencing method for the detection and comparison of all possible microbes in different endodontic pathologies.

In the fourth part of the study we identified and compared oxidative stress levels in the saliva and root canal content in patients with different endodontic pathologies and in healthy subjects.

The studies described in this dissertation were carried out in the Institute of Dentistry, University of Tartu; Kaselo Hambaravi Private Dental Clinic; Department of Microbiology, Institute of Biomedicine and Translational Medicine, University of Tartu; and Department of Biochemistry, Institute of Biomedicine and Translational Medicine, University of Tartu.

REVIEW OF LITERATURE

1. Tooth Anatomy, Histology and Physiology

A tooth is divided into two parts; the crown and the root (Figure 1). The crown is visible in the oral cavity and is situated coronal to the gums; the root is situated apical to the gums and is nested in bone. The outer layer of the crown is a specialized tissue called enamel, the layer of tissue under the enamel is another specialized tissue called dentin. The roots of the teeth are covered by tissue called cementum. The point where cementum and enamel meet is called the cemento-enamel junction (CEJ) or the cervical line (Heymann *et al.,* 2012)

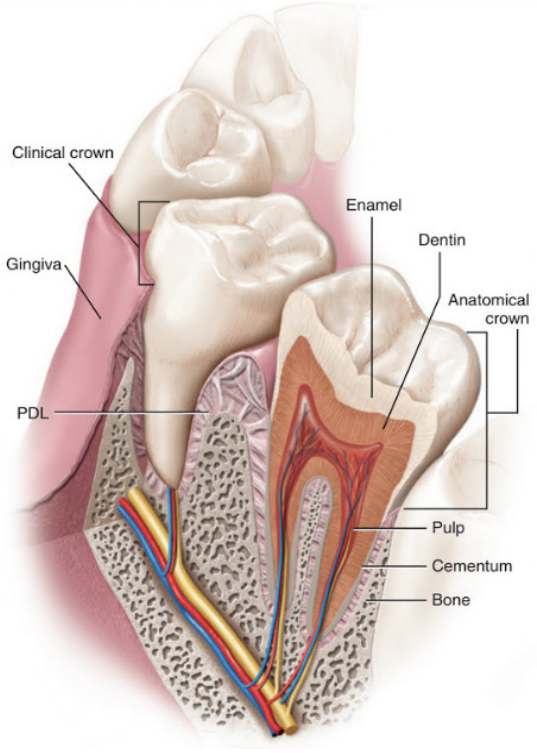

Figure 1. Longitudinal section of a molar showing the internal morphology (adapted from Ten Cate's Oral histology: Development, Structure and Function, 9th edition, Antonio Nanci, 2018, Elsivier publishing with permission).

Enamel forms the outer coating of the coronal part of tooth; it is one of the hardest tissues in the human body. It is formed by ameloblasts that have short extensions toward the dentinoenamel junction (DEJ), also called as Tomes processes. It is a highly crystalline structure of 95–98% inorganic component by mass (Nanci, 2018). The inorganic component comprises 86–95% of hydroxyapatite by volume. The organic component comprises 1–2%, while water contributes 4–12%. The rods and prisms are the main structural units of the material and are generally oriented at 90° to the external surface. Enamel is brittle and fractures easily when it is not supported by the dentine. The high

inorganic content and acellular structure of enamel means that once it is lost or damaged (either through normal attrition or trauma) it can't be replaced or regenerated.

Dentin and pulp together form the pulp-dentin complex. These connective tissues are formed from the dental papilla of the tooth bud.

Dentin is a unique, avascular mineralized connective tissue that forms the bulk of the tooth. It underlies enamel in the crown and cementum in the roots, providing structural support to these tissues and resilience to the tooth. Enamel and dentin together surround the dental pulp chamber. Dentin is yellowish in color and has a high mineral content (70%, mainly hydroxyapatite crystals), which makes it brittle. It also contains 20% organic compounds (mainly Type I collagen) and 10% water. Dentin consists of intertubular dentin being the primary structural component, comprising hydroxyapatite embedded in a collagen matrix, peritubular dentin which provides a collagen-free hyper-mineralised tubular wall, and dentinal tubules – these are filled with extended processes of odontoblasts, which form the interface between the dentin and the pulp (Fuller *et al.,* 2001; Nanci, 2018).

Dentin is classified into three types based on formation, time of appearance and structure (Kuttler, 1959). *Primary dentin* forms most of the tooth and it outlines the pulp chamber. Dentin tubules traverse the entire thickness of the primary dentin, and these contain the cytoplasmic extensions of the odonto-blasts. *Secondary dentin* is formed after root formation has been completed. It is mostly formed as a natural outcome of ageing due to the effects of normal biologic functions and as a response to dental treatment. Both primary and secondary dentin is formed by odontoblasts. The latter are part of both dentin and pulp tissues because their cell bodies are in the pulp cavity and their long, cytoplasmic cell processes (Tomes fibers) extend deep into the tubules in the mineralized dentin. There will be formed a net of capillary and nerve bundles near to the bodies of odontoblasts that will stimulate the formation of pulp tissues. Odontoblasts are also the first immune system protective cells, which carry on the information towards pulp tissue. *Tertiary dentin* (also known as reactive or reparative dentin) is deposited in response to strong noxious stimuli, such as caries or restorative dental procedures. The dentinal tubules are generally absent, but when present they are reduced in number and highly irregular compared to the primary and secondary dentin (Slootweg, 2013).

Dental pulp is loose connective tissue with an appearance similar to mucoid connective tissue containing arteries, nerves and extracellular components located in pulp space. It forms, supports, and is an integral part of the dentin that surrounds it. The pulp cavity extends down through the root and opens into the periodontium via the apical foramen. The blood vessels and nerves of dental pulp enter and leave the tooth through this foramen. This sets up a form of communication between the pulp and surrounding periodontium.

The majority of pulp (75–80%) is water but pulp contains similar compo-nents common to all connective tissues. There are fibroblasts and undifferen-tiated mesenchymal cells, and other cell types (macrophages, lymphocytes, etc.)

that are mainly required for the maintenance and defense of the dental tissues. In addition to cells, the pulp consists of fibrous matrix containing collagen fibers, mostly type I and II, that are present in an unbundled and randomly dispersed fashion, higher in density around blood vessels and nerves. Type I collagen is produced by the odontoblasts while type II by the pulp fibroblasts. Proteoglycans, glycoproteins and water dominate in the environment that surrounds both cells and fibers of the pulp.

The primary function of the pulp is formative; it gives rise to odontoblasts that not only form dentin but interact with dental epithelium, early in tooth development, to initiate the formation of enamel. Subsequent to tooth formation, pulp provides several secondary functions related to tooth sensitivity, inductivity, hydration, and protection (Torabinejad & Walton, 2008). Dental pulp is microcirculatory system that regulates local interstitial environment transporting nutrients, hormones and assures efflux of the gasses and metabolic byproducts with which it fulfills its functions (Hargreaves *et al.,* 2012).

Periodontium consists of the oral hard and soft tissues that invest and support the teeth. It can be divided into the gingival unit (consisting of free and attached gingiva and the alveolar mucosa), and the attachment apparatus (consisting of the cementum, periodontal ligament, and alveolar process). The periodontium attaches the teeth to the maxilla and the mandible and provides a continually adapting structure for the support of the teeth during function. The periodontium has two mineralized connective tissues, cementum and alveolar bone, and two fibrous connective tissues, the periodontal ligament and the lamina propria of the gingiva. The periodontium is attached to the jaws by alveolar bone and to the dentin of the tooth root by cementum (Heymann *et al.,* 2012).

2. Pulpal and periapical pathology

2.1. Classification of pulpal and periapical diseases

Till December 2009 in endodontics there was no standard diagnostic nomenclature consensus for pulpal (Levin *et al.,* 2009) and periapical (Gutmann *et al.,* 2009) status in health or disease. Instead, there has been a variety of diagnostic classification systems advocated for determining endodontic disease (Glickman *et al., 2009*). Most of them are based on histopathological but not on clinical findings (Hargreaves *et al.,* 2012) and are therefore unsuitable for everyday clinical practice.

Diseases of the pulp and periapical tissues are dynamic and progressive; symptoms can vary depending on the patient status and the stage of the disease (Newton *et al.,* 2009). Correct treatment needs complete endodontic diagnosis that must include both pulpal and periapical diagnosis (AAE, 2013) (Table 1). Moreover, the clinical and radiographic examinations in combination with a thorough periodontal evaluation and clinical testing (pulp and periapical tests) should be used to confirm the diagnosis (Schweitzer, 2009).

Table 1. Diagnostic terminology in endodontics recommended by AAE Consensus Conference (AAE, 2009).

PULPAL CONDITION	
Normal pulp	A clinical diagnostic category in which the pulp is symptom-free and normally responsive to pulp testing.
Reversible pulpitis	A clinical diagnosis based on subjective and objective findings indicating that the inflammation should resolve and the pulp return to normal.
Symptomatic irreversible pulpitis	A clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing. Additional descriptors: lingering thermal pain, spontaneous pain, referred pain.
Asymptomatic irreversible pulpitis	A clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing. Additional descriptors: no clinical symptoms but inflammation produced by caries, caries excavation and trauma.
Pulp necrosis	A clinical diagnostic category indicating death of the dental pulp. The pulp is usually nonresponsive to pulp testing.
Previously treated	A clinical diagnostic category indicating that the tooth has been endodontically treated and the canals are obturated with various filling materials other than intracanal medicaments.
Previously initiated therapy	A clinical diagnostic category indicating that the tooth has been previously treated by partial endodontic therapy (eg, pulpotomy, pulpectomy).
APICAL CONDITION	
Normal apical tissues	Teeth with normal periradicular tissues that are not sensitive to percussion or palpation testing. The lamina dura surrounding the root is intact, and the periodontal ligament space is uniform.
Symptomatic apical periodontitis	Inflammation, usually of the apical periodontium, producing clinical symptoms including a painful response to biting and/or percussion or palpation. It might or might not be associated with an apical radiolucent area.
Asymptomatic apical periodontitis	Inflammation and destruction of apical periodontium that is of pulpal origin, appears as an apical radiolucent area, and does not produce clinical symptoms.
Acute apical abscess	An inflammatory reaction to pulpal infection and necrosis characterized by rapid onset, spontaneous pain, tenderness of the tooth to pressure, pus formation, and swelling of associated tissues.
Chronic apical abscess	An inflammatory reaction to pulpal infection and necrosis characterized by gradual onset, little or no discomfort, and the intermittent discharge of pus through an associated sinus tract.
Condensing osteitis	Diffuse radiopaque lesion representing a localized bony reaction to a low-grade inflammatory stimulus, usually seen at apex of tooth.

2.2. Definition and terminology of apical periodontitis

Apical periodontitis (AP) also termed as periapical periodontitis or periradicular periodontitis is an inflammatory disease around the apex of a tooth root that is caused by infection in root canal system (Kakehashi *et al.,* 1965; Siqueira & Rocas, 2007; Segura-Egea *et al.,* 2012). The term is constructed of *peri-* ("around"), *apical* (referring to the apex of the root) and *-itis* (denoting the inflammation). AP can be considered a sequela of dental caries, irreversible pulpitis and pulpal necrosis; therefore AP is the most usual outcome of untreated dental caries. In rare occasions, AP can occur due to occlusal height disparities after restoration of the tooth, extrusion of endodontic root filling material or bacterial invasion from a gingival-periodontal communication. AP can be acute or chronic in nature. Acute AP is usually symptomatic, characterized by tenderness when tapping or chewing on it. Chronic AP is frequently asymptomatic while characterized by periapical bone destruction and degradation of extracellular matrix (Graunaite *et al.,* 2011). AP may also develop into a periapical abscess, or into a periapical cyst (Hargraves *et al.,* 2016).

To homogenize and abbreviate recording both pulpal and periodontal diagnoses at the same time we used simplified diagnostic terminology during this research by dividing AP into two main subcategories. First, teeth with visible periradicular radiolucency and with no previous root canal treatment and filling were categorized as primary apical periodontitis (pAP) (Siqueira *et al.,* 2009). Secondly, teeth with visible periradicular radiolucency with visible previous root canal treatment and filling on radiographs were categorized as secondary apical periodontitis (sAP).

2.3. Prevalence of apical periodontitis

Different epidemiological cross-sectional studies show AP being a widespread condition in many countries (**Paper I Table S1**). Reported prevalence of AP ranges from 27% in Finland (Huumonen *et al.,* 2017) up to 83% in Jordan (Al-Omari *et al.,* 2011) if a patient as a person is a subject under investigation. When considering teeth as subject under investigation the percentage of AP ranges from 1.4 to 8.0% from all teeth (Eriksen *et al.,* 1991). Data about most post-Soviet countries have been largely missing, being present only for Lithuania (Jersa & Kundzina, 2013), Latvia (Sidaravicius *et al.,* 1999) and Belarus (Kabak & Abbot, 2005).

Prevalence of AP has positive correlation with age, gender, level of previous dental treatment and socioeconomic status of the region (Kirkevang, 2001). The prevalence of both AP and root-filled teeth increases with age (Hamedy *et al.,* 2016). With aging, the comorbidity increases, also the immune system deteriorates. Additionally, oral disease experience in the elderly is higher than in younger persons (Guiglia *et al.,* 2010).

Several studies have shown **(Paper I Table S2)** that previous root canal treatment is one of the biggest risk factors for AP as the latter is always more frequent in treated than non-treated teeth (Jimenez-Pinzon *et al.,* 2004). At the same time several studies have indicated that AP is closely related to the quality of dental treatment. Because of that, the determination of the treatment quality alongside the prevalence of AP is utmost important (Farzaneh *et al.,* 2004). Knowing the distribution and prevalence of AP within a certain population helps to plan the treatment need and evaluate the success of endodontic interventions (Eriksen *et al.,* 2002; Huumonen & Ørstavik, 2002).

2.4. Etiopathogenetic aspects of apical periodontitis

AP is a inflammatory disease of periradicular tissues that is caused by etiologic factors of endodontic origin. It is a host defense reaction to microbial invasion from the root canal system and it manifests as local inflammation, resorption of hard tissues, destruction of other periapical tissues, and formation of different histopathological categories of AP, such as reactive granulomas and cysts (Nair, 2004).

The destruction of periapical tissues occurs due to counteraction of bacterial factors with host immune system defence components (Figure 2). Bacteria in endodontic infections produce different metabolic byproducts, exo-and endotoxins, including lipopolysaccharide (LPS) – an endotoxin that activates the complement system and consequently the two lines of phagocytic cell defense: an inner area where the polymorphonucleate phagocytes dominate, and the outer area with phagocytic macrophages. There is a dense infiltrate of T and B lymphocytes, macrophages, dendritic and plasma cells, polymorphonuclear leukocytes in periapical lesions histologically. The number, morphology and properties of these cells can vary, but T –, B lymphocytes and macrophages comprise the majority of the identified cells. The imbalance between the activity of osteoclasts and osteoblasts leads to bone destruction (Graunaite *et al.,* 2011).

Persistent AP develops when the initial root canal treatment has not effectively eliminated intraradicular infection (Pereira *et al.,* 2017). In the 1990s it was shown that there are six biological factors that could act as causative agents in development of asymptomatic apical radiolucencies after root canal treatment procedures. These include persisting intraradicular infection; extraradicular infection (periapical actinomycosis); extruded root canal filling material (causing foreign body reaction); accumulation of cholesterol crystals (irritating periapical tissues); true cystic lesions; scar tissue healing of the lesion (Nair, 2006).

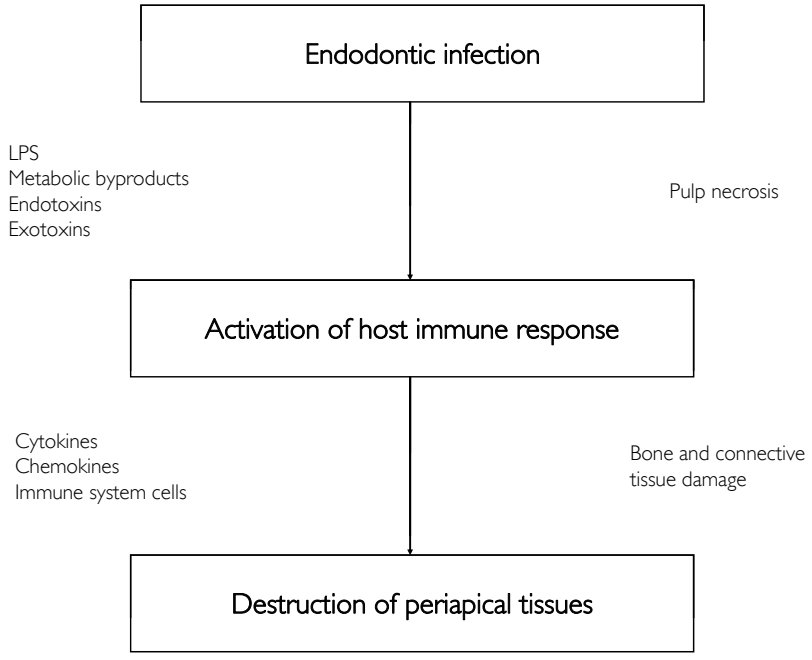

Figure 2. Overview of general pathogensis of apical periodontitis (adapted from Yucel-Lindberg T, Båge T. Inflammatory mediators in the pathogenesis of periodontitis. Expert Rev Mol Med. 2013 Aug 5;15:e7 with permission).

2.4.1. Causative agents of apical periodontitis

The main etiological role of microorganisms in the development of AP was uncertain for many years. In 1965 it was shown that no AP developed in germ-free rats' teeth that had had pulpal tissue exposed to the oral cavity. Periapical radiolucencies did occur in control rats with conventional oral microbiota. Today, the role of intraradicular microorganisms in the etiology of AP is more clear (Nair, 2004). By using massive parallel pyrosequencing analysis, 187 bacterial species-level phylotypes, 84 genera and 10 phyla were found in the apical part of root canals of teeth with AP indicating highly diverse bacterial communities (Siqueira *et al.,* 2011).

The intracanal microbiota of necrotic teeth with clinically intact crowns is dominated (>90%) by obligate anaerobes, the most abundant phyla being Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria (Yun *et al.,* 2016; Hargreaves *et al.,* 2016) (Table 2). The microbiota of non-vital teeth exposed to the oral cavity is different and less dominated (<70%) by strict anaerobes (Nair, 2004). Culture techniques, dark-field and transmission electron microscopy helped find spirochetes in necrotic root canals. Several different study methods have also revealed fungi in the canals in pAP (Baumgartner *et al.,* 2000). Intraradicular viruses have only been found in non-inflamed pulps of patients with immunodeficiency virus (Nair, 2004).

Microorganisms usually enter the tooth via openings in the dental hard tissues. These can occur due to caries, clinical procedures or dental traumas. Various traumatic injuries can lead to the formation of cracks and fractures. However, microbes have also been isolated from clinically intact teeth with necrotic pulps. The teeth might seem clinically sound; however, micro-cracks that provide an entry portal for the bacteria might still be present. Microbial colonisation is preceded by pulpal necrosis (Nair, 2004). It was also suggested that microorganisms from the gingival sulci or periodontal pockets could reach the root canals of necrotic teeth via severed periodontal blood vessels. Dentinal tubules can also be exposed at the cervical root surface due to gaps in the cementum. Another theory is called „anachoresis" and is described as bacterial „seeding" in the necrotic pulp via the blood circulation. However, no up-to-date study confirms this theory, and the exposure of the dental pulp to the oral cavity is considered to be the most important causative factor of endodontic infection (Nair, 2004).

Any microorganism that invades the root canal has the potential to cause inflammation. However, the pathogenicity and virulence of different species can vary and be affected by the presence of other species. The pathogenic properties are mostly influenced by: (1) interactions and synergism with other micro-organisms in the root canal; (2) the ability to interfere with and evade host defenses; (3) the release of lipopolysaccharides (LPS) and other bacterial modulins; and (4) the synthesis of enzymes that damage host tissues (Nair, 2004). Most microorganisms in the root canal system congregate in the form of biofilm – a sessile multi-cellular microbial community characterised by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extra-cellular polymeric substances (EPS) (Jhajharia *et al.,* 2015; Aw, 2016).

Table 2. Common causative agents of periodontal pathology (Hargreaves *et al.,* 2016)

Bacterial phyla	**Common endodontic representatives**
Firmicutes	*Dialister* spp, *E. faecalis*, *Eubacterium* spp.
Actinobacteria	*Olsenella uli, Actinomyces* spp.
Synergistetes	*Pyramidobacter piscolens*, clone W090
Spirochaetes	*Treponema denticola, Treponema socranskii*
Fusobacteria	*Fusobacterium nucleatum*
Proteobacteria	*Eikenella corrodens, Campylobacter rectus*
TM7	Clone l025
Bacteroidetes	*Tannerella forsythia, Porphyromonas gingivalis*

2.4.2. Oxidative stress in apical periodontitis patients

In a state of health, there is a dynamic balance between pro-oxidants and antioxidant defense system (Halliwell & Gutteridge, 2015).

In certain situations, like in case of inflammation, such a balance becomes disturbed in favor of the pro-oxidants. Thus, oxidative stress (OxS) could be

defined as a situation of profound disturbance in the pro-oxidant and anti-oxidant balance in favour of the former (Pincemail *et al.,* 1996). OxS is involved in the pathogenesis of a variety of inflammatory disorders. Prolonged high-grade OxS leads to tissue damage (Halliwell & Gutteridge, 2015).

Principal **pro-oxidants** are reactive species (including free radicals) divided into reactive oxygen species (ROS) and reactive nitrogen species (RNS) and they mediate the main effects of other pro-oxidative factors (Halliwell & Gutteridge, 2015). In the organisms the crucial ROS are superoxide radical, hydroxyl radical, lipid peroxyl radical and non-radical hydrogen peroxide (the latter is produced from superoxide by superoxide dismutase) and the principal RNS are nitric oxide and non-radical peroxynitrite.

At the same time, the human body has developed **antioxidant** defense mechanisms involving a number of enzymatic and nonenzymatic systems, such as the peroxidase system, certain proteins, vitamins, uric acid, etc. The latter has been characterized as the most important antioxidant molecule found in saliva (Liskmann *et al.,* 2007; Inchingolo *et al.,* 2014).

In case of health, the amounts of pro- and antioxidants are well balanced. In case of infection, the generation of ROS is a major pathogenic mechanism against the invading pathogen (Dezerega *et al.,* 2012). However, in some occasions excessive formation of the reactive species (RS) can occur *in vivo* causing an imbalance in the pro-oxidants/antioxidants system. That leads to the damage of lipids, proteins, nucleic acids and carbohydrates of cells and tissues, resulting in tissue injury. Any imbalance in favour of the pro-oxidants potentially leading to damage was termed 'oxidative stress' (Rahal *et al.,* 2014; Halliwell & Gutteridge, 2015). Recently, an additional conception of OxS was advanced as "a disruption of redox signaling and control" (Jones, 2006).

A large body of evidence exists that high-grade OxS has one of the crucial roles in the pathogenesis of several disorders/diseases. Although research on the role of ROS and the protective capacity of salivary antioxidants in marginal periodontitis has been extensive (D'aiuto *et al.,* 2010), very few studies have been carried out concerning the associations between OxS and AP. These studies have described the pro-oxidant status in apical lesions of patients with AP (Dezerega *et al.,* 2012), and influence of endodontic treatment on systemic OxS (Inchingolo *et al.,* 2014).

Activated phagocytosis takes place during endodontic infection with production of ROS, activation of immunological response, and production of inflammatory mediators including matrix metalloproteinases (MMP). All this disturbs normal redox balance and shifts cells into the state of OxS. ROS induce molecular damage and disturbed redox signaling, that result in the loss of bone homeostasis, increased pro-inflammatory mediators, and MMP overexpression and activation, leading to apical tissue breakdown. ROS produced by phagocytic cells in response to bacterial challenge represent an important host defense mechanism, but disturbed redox balance results in tissue injury (Hernández-Ríos *et al.,* 2017).

2.5. Clinical evaluation and diagnosis of apical periodontitis

Symptomatic AP is usually diagnosed from its clinical presentation while the diagnosis of asymptomatic AP is commonly dependent on the presence of radiographic signs of the disease (Gutmann & Lovdahl, 2010). Endodontic diagnosis cannot be made from a single isolated piece of information. The clinician must systematically gather all of the necessary information by using different methods to make a "probable" diagnosis (Table 3). When the clinical and radiographic examinations are inconclusive or give conflicting results, definitive pulp and periapical diagnoses cannot be made. It is also important to recognize that treatment should be rendered only with a definitive diagnosis (AAE, 2013).

Table 3. Examination procedures required to reach an endodontic diagnosis (Abbott, 2007)

Procedure	Description
Medical/dental history	Past/recent treatment, drugs
Chief complaint (if any)	Duration of the complaint, symptoms, duration of a pain episode, location, onset, stimuli, relief, referred, medications
Clinical examination	Facial symmetry, sinus tract, soft tissue, periodontal status (probing, mobility), caries, restorations (defective, newly placed)
Clinical testing Pulpal tests	Thermal test (cold, heat), electric pulp test (EPT)
Clinical testing Periapical tests	Percussion, palpation, bite test
Radiographic analysis	New periapical, bitewing, Cone beam-computed tomography (CBCT)
Additional tests	Transillumination, selective anesthesia, test cavity

2.5.1. Diagnostic methods in case of endodontic pathology

Establishing a differential diagnosis in endodontics requires a certain blend of information, which contains medical and dental history, chief complaint (if present), clinical extra- and intraoral examination, clinical pulp and periapical tests, radiographic analysis and additional tests if needed (AAE, 2013).

Extraoral examination evaluates swelling, changes in color on soft tissue of the face, temporomandibular joint problems, lymph nodes palpation may lead to diagnosing spread infection or malignant disease (Ingle *et al.,* 2013).

Intraoral examination starts with soft tissues, to register any inflammation, sinus tract or intraoral swelling. After that clinician starts to seek for dental hard tissue problems (Ingle *et al.,* 2013). The following are clinical pulp and periapical tests.

a. Clinical pulp tests involve the attempt to make a determination of the responsiveness of pulpal sensory neurons. The tests involve thermal and electrical stimulation of a tooth to determine whether the pulpal nerves are functional (Jafarzadeh & Abbott, 2010). Abnormal responses include lack of response, lingering or intensification of a pain, or an immediate painful sensation (Hagreaves *et al.,* 2016). Symptomatic AP may or may not respond to pulp vitality tests, on the other hand asymptomatic AP does not respond to vitality tests (Hargreaves *et al.,* 2016).

- *Heat test* is appropriate when the patient cannot identify the sensitive tooth and the main complaint is dental pain to hot liquid or food. Typically the tooth that responds to heat could be relieved by cold (Hagreaves *et al.,* 2016). Typical methods used include gutta-percha or compound material heated to melting temperature and directly applied to the tooth (Chen & Abbott, 2009).
- *Cold test* is the most reliable thermal pulp test. If the mature, untraumatized tooth does not respond to electric nor cold test, the pulp should be considered necrotic (Hagreaves *et al.,* 2016). Cold test could be performed with frozen carbon dioxide, either spray or dry ice form, or refrigerant spray- propane/ butane/ isobutane gas mixture stored in pressurized can. With both thermal tests adjacent and contralateral teeth should be tested (Hagreaves *et al.,* 2016).
- *Electric pulp test (EPT)* does not give information about the health or disease of the pulp tissue. It gauges the ability of nerves to respond to electrical stimulation (Ingle *et al.,* 2013). EPT works on the premise that electrical stimuli cause an ionic change across the neural membrane in myelinated nerves. A „tingling" sensation will be felt once the increasing voltage reaches the pain threshold. EPT is known to be unreliable in healthy immature teeth, in teeth that are undergoing orthodontic treatment and in teeth after dental trauma (Chen & Abbott, 2009).
- *Cavity test.* To accomplish the cavity test, the patient is not anesthetized and asked to respond if any painful sensation is felt during the drilling procedure. Once the bur contacts sound dentin, pain occurs, and the procedure is terminated. Prepared site is closed with composite filling. This sensation signifies only that there is some viable nerve tissue remaining in the pulp (Hargreaves *et al.,* 2016).

b. Clinical periapical tests, as apical palpation and percussion are two diagnostic tests, which are most often used when diagnosing AP.

- *Percussion test.* Positive percussion test often indicates the periapical lesions associated with necrotic pulp. False positive results can occur if the clinician is evaluating a cracked tooth or a „high" restoration. Percussion test could be done with a operator finger or end of the mirror handle (Ingle *et al.,* 2013). When AP occurs unrelated to marginal periodontal lesions, it is usually a sequel to pulpal necrosis (Baig, 2016), and results in a sharp painful sensation, causing a withdrawal response

(Torabinejad, 2008). When AP is present as a sequel to periodontal lesion, the pulp is usually vital (Baig, 2016), and pain is mild to moderate range. (Torabinejad & Walton, 2008).

– *Palpation test* determines how far the inflammatory process has extended periapically. Palpation is a firm pressure on the mucosa overlying the apex (Torabnejad & Walton, 2008). Absence of discomfort during palpation does not mean that disease is not present (Ingle *et al.,* 2013).

Both periapical tests should be done on control teeth on the same side and contralateral side (Ingle *et al.,* 2008). Symptomatic AP teeth will have an acutely painful response to biting pressure and percussion. Teeth with asymptomatic AP are generally not sensitive to biting pressure, but may „feel different" to the patient on percussion (Hagreaves *et al.,* 2016).

2.5.2. Radiographic diagnostics of endodontic pathology

Within the scope of endodontics, radiographic images serve a double purpose: the confirmation of normality and indication of pathosis. It should not be the expected that every pulpally involved tooth would have radiographic signs of pathosis. The change to be discovered radiographically is the loss of hard tissue, usually bone surrounding the apex. Periapical pathosis is, in turn, a consequence of pulpal necrosis and extreme pulpal inflammation. Routinely, teeth with pulpal symptoms (discomfort to thermal changes) will not have two-dimensional radiographic changes, yet they still may require a root canal procedure. This is of crucial diagnostic significance. Some teeth require root canal treatment because of symptoms (pain and or discomfort feeling) other teeth do not have any symptoms but do have visible periapical pathosis on radiograph (Gutmann & Lovendahl, 2010).

In some cases of acute periapical abscesses, there are no radiographic changes even though there may be swelling and an acute pain. Radiographic evidence of apical bone resorption will require usually as much as 7 days or more to occur after the onset of an acute periapical abscess (Gröhndahl & Huumonen, 2004). In these cases, while no lesion is observed at the time of emergency treatment, a lesion may be present at a subsequent appointment when the tooth is comfortable and ready for completion of the treatment. The ability of radiographs to accurately detect signs of AP is essential for diagnosis and treatment planning (Gutmann & Lovendahl, 2010). Overview of the radiographic methods used for diagnosing of endodontic pathologies is given in Figure 3.

Conventional radiography

In producing radiographic images, before exposing the receptor film the X-ray beam passes mineralized tissues that have a differential absorption of radiation

(Masri & Driscoll, 2015). One technique for controlling the many variables in the diagnostic quality of conventional radiography has been the advent of digital radiography, in which a sensor is used to capture the image created by the radiation source. This sensor is attached to a local computer, which interprets this signal and, using specialized software, translates the signal into a two-dimensional digital image that can be displayed and enhanced (Hargreaves *et al.,* 2010). With digital periapical radiograph systems, the image is dynamic and can be enhanced by contrast and brightness to improve its diagnostic information. Single digital periapical radiographs were found to be more accurate than single conventional radiographs (Kanagasingam *et al.,* 2015). There are several advantages of digital X-ray imaging over analog film imaging that can benefit the clinician: reduced time, reduced radiation, ability to make multiple exposures without repositioning the sensor, storage and maintenance of the images, and electronic transmission of images (Gutmann & Lovendahl, 2010). Radiographic assessment is essential in every aspect of endodontics. Before a meaningful discussion of the diagnostic interpretation of radiographs begins, the technical quality of the image must first be assessed (Delis *et al.,* 2017).

Intraoral periapical radiography

Intraoral periapical radiography has been accepted as the suitable imaging system. A good radiographic image for endodontic diagnostic and treatment purposes is obtained when the center of the X-ray beam passes through the apex and projects the tooth on the receptor film or sensor at a right angle (Gutmann & Lovendhal, 2010).

Intraoral periapical radiographic image must visualize the accused tooth as a whole with crown, root and periapical area. The image should ideally be taken with a paralleling technique (Langland & Langlais, 2002). Anatomical limitations, such as shallow palatal vault, may prevent the ideal positioning of the intraoral image receptor. In case of the bisecting-angle technique, the X-ray beam is directed perpendicular to an imaginary line, which bisects (divides in half) the angle formed by the long axis of the tooth and the long axis of the film. And yet an accurate image is obtained when the image receptor (X-ray film or digital sensor) is parallel to the long axis of the tooth, and the X-ray beam is perpendicular to both the image receptor and the tooth undergoing examination (Forsberg, 1987). More ideal positioning of the image receptor may be possible when the roots are relatively straight or when there is sufficient space to position the image receptor correctly. If these objectives are not achieved there will be a degree of geometric distortion and magnification (Lofthag-Hansen *et al.,* 2007).

Pulpal conditions that cause periapical pathosis are pulpal necrosis and irreversible pulpitis. The radiographic changes usually follow within a few days after the time of onset of apical inflammation. Alterations to structures of apical periodontium, such as the medullary bone trabeculae, the width of the

periodontal ligament (PDL), and the lamina dura are early indicators of AP (Riccucci *et al.,* 2006). By itself, it is important to distinguish normality from pathosis (Gröhndahl & Huumonen, 2004).

A widened PDL space will be localized to the apex of the tooth (or the affected portal of exit, for instance, lateral canal) and the adjacent areas. The PDL space coronal (and/or apical, if for example a lateral canal is involved) to this area will be unaffected, and there will be a marked transition between the affected and unaffected sites (Patel *et al.,* 2016).

Another relatively early radiographic feature of apical pathosis is disruption of the lamina dura, which appears to lose density (Riccucci *et al.,* 2006). The integrity of lamina dura on the radiograph should be viewed with caution. Within the variations in normal lamina dura radiodensity and thickness, as well alterations by the angle of radiographic exposure, however, this feature can present diagnostic challenges for clinicians (Gutmann & Lovdahl, 2010).

Structural changes like a disorganization of the normal trabeculae around the apex of the affected tooth may be seen as a well-defined radiolucency on the radiograph. Depending on the X-ray beam angulation and the site of the tooth, adjacent anatomical features (the maxillary sinus, mental foramen, incisive canal) may impair interpretation of the radiograph; therefore the radiolucency may not be readily identified or may look larger (Hargreaves *et al.,* 2012).

Additional parallax radiographic images taken by changing the horizontal and/or vertical angulation of the X-ray beam in relation to the area under examination may be used to enhance assessment of the spatial relationships of the imaged anatomical structures (Davies *et al.,* 2015). Mesial and distal angulations parallax images (SLOB technique, Same Lingual Opposite Buccal rule) improve the detection of AP lesions compared to a single view (Kanagasingam *et al.,* 2015). SLOB principle states that the objects closest to the lingual surface appears to move in the same direction that the cone moved, and the object closest to the buccal surface appears to move in the direction opposite the movement of the cone or tube head (Ingle *et al.,* 2013). This technique prevents the X-ray beam to superimpose the canals of the affected tooth, and helps to distinguish other destructions associated with AP (resorption, fractures). Also the occlusal (standard, oblique, vertex) radiographic film is used to identify the extent of lesions in a buccolingual direction (Gutmann & Lovdahl, 2010).

Condensing osteitis is presented radiographically as an area of increased density (radiopacity) around the root apex. This type of lesion is a reactionary production of dense bone in the periapical area in response to low-grade pulpal irritation (Patel *et al., 2016).*

The area of bone destruction associated with AP is underestimated on conventional radiographs (Paula-Silva & Wu, 2009). The lesions may be masked by the overlaying denser cortical bone or superimposed of other adjacent structures. Assessment of periapical status of the tooth is also limited by the fact that the intraoral radiograph is a two-dimensional representation of a three-dimensional object (Gutmann & Lovdahl, 2010).

Extraoral radiography

Panoramic radiography

Panoramic radiograph is a single image of facial structures that includes maxillary and mandibular arches and their supporting structures. The panoramic radiograph is based on the principle of reciprocal movement of X-ray source and an image receptor around a central point or plane called the image layer, in which the object of image is located (Fuhrmann, 2015).

No significant difference between panoramic radiographs and full-mouth intraoral periapical surveys in the detection of periapical radiolucencies has been found (Muhammed *et al.,* 1982). The main advantage of panoramic radiograph is that all teeth are visible in a single image. This method also results in relatively lower patient radiation doses (Molander *et al.,* 1995). The convenience of panoramic radiograph and the speed with which this can be obtained are advantageous (López-López *et al.,* 2012). In traumatic fractures involving the alveolar process, a distinct fracture line should be evident on intraoral and extraoral radiographs. When assessing mandibular fractures, generally panoramic and a lateral cephalometric radiograph are used to provide two views of the fracture line from opposing perspectives. Postero-anterior and lateral views of the skull, and sometimes occlusal and submentovertex, are generally the conventional radiographs used to diagnose maxillary fractures (Patel *et al.,* 2016).

CBCT *(cone-beam computed tomography)*

Three-dimensional radiography is represented in the form of cone-beam computed tomography (Patel *et al.,* 2016). Cone-beam computed tomography uses cone- or pyramid-shaped beam and acquires all the data in a single rotation. It also enables a small area or volume in the center of the patient to be imaged. AP in its early stages presents on a CBCT scan as a well-defined widening of PDL space or clearly defined periapical radiolucency (Tsai *et al*., 2013). CBCT is a more accurate diagnostic tool than conventional intraoral periapical or panoramic radiograph diagnosing AP. The sensitivity for CBCT (<0,91) is much higher than for PA radiographs (0,77) for detecting existing periapical lesion (Paula-Silva & Wu, 2009).

Several clinical studies have concluded that the diagnostic accuracy of CBCT in the detection of AP is superior to that of periapical radiography (Low *et al.,* 2008). Comparing the outcome of endodontic treatment 2 years after treatment, it was found that failure outcome was reached in 13% of cases assessed with PA radiography and 26% of cases evaluated by using CBCT. Comparing the periapical status of teeth with suspected endodontic disease using CBCT and PA radiography, 38% more apical lesions were detected with CBCT (Lofthag-Hansen *et al.,* 2007). Also the outcome of secondary (re-treatment) AP cases carried out in 98 teeth 1 year after re-treatment showed a

significantly different success rate between intraoral radiographs (93%) and CBCT (77%) (Davies *et al.,* 2015).

When combined with digital radiography, however, CBCT can significantly enhance the diagnosis and treatment planning of AP (Gutmann & Lovdahl, 2010).

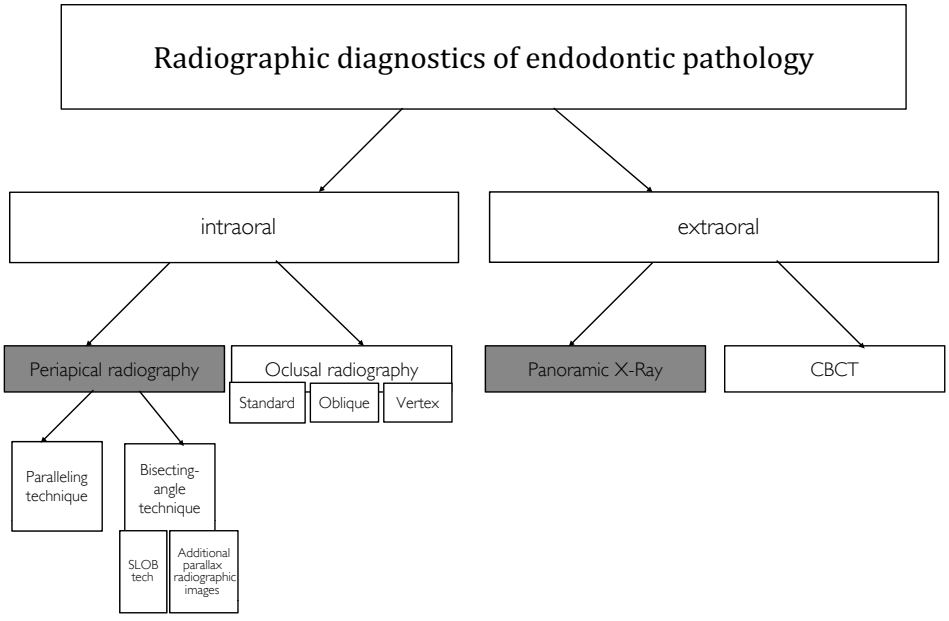

Figure 3. Overview of radiographic diagnostics methods used for diagnosing dental pathologies. Primary options are indicated as gray.

2.5.3. Other methods

When classical pulpal and periapical tests do not give a definitive diagnosis, additional tests should be considered for evaluation (AAE, 2013).

Periodontal tests

Periodontal defects, evaluated during probing, could be a sign either an endodontic or a periodontal problem. If the periodontal probe sinks abruptly into an isolated periodontal defect, the level of suspicion for vertical root fracture increases (Ingle *et al.,* 2013). During periodontal examination the clinician should register the level of tooth mobility, which determines the health status of periodontal ligament (Ingle *et al.,* 2013).

Alternative pulp tests

Alternative pulp tests have been applied for scientific purposes; they are not used in everyday clinical practice.

- *Laser Doppler flowmetry* is a method used to assess blood flow in micro-vascular system. A diode is used to project an infrared light beam through the crown and pulp chamber of the tooth. LDF is an accurate, reliable and reproducible method, but is not comfortable enough to use on a routine basis (Hargreaves *et al.,* 2016). Multi-rooted tooth might have inflamed pulp tissue in one canal, whereas the pulp chamber and other canals might be necrotic and infected (Salgar *et al.,* 2017). A calcified tooth structure might also be capable of conducting electrical current to tissue apical to an area of pulp necrosis (Salgar *et al.,* 2017).
- *Pulse oximetry data* are based on objective findings rather that patient objective response. It is designed to measure oxygen level in the blood (Hargreaves *et al.,* 2016).

Additional tests in endodontics

Additional tests are used when conventional test give inconclusive answers for correct diagnosing.

- *Biting test.* Tooth may be sensitive to biting when pulpal pathosis has extended into the periodontal ligament space or secondary to a crack in the tooth. If periradicular periodontitis is present, the tooth will respond with pain to percussion and biting test regardless of where the pressure is applied. A cracked tooth will elicit pain only when stimulation is applied in certain direction to one cusp of the tooth. For the bite test a device should allow to apply pressure to individual cusps. The pressure should be applied slowly until full closure and then quickly released. A common finding with cracked tooth is the presence of pain on release of biting pressure. As with all pulp tests, adjacent teeth should be used as controls (Hargreaves *et al.,* 2016).
- *Transillumination test.* This is an effective test for detecting caries, calculus and soft tissue lesions. It is primarily used to help determine the presence of crown or root fracture. If the crack is present in the dentin, the light will be interrupted at that crack and a dark line will be marking its location (Hargreaves *et al.,* 2016).

2.6. Treatment options for apical periodontitis

The general goal of endodontic therapy is to prevent or cure periradicular periodontitis. During the treatment, the root canal system is cleaned and disinfected in order to reduce the number of microorganisms. Necrotic tissue is removed, and finally, the system is sealed to prevent recontamination. Endodontic therapy can be divided into surgical endodontics (also called endodontic

surgery, periradicular surgery, apicoectomy, retrograde therapy, etc) and non-surgical (also known as orthograde therapy, conventional root canal treatment etc) (Rosenberg et al., 2009). Treatment planning must always include a careful evaluation of periapical condition, so that a decision can be made among non-surgical procedure, surgical procedure or tooth extraction (Ørstavik & Pitt Ford, 1998; Del Fabbro *et al.,* 2016).

There are many nonsurgical and surgical treatment options that help to perform the debridement of the root canals (Table 4; Figure 4). **Nonsurgical treatments** include: conservative root canal therapy/treatment, RCT with using calcium hydroxide, aspiration-irrigation technique, lesion sterilization and tissue repair therapy, active nonsurgical decompression technique (Mejia, 2004; Burrus *et al.,* 2014). New techniques which use drug-loaded injectable scaffolds, simvastatin, and epigallocatechin-3-gallate, pulp revascularization, pulp regeneration have been tried as well (Karunakaran *et al.,* 2017). **Surgical treatments** include: incision for drainage, apicoectomy, decompression technique, root amputation, bicuspidization, hemisection, intentional replantation, trepanation, the apexum procedure (Hagreaves *et al.,* 2016).

Endodontic treatment is reported to be a highly successful procedure and should be considered before tooth extraction. In primary endodontic treatment the success rates were reported to be between 73–97%, for the secondary treatment the success rates are between 59.5–95%, and for surgery treatments 27.8 to 80% (Elemam & Pretty, 2011).

2.6.1. Non-surgical treatment

Non-surgical treatment can be divided into the following steps: diagnostics and treatment planning, anesthesia, tooth isolation, shaping and cleaning of the root canals, root canal filling with biocompatible materials and preparation of the final restoration (Hagreaves *et al.,* 2016).

Anesthesia and tooth isolation

Nonsurgical treatment is routinely performed under local anesthesia. There are different anesthetic solutions available, such as mepivacaine, articaine, bupivacaine, lidocaine and others. Different anesthetizing techniques are used for different groups of teeth and clinical situations.

Before initiation of chemomechanical debridement old leaking restorations and carious lesions should be removed. The tooth is then isolated from the oral cavity with the help of a rubber dam. Rubber dam placement improves access and visibility, reduces operative time, secures the patient from the instruments and chemical materials used during the procedure, helps to control cross infection (Kulild, 2013; Hagreaves *et al.,* 2016).

Chemomechanical debridement

Nonsurgical treatment is carried out by the means of chemomechanical debridement (Regan & Fleury, 2006). The main objectives in cleaning and shaping the root canals can be defined as following: (1) removal of infected tissue, both soft and hard, (2) creation of access for the irrigants to the apical portion of the canal, (3) space creation for the medicaments and obturation materials, and (4) saving the integrity of radicular structures (Hargreaves *et al.,* 2016).

Ideally, when mechanical root canal instrumentation is complete it should be centered incorporating of the original canals into the prepared shape, however it is unlikely to be achieved with currently available instruments (Paqué *et al.,* 2009). It is widely accepted that a continuous taper should be created within the canal, however there is still discussion in the optimal final apical preparation size and the final taper (Baugh & Wallace, 2005). The techniques of instrumentation include the standardized technique (the same working length is remained for all the instruments), the step-back technique (a stepwise reduction of the working length for larger files), step-down technique (shaping the coronal aspect of a root canal first, before apical instrumentation commenced), crown-down technique (the modification of original step-down, the bigger reliance on the coronal flaring), and balanced force technique (specific clockwise and counterclockwise instrument rotation). There are also specific techniques for each NiTi rotary instruments (Hargreaves *et al.,* 2016). The main chemomechanical goals of irrigation in endodontics are following: (1) flush out debris, (2) lubricate the canal, (3) dissolve organic and inorganic tissue, and (4) prevent the formation of a smear layer or its dissolution. Biologically the irrigants should (1) be effective against microorganisms both in planktonic and biofilm state, (2) inactivate endotoxin, and (3) be nontoxic to vital tissues, (4) not cause an anaphylactic reaction (Hargreaves *et al.,* 2016).

Root canal filling and final restoration

Most obturation techniques employ a core material and sealer. Ideal filling material should be easily manipulated and provide ample working time, be dimensionally stable with no shrinkage once inserted, seal the canal laterally and apically, be non-irritating to the periapical tissues, impervious to moisture and nonporous, unaffected by tissue fluids – no corrosion or oxidization, inhibit bacterial growth, be radiopaque and easily discernible on radiographs, not discolor tooth structure, be sterile and easily removed from the canal if necessary (Hargreaves *et al.,* 2016).

Adequate nonsurgical treatment per se does not guarantee long-term retention of root canal treated teeth; there is good evidence that this outcome is closely related to placement of an adequate coronal restoration (Aquilino & Caplan, 2002).

2.6.2. Surgical treatment options

Apical periodontitis may need some kind of surgical treatment options. Indications for apical surgery usually include cases in which canal obstructions do not allow the retreatment or those in which it would not be advisable to do so. The main purpose of this surgery is to clean and seal all communication of the root canal near the apex (Torabinejad & Rubinstein, 2017).

Periradicular surgery

This is the most commonly used method aiming to remove etiological factors, to prevent recontamination of periradicular tissues (Hargreaves *et al.,* 2016). The main goal should be obtained by root-resection, root-end cavity preparation, and a bacterial-tight closure of the root-canal system. At one year follow up 95–97% of cases are classified as successful (von Arx, 2011).

Indications for apical surgery are (1) radiological findings of apical periodontitis and/or symptoms associated with an obstructed canal, (2) extruded material with clinical or radiological findings of AP over extended period, (3) persisting or emerging disease, (4) perforation of the root or the floor of the pulp chamber. In case of hopeless prognosis, the tooth should be extracted. For diagnosis and surgical planning, use of CBCT is recommended (von Arx, 2011). The typical sequence of procedures used specifically in periradicular surgery are mucoperiosteal flap design, incision and reflection of soft tissues, osteotomy to prepare apical access through hard tissue, periradicular curettage, root-end resection, root-end cavity preparation, root-end filling, flap replacement and suturing, postoperative care and instructions and finally suture removal and evaluation (Torabinejad & Walton, 2008).

With the use of the operating microscope, dentists can distinguish and deal with difficult anatomical details, examine roots for fractures or perforations and remove injured tissues precisely and completely (Kim & Kratchman, 2006). To access the bone, a full thickness flap, which consists of gingival, mucosal tissue and periosteum, must be raised (Dhingra *et al.,* 2014). The incision technique and flap design should be chosen according to clinical and radiographic parameters (von Arx & Salvi, 2008). The wide variety of flap design have been introduced such as marginal mucoperiosteal flaps with one (triangular) or two (trapezoidal or rectangular) releasing vertical incisions, submarginal mucoperiosteal flaps with horizontal incisions within the attached gingival and its modification, and semi-lunar flaps (Dhingra *et al.,* 2014). Combination of the forenamed designs can be used as well.

Once the mucoperiosteal flap is raised, the cortical bone over the root end is removed (von Arx, 2010). The osteotomy should be as small as possible but as large as necessary to accomplish the clinical objective (Kim & Kratchman, 2006). During the removal of hard tissue heat generation must be minimized, because it causes irreversible cellular damage (Hargreaves *et al.,* 2016). Osteotomy enlargement coronally could cause perio-endo communication. The

optimal size is 3–4 mm in diameter (Dhingra *et al.,* 2014). An apical resection of 3mm should include most accessory and lateral canals and thus eliminate most residual irritants. Ideally root-end preparation is made along long axis of the tooth to a depth of at least 3mm, which is performed with an ultrasonic tip or microhandpiece bur. The ideal root-end filling materials seals the contents of the root-canal system, preventing egress of any bacteria, bacterial by-products or toxic material into surrounding periradicular tissue. Most commonly used materials for that reason are ZOE cements, GI cements, composite resin, resin-glass ionomer hybrid, MTA or newer bioceramics (Debelian & Trope, 2016).

After these procedures the osteotomy site is sutured. The first to be sutured are corners, after that the center of the flap is positioned and sutured. Suturing technique can be interrupted or splint (Hargreaves *et al.,* 2016) or a combination of different techniques.

Other methods

Incision for drainage. AP can develop into an acute apical abscess. The abscess consists of a collection of pus into a cavity formed by tissue liquefaction. Treatment of acute abscesses involves incision of the soft tissues to enable the collection of pus to be drained. Incision for drainage gives only temporary relief and therefore must be followed by root canal treatment or extraction of the involved tooth to remove the source of infection (Castellucci, 2004).

Decompression. Creating a small fenestration in the cystic wall allows the lining of the cyst lumen to become confluent with that of the oral cavity. The insertion of a decompression stent or drainage tube is necessary so that continuity between the cystic lumen and the oral cavity is maintained. Such continuity establishes free draining of cystic contents and equalization of the intra- and extracystic pressures (Sammut *et al.,* 2012).

Hemisection and root amputation. This is the treatment option in multi-rooted teeth when one root with persisting apical pathosis is separated from other root and extracted. Other roots with healthy periapex are root canal treated and restored with suitable restorative technique (Babaji *et al.,* 2015).

Bicuspidization. This is an infrequently used surgical procedure when mesial and distal roots of mandibular molars are separated with their respective crown portions. Separation eliminates previously infected furcation area and therefore eliminates infection and facilitates oral hygiene (Muhamad *et al.,* 2015).

Apexum procedure. This is another marginal method that means minimally invasive removal of periapical chronically inflamed tissues through a root canal access (Raisinghani, 2011). For this procedure a special tube that contains a NiTi wire is protruded through the apical foramen. The wire takes a specially designed form and is rotated at 300 rpm, mincing the soft tissue, while being deflected from the surrounding bone. Most of the minced tissue is sucked out through the tube (Ingle *et al.,* 2008).

Table 4. Summary of treatment options for AP

Nonsurgical treatment	Surgical treatment
Frequently used	
Conservative root canal treatment (cleaning, shaping, disinfecting and obturation)	Incision for drainage
RCT with the use of calcium hydroxide	Apicoectomy with or without retrograde filling
Rarely used	
Aspiration-irrigation technique	Decompression technique
Lesion sterilization	Root amputation
Tissue repair therapy	Lower molar bicuspidization
Active nonsurgical decompression technique	Hemisection
Drug-loaded injectable scaffolds	Intentional replantation
Simvastatin	Trepanation
Epigallocatechin-3-gallate	Apexum procedure
Pulp revascularization	
Pulp regeneration	

All the procedures can be done individually or combined with one ore more procedures.

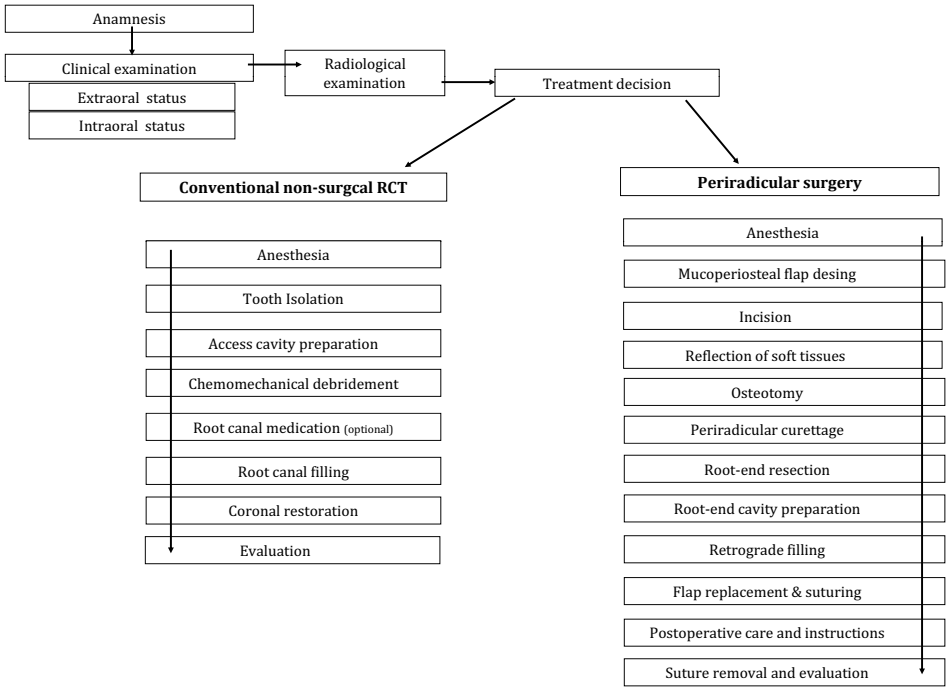

Figure 4. Flow chart of treatment stages in case of AP.

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The prevalence of apical periodontitis is widely investigated all around the world, mostly in Western European and Scandinavian countries. At the same time the data is lacking in most of the former Soviet Union countries. As the prevalence of AP has never been investigated in Estonia, this information gap needs to be filled in order to give important information to Estonian dentists and health care administrators but also to contribute to the worldwide knowledge about the prevalence of AP.

It has been demonstrated that the presence of different dental diseases and the quality of previous dental treatment are among important determining factors for AP. To date, this information is missing for Estonian population that is the last uninvestigated Baltic state.

Bacteria have been declared as the main etiologic factor for developing AP but differences in microbial communities in different endodontic pathologies need to be elucidated applying novel methods like next generation sequencing, especially in different geographic areas and populations.

Previous studies have indicated tight association between inflammatory diseases and local and systemic oxidative stress. However, this is not thoroughly researched in case of oral diseases and there is limited data about OxS in case of apical periodontitis.

AIMS OF THE RESEARCH

The general aim of the present thesis was to clarify the prevalence, determining factors and etiopathogenetic aspects of apical periodontitis among Estonian population.

Therefore, the specific objectives of the present thesis may be summarized as the following:

1. To describe the prevalence of apical periodontitis for the first time in Estonia by using panoramic radiographs.
2. To specify the determining factors of apical periodontitis in Estonian population and to find a correlation between different dental diseases/previous dentistry and signs of apical periodontitis on panoramic radiographs.
3. To identify dental microbial communities as main etiologic factor for endodontic pathologies using next generation sequencing method (Illumina sequencing).
4. To compare oxidative stress levels in the saliva and root canal content of patients with different endodontic pathologies and of healthy subjects.

MATERIAL AND METHODS

4. Subjects and study design

The current thesis is based on the results of three studies to get more thorough understanding of AP. The **first study** of the thesis (Paper I) was conducted as a cross-sectional study on the panoramic radiographs and focused on the epidemiology of the AP in Southern Estonian population. In this study the prevalence of AP was ascertained, and the determining factors of AP were elucidated. The **second study** of the thesis (Paper II) investigated the microbial aspect as the main causative factor of the AP. The **third study** of the thesis (Paper III) investigated the biochemical aspects of AP and the related more systemic bodily reactions.

In general, subjects included into the investigations had to have endodontic pathology. In addition to diseased patients, the subjects without any kind of local or systemic pathologies were also added as control group (Table 5).

Table 5. Study subjects and objects

Study	Subjects	Type of investigation
Prevalence of AP in Southern Estonia **(Paper I)**	Total of 6552 panoramic radiographs of 6552 subjects	Radiological evaluation of the panoramic radiographs, investigating presence or absence of periapical radiolucencies
Determining factors of AP in Estonian population **(Paper I)**		Radiological evaluation of the panoramic radiographs , investigating presence or absence of dental hard tissue diseases and previous dentistry
Investigation of root canal microbiota (**Paper II**)	Total of 12 patients with endodontic pathologies	Clinical investigation and microbiological investigation using next generation sequencing
Biochemical investigation of root canal and saliva (**Paper III**)	Total of 86 patients of which: 22 with primary apical periodontitis 26 with secondary apical periodontitis 8 with periapical abscess; 13 with irreversible pulpitis; 17 healthy controls	Clinical and biochemical investigation. Biochemical investigation of systemic (saliva) and local (root canal sample) oxidative stress included the following markers: myeloperoxidase, 8-isoprostanes and oxidative stress index

4.1. Subjects undergoing to panoramic radiographic examination

The sample for this cross-sectional study consisted of panoramic radiographs of 6552 patients, which is 0.5% of the total Estonian population and about 7% of the population of Tartu. The initial sample contained 6574 radiographs but 22 duplicates were excluded before evaluation. All the panoramic radiographs were taken for the first time at the radiology department at the Clinic of Dentistry, Tartu University Hospital, Estonia between November 2010 and May 2012 only for the consultation and/or treatment purposes. Since this is the only higher lever dental clinic in South Estonia, it draws a large amount of patients from all over Estonia, especially the southern part.

4.2. Subjects attending to root canal treatment

The study material was collected from patients who were seeking consultation and/or dental treatment from the Tartu University Dental Clinic or Kaselo Private Dental Clinic in Põltsamaa, Estonia.

4.2.1. Participants of microbiological study

The subject population was composed of 12 antibiotic-naive consecutive patients (ages ranging from 27–66 years) attending the Clinic of Stomatology at the University of Tartu, Estonia (Paper II Table 1) who met the criteria and agreed to participate in the study. Patients came to the clinic for root canal treatment or tooth extraction between September 2010 and April 2011. Thorough anamnesis (systemic and local diseases, previous treatment, hygiene habits, allergies), intraoral status, and periapical radiographs were taken, which were all necessary for the upcoming treatment. All the patients had to go through meticulous clinical examination (5.1 Clinical examination). Twelve subjects included in this study were divided under three groups as follows: five subjects with pAP, three subjects with sAP, and four subjects with periradicular abscess (evolved from AP).

All subjects who received antibiotic or anti-inflammatory therapy in the previous 6 months were excluded. None of the sampled teeth presented posts, crowns, or bridges. Inclusion and exclusion criteria are presented in Table 6.

4.2.2. Participants of biochemical study

The patients with endodontic pathology and the healthy controls were recruited between 09. January 2015 and 19. August 2016 from the Clinic of Dentistry at Tartu University Hospital, Estonia and a private dental clinic Kaselo Dental Clinic in Põltsamaa, Estonia. There was no difference between the patients

since adult patients have to pay for their dental care in Estonia in both settings. In addition, the inclusion and exclusion criteria were similar in both clinics (Table 6) and all the patients were recruited by the same dentist (V.V.). Altogether 124 subjects were initially recruited. From 86 subjects sufficient amount of specimens for all biochemical studies was available, therefore final study group included 86 subjects, of which 69 with and 17 without endodontic pathology (Figure 5; Paper III Table 1).

4.3. Subjects attending the extraction of healthy control teeth

The analyzed 17 control teeth in biochemical study were totally intact wisdom or premolar teeth that were extracted due to prophylactic or orthodontic indications from generally healthy people aged 19–41 years (Figure 5; Paper III Table 1).

Table 6. Inclusion and exclusion criteria for microbiological and biochemical studies

Endodontic treatment groups				**Control group**
pAP group	**sAP group**	**Pulpitis group**	**Abscess group**	
Inclusion criteria				
Age at least 10 year old				
Tooth with AP lesion wo RCF	Tooth with AP lesion with RCF	Tooth wo AP lesion	Tooth with AP lesion	Excellent oral health
Objective and subjective criteria for AP		Objective and subjective criteria for pulpitis	Objective and subjective criteria for abscess of endodontic origin	
Lesion on radiograph with PAI >2		No lesion on radiograph (PAI<2)	Lesion on radiograph with PAI >2	Intact teeth
Excellent general health			Good general health	Excellent general health
Exclusion criteria				
Age <10 y				
Diseased primary teeth				Bad oral hygiene
Vertical root fracture				Any kind of tooth disease
Horizontal root fracture				Any kind of gingival or periodontal disease
Tooth with endo-perio lesion				Restored teeth
Tooth with perio-endo lesion				Any kind of general acute disease

Endodontic treatment groups				Control group
pAP group	**sAP group**	**Pulpitis group**	**Abscess group**	
Other conditions and clinical situations that require RCT				Any kind of chronic general disease
Tooth with gingival pocket more than 3,5mm				Any kind of infectious disease in present or past history
Unrestorable tooth (deeply fractured or highly carious tooth which is impossible to restore and therefore must be extracted)				
Tooth that has undergone apical surgery with or without retrograde filling			Tooth that has undergone apical surgery with or without retrograde filling	
Any type of smoking or tobacco abuse				
Antibiotic or any other medicament usage in previous 3 months				Antibiotic or any other medicament usage in previous 6 months
Diabetes type I or II				
Immune suppression (HIV, AIDS, chemotherapy)				

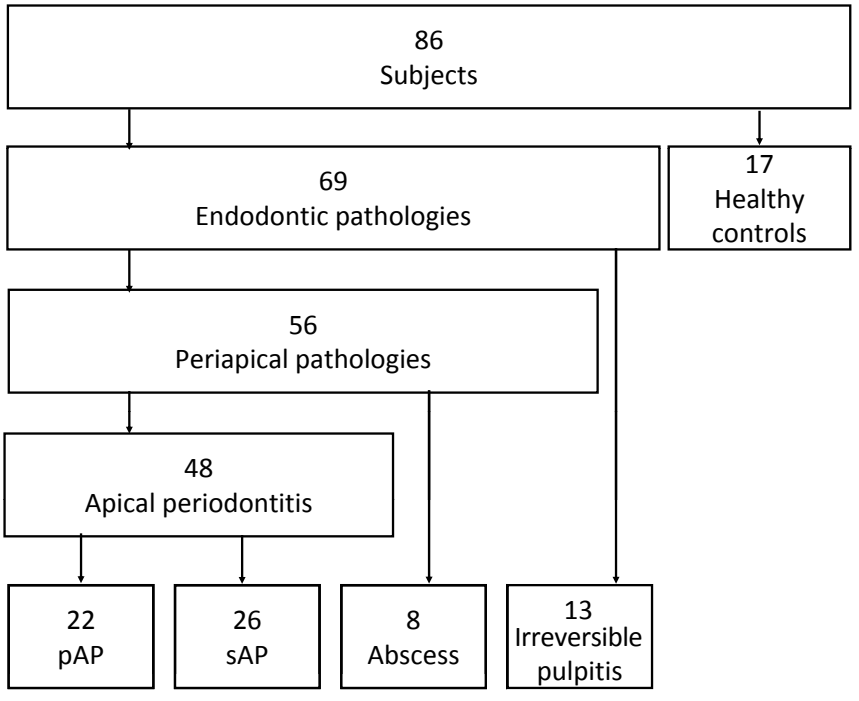

Figure 5. Participants of biochemical study.

5. Methods

5.1. Clinical examination

Clinical examination consisted of taking anamnesis and conducting different clinical tests (Hargreaves *et al.,* 2016) after which the patients could be categorized into the certain clinical study groups.

5.1.1. Anamnesis

Clinical examination started with taking meticulous anamnesis from study subjects. Written anamnesis was collected using self-evaluation autoanamnesis chart. Written charts and oral questioning included general anamnesis, general oral and dental anamnesis, intra- and extraoral *status praesens*, complete dental anamnesis including cariology, periradicular diseases and mucosal diseases.

General anamnesis included: gender, age, family status, occupation, general health, present or previous illnesses, medicine intake, systemic harmful habits like tobacco, alcohol, carbohydrates, drugs, food supplements, local harmful habits like nail biting or writing instrument chewing, lip licking etc.

Oral and dental anamnesis included: chief complaint if present, oral and/or dental pain or discomfort, known previous dentistry, oral hygiene habits including brushing and flossing habits, fluoride intake, present or previous traumas in head and neck region, present or previous inflammation in oral region, previous or present orthodontic treatment, previous or present prosthetic treatment, previous or present periodontal treatment, previous or present cariologic dental treatment with fillings.

Present extraoral status included: facial asymmetry, skin structure and changes, extraoral palpation of lymph nodes, temporomandibular joints and masticatory muscles etc.

Present intraoral status included: lips, mucous membrane, gingiva, bite registration by Angle classification, general tooth wear etc.

Dental anamnesis included: presence or absence of any kind of dental disease or previous dental treatment (caries, filling, root canal treatment, orthodontic appliances, prosthetic fixed and/or removable appliances etc.).

5.1.2. Clinical tests

Pulp vitality tests: thermal tests were performed using cold, hot; and electric pulp test.

A cold test was performed with Endo-Frost (50ºC) (*Roeko, Langenau, Germany*) and a cotton pellet (size 00) (*Roeko*); the frozen cotton pellet was held on the isolated and dried tooth on the restoration-free surface for about 2–5 seconds or until discomfort or pain was felt.

The hot test was performed using silicone polisher (*HiLuster; Kerr Corp, Orange, CA*) with a 1:1 contra-angle handpiece (*W&H, Bürmoos, Austria*)

without air and water cooling by touching the restoration-free tooth surface with about 4000 rpm for about 5 seconds or until the patient felt discomfort or pain.

The electric pulp test or the vitality test was performed with the Elements Diagnostic Unit (*Sybron Endo, Orange, CA*) according to the manufacturer's instructions or until pain was felt. The probe was touched to the restoration-free part of the isolated tooth until discomfort or pain was felt.

Periapical tests including percussion and palpation were used.

A percussion test was performed with a mirror handle using gentle and uniform tapping on the occlusal and horizontal side of each tooth, and sound teeth were registered as zero feeling; the same kind of tapping was performed on the accused tooth, and the feeling of the patient was compared and described.

The palpation test was performed using uniform and solid pressure with the right index finger on the tip area of the root on both sides of the alveolar bone; the tooth was palpated by applying pressure on the tooth both vertically and horizontally. This was done bilaterally on both sides of the jaw to consider anatomic differences.

5.2. Radiographic examination

5.2.1. Radiographic examination of patients participating in microbiological and biochemical studies

Radiological analyses were conducted by taking periapical radiograph from accused teeth. The images were taken by an experienced radiologist or experienced dentist (V.V.) using Kodak 2100 Intraoral X-Ray System (*Carestream Dental Rochester, NY, USA*) with the RVG 6100 sensor (*Carestream Dental LLC, Atlanta, GA*) at a parallel angle with RINN yellow (posterior) or blue (anterior) sensor holder (*Dentsply Rinn, Elgin, IL*). PA radiographs were analyzed using the Trophy DICOM program (*Carestream Dental LLC, Atlanta, GA*).

Abnormalities were registered and results were written in anamnestic charts. Periapical index (PAI) was determined as described previously (Østravik *et al.,* 1986) using a 5-point scale designed to determine the absence, presence, or transformation of a diseased state (Table 7).

In addition, patient dental charts were used in case of sAP patients to assure that previous root canal treatment (RCT) in the accused tooth was performed more than 4 years ago.

Table 7. Description of Periapical Index scores (Østravik *et al.,* 1986)

PAI Score	Description of Radiographic findings
1	Normal Periapical Structures
2	Small changes in Bone Structures
3	Change in Bone Structure with Mineral Loss
4	Periodontitis with well–defined radiolucent area
5	Severe periodontitis with exacerbating features

5.2.2. Radiographic examination of patients participating in prevalence study

All radiographs were carried out by the same experienced dental radiologist using a panoramic digital radiography device Planmeca ProMax 3D Plus (*Planmeca Oy, Helsinki, Finland)* with imaging values between 54–96 kV and 1–14 mA, depending on the subject's size. For assessment of the radiographs, a HP 22uh 21.5-inch LED Backlit Monitor 1920 x 1080 @ 60 Hz using Romexis Imaging Software *(Planmeca Oy, Helsinki, Finland)* was used. For better assessment the observers were able to use all software image enhancement functions whenever they felt the necessity for it.

At first the author of this dissertation examined all radiographs. All teeth were recorded according to FDI nomenclature (Leatherman, 1971). In all teeth the periapical and coronal status as well as previous treatment and its quality were recorded (Table 8).

To identify the teeth with AP only the presence or absence (strict rule) of periapical radiolucency was used (Strindberg, 1956; Ng *et al.,* 2008). The root with the lowest quality of treatment was included into study in case of multirooted teeth (Ng *et al.,* 2007). Thereafter the second observer from department of oral and dental diseases examined all radiographs. She also filled all the variables for all the teeth in all radiographs the same manner as first observer. In cases of disagreement the third experienced observer resolved the discrepancy.

Table 8. Evaluation criteria for radiographic analysis

Evaluation criteria	Coded- value
Periapical pathology (PA)	0 – without PA radiolucency (PAI ≤ 2)
	1 – visible PA radiolucency (PAI >2)
Method of RCT	0 – pulpotomy
	1 – pulpectomy
RCF length	0 – adequate (no more than >2mm shorter from radiographic apex)
	1 –shorter than >2mm from radiographic apex
	2 – overfilled RCF
RCF density	0 – adequate (dense and homogenous in all aspects of RCF)
	1 – inadequate (sparse, voids, gaps in RCF or between RCF and RC)
Type of restoration	0 – missing restoration
	1 – filling
	2 – crown
	3 – bridge abutment tooth

Evaluation criteria	Coded- value
Presence of caries	0 – no visible caries
	1 – primary caries 2 – secondary caries
Post in RC	0 – no post
	1 – fiber post
	2 – prefabricated screw or post
	3 – cast post and core
Quality of prosthetic restoration	0 – adequate (no gap, no overhanging)
	1 – inadequate (gap between core and crown, overhanging crown)

The following parameters were also evaluated:

- presence, type and situation of dental implants;
- presence, type of orthodontic appliances;
- presence, angulation, level of eruption of wisdom teeth;
- presence, coronal and radicular situations of all deciduous teeth.

5.2.3. Calibration of observers

Calibration of observers was carried out on a selected set of 104 panoramic radiographs from an unpublished pilot study from the same investigation group. The inter-observer agreement scores gave Kappa-value of 0.51 for presence of periradicular radiolucency, 0.55 for length of root canal filling (RCF), 0.44 for quality of RCF density, 0.56 for quality of coronal fillings, 0.63 for quality of crowns. Because the inter-observer agreement was moderate (0.44–0.63), a highly experienced dentist served as the third observer (M.S.) for cases where disagreement occurred.

5.3. Sample collection

5.3.1. Microbiological study

Samples were collected from each of the 12 teeth under strict aseptic conditions as described previously (Fouad, 2009. Briefly, the tooth was cleaned with pumice and isolated with a rubber dam. The tooth and the rubber dam were cleaned with a solution of 3% hydrogen peroxide and then disinfected with 2.5% sodium hypochlorite (NaOCl) solution. The coronal access was made with the use of sterile round burs without water spray. The pulp chamber and the operatory field were disinfected again using a swab soaked in 2.5% NaOCl. This solution was inactivated with sterile 5% sodium thiosulfate. Samples were

collected from the root canal by means of a sterile #08-25 H-type file (*Dentsply Maillefer, Ballaigues, Switzerland*) with a firm filing motion introduced as apically as possible but 1 mm short of the apical foramen. This length was determined by means of a periapical radiograph and a sterile plastic ruler (*Dentsply Maillefer*) and apex locator (*Root ZX, Morita, Japan*). Subsequently, 1–4 sterile paper points were introduced in the root canals at about the same level of the file, and each was left in place for 20 seconds to soak up the fluid. Both the file and paper points were then transferred to Eppendorf tubes containing 1 mL Brucella broth (*Oxoid, Basingstoke, UK*) as the transport medium. Samples were transported to microbiology laboratory within 2 hours for immediate processing. Thereafter the samples were frozen to –80 °C and kept in sterile Eppendorf tubes for further research.

5.3.2 Biochemical study

Resting saliva samples were collected and handled before clinical treatment as described previously (Salimetrics LLC, 2015). Whole saliva samples were obtained before clinical treatment. During the saliva collection the subjects were requested not to eat and drink (except water) or chew gum. After rinsing the mouth with tap water, unstimulated saliva was collected by expectoration into sterile polypropylen tubes with the patient's head tipped forward and the nose pointing to the floor (Wolfram *et al.,* 2006). The time period of sample collection was recorded in minutes and the collection time did not exceed five minutes (Arunachalam, 2015). All procedures related to collection of saliva specimens were performed by the same investigator/dentist. Before the biochemical analyses the samples were unfrozen and centrifuged for 15 minutes at 3000 rpm and the supernatant removed for immediate analysis. All procedures related to the collection of saliva specimens were performed by the same investigator/dentist.

RC content samples were collected under strict aseptic conditions as described previously in paragraph 5.3.1. In addition, exactly 6 ling stokes from each tooth (RC) were done. If a tooth had more than one root, sampling was done from the more diseased root. Remnants of the pulp, tooth tissue, and/or previous root filling material were collected using the Hedström file (*Dentsply/ Maillefer, Ballaigues, Switzerland*). The file was advanced to the working length – the "0" mark in the electronic apex locator (*Root ZX; Morita, Osaka, Japan*) minus 0.5 mm. The file was thereafter inserted into an Eppendorf tube containing 1 mL of phosphate-buffered saline as a transport medium. The tube was then vortexed until all the remnants from the file were loose, after which the file was immediately removed from the tube.

Saliva and pulp samples for the measurement of 8-isoprostanes (8-EPI) were stored at –80°C in the presence of 0.005% butylated hydroxytoluene (10 μL of 5 mg/mL solution in ethanol per 1 mL sample) to avoid auto-oxidation before assay using the chosen method (kit no 516351; Cayman Chemical, Ann Arbor,

MI, USA) (Dahl & Van Breem, 2010). The saliva and pulp samples for other biochemical analyses were frozen and stored at –80 °C until the analysis. Samples were thawed before the biochemical analyses, homogenized, and centrifuged for 15 minutes at 3000 rpm, and the supernatant removed for immediate analysis.

5.4. Microbiological analyses

Genomic DNA was extracted from the samples using the DNA QIAamp DNA Mini Kit (*Qiagen, GmbH, Germany*) according to the manufacturer's instructions and stored at –80 °C.

The samples were characterized by profiling the microbial community on the basis of the 16S rRNA gene by using the Illumina HiSeq2000 sequencing combinatorial sequence-tagged PCR products. Forward (5'-CAACGCGARG AACCTTACC-3') and reverse (5'-ACAACACGAG CTGACGAC-3') primers were used to amplify the bacterial-specific V6 hypervariable region of the 16S rRNA gene (Gloor *et al.,* 2010). Taxonomic identification of sequences was performed with the RDP Classifier using Greengenes and HOMD 16S rRNA RefSeq (Human Oral Microbiome Database). In total 43969 sequences were obtained with an average of 3140 reads per sample. Details of the sequencing method and data analysis are provided in Paper II Table S1.

5.5. Biochemical analyses

5.5.1. Oxidative stress index (OSI)

OSI is an indicator of the redox balance between oxidation and antioxidation. It is expressed as the ratio of total peroxide concentrations (TPX) to total antioxidant capacity (TAC) (Halliwell & Gutteridge, 2015). An OxyStat colorimetric assay kit (*Biomedica, Wien, Austria; cat no BI-5007*) was used for measuring TPX as described (Salum *et al.,* 2013). TAC was measured using a colorimetric assay (Erel, 2004) based on the decolorization of 2,2′azinobis(3-ethylbenzothiazoline-6-sulfonate) radical action. Decolorization by antioxidants according to their concentration and antioxidant capacity was measured as the change in absorbance at 660 nm. The assay was calibrated using Trolox (a soluble vitamin E analog). TAC values were expressed as an equivalent of the millimolar concentration of Trolox solution.

5.5.2. Myeloperoxidase (MPO)

Plasma levels of MPO were measured using the MPO-ELISA kit (BioCheck, Inc., Foster City, CA, USA; cat no 1129). The assay utilized a unique monoclonal

antibody directed against a distinct antigenic determinant on the MPO molecule. This monoclonal anti-MPO antibody was used for solid phase immobilization (on the micro-titre wells). Another mouse monoclonal anti-MPO antibody conjugated to horseradish peroxidase was in the enzyme conjugate solution. The test samples were allowed to react sequentially with these two antibodies, resulting in MPO molecules being sandwiched between the solid phase and enzyme-linked antibodies. After two separate 90-min incubation steps at room temperature with shaking, the wells were rinsed with wash buffer to remove unbound labeled antibodies. Tetramethylbenzidine (TMB) reagent was added and incubated for 20 min with shaking, resulting in the development of color. The color development was stopped with the addition of stop solution, changing the color to yellow. The concentration of MPO was directly proportional to the color intensity of the test samples. Absorbance was measured spectrophotometrically at 405 nm. MPO level was determined using the standard curve data using a four-parameter regression model and expressed as ng/ml. For each assay, the reagents used were analytical grade, prepared each day, and stored in the refrigerator. Reagents were equilibrated at room temperature for 0.5 h before use. Levels and activities of antioxidants were measured in duplicates or triplicates. The reproducibility calculated as the coefficient of variation was <5% for all assays. No storage-related changes in enzyme activities were observed up to 6 months at −80 °C. (Shen *et al.,* 2010).

5.5.3. 8-isoprostanes (8-EPI)

A commercially available enzyme-linked immunosorbent assay (*Cayman Chemical; kit no 516351*) was used for the measurement of 8-EPI as described (Dahl & Van Breem, 2010). The assay used is based on the competition between an 8-EPI-acetylcholinesterase (AChE) conjugate (8-EPI tracer) and 8-EPI for a limited number of 8-EPI-specific rabbit antiserum binding sites. The concentration of 8-EPI varies while the concentration of 8-EPI tracer is kept constant. The amount of 8-EPI tracer capable of binding to the rabbit antiserum is inversely proportional to the concentration of 8-EPI in the well. The resulting rabbit antiserum-8-EPI (either free or tracer) complex binds to the rabbit IgG mouse mAb previously attached to the well. The plate is then washed to remove any unbound reagents, after which Ellman's reagent (containing the substrate to AChE) is added to the well. The product of this enzymatic reaction is yellow in color and absorbs strongly at 412 nm. The intensity of this color is proportional to the amount of 8-EPI tracer bound to the well.

5.6. Statistical analysis

For statistical analyses, Statistical Package for Social Sciences (SPSS), Version 17.0 (*SPSS Inc., Chicago, Illinois, USA*) and SigmaStat software (*Systat Software,*

Chicago, IL) were used. The differences between the groups in microbiological and biochemical studies were analyzed using the Kruskal-Wallis one-way analysis of variance by ranks and the Fisher exact test. A principal coordinate analysis performed in PAST software (Hammer, Harper & Ryan, Oslo, Norway) was used to visualize the similarities between samples. Spearman rank order correlation analysis was used to find associations between the biochemical markers.

Significant differences in prevalence study regarding distribution of AP and RCF by gender and age were tested using the Chi-square test. Binary logistic regression was used to analyze the associations between clinical characteristics and AP. OR with 95 CI was used as measure of effect. A confidence level of 95% and a two-sided P value of <0.05 were used to reveal significant differences.

5.7. Ethical considerations for studies

All studies were approved by the Ethics Review Committee on Human Research of the University of Tartu (protocols no 195/T-11, 234/T-6, 246/T-19, 253/T-1). All subjects of the microbiological and biochemical studies entered the study after signing an informed consent form. In the prevalence study only information about the age and gender information was available in addition to radiographs.

RESULTS AND DISCUSSION

6. Apical periodontitis in Southern Estonian population

6.1. Prevalence of apical periodontitis

6.1.1. Patients as the research objects

We evaluated panoramic radiographs of 6552 subjects ranging in age from 3 to 93 years (mean 35.5 ± 19.2 years), of them 2563 (39.1%) were male and 3989 (60.9%) were female. The mean age of our study group, 35.5 years, was similar to some other studies (Sidaravicius *et al.,* 1999; Timmerman *et al.,* 2017), but lower than those reported by Tsuneishi *et al.* (2005) (50.8 y), Georgopoulou *et al.* (2005) (48.0 y) and Huumonen *et al.* (2017) (50.2 y). Majority of the subjects (62.6%) were 10 to 44 years old and the biggest age-group (11.3%) included the patients in the age of 15–19 years (Paper I Table 1).

Of the total sample only 209 subjects (3,2%) had completely intact teeth. At the same time 54.7% (3584 patients) had AP and 58.2% (3815 patients) had root canal treatment (RCT) (Figure 6). The prevalence of AP in our study (54.7%) was higher than generally outlined in other European studies. Higher figures (63–80%) have only been reported in Belgium, Lithuania, Latvia, Austria, and Belarus in Europe (Jersa & Kundzina, 2013; Kabak & Abbott, 2005; Sidaravicius *et al.,* 1999; DeMoore *et al.,* 2000; Peršić *et al.,* 2011) while even as high as 87% in Jordan (Al-Omari *et al.,* 2011). At the same time, 58.2% of the individuals in our sample had at least one RCT tooth, a result similar to Western European countries – 56.3% in Germany (Weiger *et al.,* 1997), 59% in Spain (López-López *et al.,* 2012), 61% in Finland (Huumonen *et al.,* 2012), and 58.8% in Denmark (Kirkevang *et al.,* 2001); while studies conducted in neighboring Baltic countries (Latvia and Lithuania) reported an even higher prevalence of endodontic treatment among 35–44-year-old patients (87% and 84%, respectively) (Jersa & Kundzina, 2013; Sidaravicius *et al.,* 1999) (Paper I Table S1).

Periapical finding in endodontically treated teeth (secondary AP) was present in 2920 subjects (76.5% of subjects with previous RCT, 44.6% of total subjects), including 1357 patients with pAP and sAP, and 1563 patients with sAP and without pAP. At the same time 2021 patients (30.8% of total subjects) had AP in non-endodontically treated teeth (primary apical periodontitis, pAP), of them 664 patients with pAP and without sAP, and 1357 patients with pAP and sAP (Figure 6).

Male gender was associated with risk for AP (OR = 1.44) (Table 10). Prevalence of pAP appeared to be constantly increasing along the age while the prevalence of sAP was the highest in middle-aged people (Figure 7). The reasons are more thoroughly discussed in General Discussion.

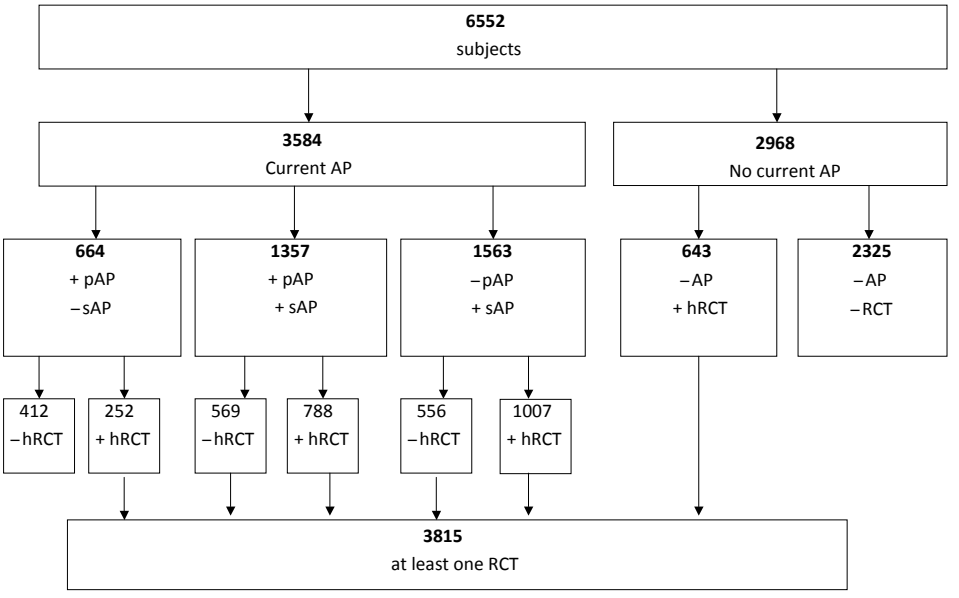

Figure 6. Overview of the study subjects in epidemiological study.

Altogether 3584 subjects with AP were investigated (of them 2021 had pAP at least in one tooth and 2920 had sAP at least in one tooth) as well as 2968 subjects without current AP.
Legend: AP, apical periodontitis; pAP, primary AP; sAP, secondary AP; RCT, root canal treatment; hRCT, healthy RCT in other teeth; +, present; −, absent.

6.1.2. Teeth as the research objects

The 6552 patients had altogether 181 495 teeth (Table 9) and the mean number of teeth per patient was 27.7 ± 2.3.

Out of 181 495 teeth, 52.7% were intact while 47.3% of teeth were impacted by caries, restoration, endodontic treatment, and/or AP. There were 36 452 teeth with visible caries (primary or secondary type or both; at the same time the caries-affected tooth could have any type of restoration, AP and/or endodontic treatment) and 49 303 teeth that had any type of restoration (filling, crown, bridge abutment etc.) (Paper I Table 2).

In total, AP was diagnosed in 6.3% of teeth (of them 4898 pAP and 6540 sAP). This figure is lower than reported in studies carried out in some other Eastern and Southeast European countries that showed results ranging from 7% in Latvia to 12,3% in Kosovo (Jersa & Kundzina, 2013; Kamberi *et al.,* 2011) but still higher than in some Western European countries where periapical lesions were detected in 2–5.2% of teeth (López-López *et al.,* 2012; Lupi-Pegurier *et al.,* 2002).

Periradicular radiolucencies were observed most often in the mandibular first molars (in 26.7% of patients) and least often in the mandibular canines (0.4% of patients). The highest prevalence of pAP was seen in lower molars (6.6%), followed by upper molars (3.0%) and lower premolars (2.5%) (Table 9).

Out of all teeth, 6.9% (12 605) were root canal treated. The most RCT teeth were the right mandibular first molars (19.9%), followed by the left mandibular first molars (19.4%) and the right maxillary first molars (15.2%). The least treated teeth were the left mandibular lateral incisors (1.0%) closely preceded by right mandibular central and lateral incisors (1.1% both) (Paper I Table S5).

Of the RCT teeth, 51.9% were associated with periradicular radiolucencies, hence, having sAP. Similarly high incidence of periradicular lesions in RCF teeth (50% to 62%) has been reported in Brazil (Siqueira & Rocas, 2005), Senegal (Touré *et al.,* 2008), Croatia (Matijević *et al.,* 2011), Palestina (Mukhaimer *et al.,* 2012) and Cyprus (Kalender *et al.,* 2013).

There was a 2.30 times higher risk for AP for the teeth of mandible than that of maxilla. Molars had 1.79 times higher risk for AP in comparison with incisors. Teeth with caries had 2.30 times higher risk for AP compared to teeth without caries.

Table 9. Distribution of teeth as concerns pAP, sAP and RCT.

		Total	Teeth Present		pAP teeth		P value	RCT teeth		P value	sAP teeth		P value
			No of teeth	%	No of teeth	% *		No of teeth	% *		No of teeth	% #	
Upper teeth	**Total**	**104832**	**90214**	**86.1**	**2011**	**2.2**	<0.001	**7305**	**8.1**	<0.001	**3163**	**43.3**	<0.001
	Molars	39312	30415	77.4	923	3.0		2656	8.7		1248	47.0	
	Premolars	26208	22364	85.3	479	2.1		2376	10.6		949	39.9	
	Canines	13104	12630	96.4	160	1.3		500	4.0		189	37.8	
	Incisors	26208	24805	94.6	449	1.8		1773	7.1		777	43.8	
Lower teeth	**Total**	**104832**	**91281**	**87.1**	**2887**	**3.2**	<0.001	**5300**	**5.8**	<0.001	**3377**	**63.7**	<0.001
	Molars	39312	28689	73.0	1904	6.6		3281	11.4		2318	70.6	
	Premolars	26208	24197	92.3	600	2.5		1563	6.5		818	52.3	
	Canines	13104	12933	98.7	108	0.8		175	1.4		81	46.3	
	Incisors	26208	25462	97.2	275	1.1		281	1.1		160	56.9	
Total		**209664 &**	**181495 ¤**	**86.6**	**4898**	**2.7**		**12605**	**6.9**		**6540**	**51.9**	

% * – pAP and RCT percentages are calculated of total number of teeth present
% # – sAP is calculated from RCT teeth
& – maximum nominal number of teeth (32 teeth x 6552 patients).
¤ – number of teeth actually present in 6552 patients (including wisdom teeth).
AP – apical periodontitis; pAP – primary AP; sAP – secondary AP; RCT – root canal treatment.

Table 10. Associations between clinical characteristics and AP (Odds Ratios [OR] and 95% confidence intervals [CI] are presented).

Variables		OR	*P*	95% CI
Length of root filling	0–2mm from radiographic apex	1.00		
	More than 2mm short from the radiographic apex	1.76	≤0.001	1.62 – 1.91
	Extrusion of material through the apex	2.51	≤0.001	2.12 – 2.97
Density of root filling	Homogenous	1.00		
	Inhomogenous	1.61	≤0.001	1.47 – 1.76
Restoration	No restoration	1.00		
	Filling	0.45	≤0.001	0.39 – 0.52
	Crown	0.34	≤0.001	0.29 – 0.41
	Bridge	0.33	≤0.001	0.27 – 0.40
Quality of prosthetic restoration	Adequate	1.00		
	Inadequate	1.63	0.020	1.45 – 1.81
Post in root canal	No post	1.00		
	Fiber post	0.95	NS	0.90 – 1.24
	Prefabricated	1.68	NS	0.89 – 2.89
	Cast post	1.34	NS	0.69 – 2.35
Caries	Absent	1.00		
	Present	2.30	≤0.001	2.14 – 2.48
Orthodontic appliance	Absent	1.00		
	Present	0.74	NS	0.42 – 1.34
Tooth localization	Maxilla	1.00		
	Mandibula	2.30	≤0.001	2.14 – 2.47
Tooth type	Incisor	1.00		
	Canine	0.79	0.012	0.67 – 0.95
	Premolar	0.97	NS	0.87 –1.08
	Molar	1.79	0.000	1.62 – 1.98
Gender	Female	1.00		
	Male	1.44	≤0.001	1.34 – 1.55

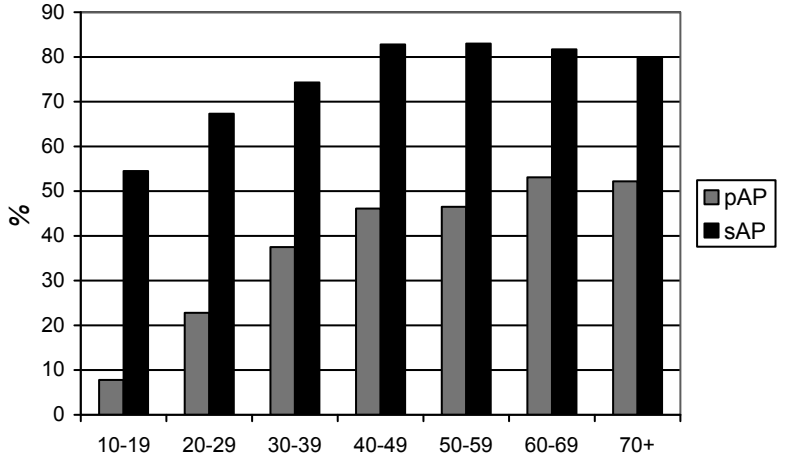

Figure 7. Prevalence of AP in different age groups.

6.2. Quality of root canal treatment

Out of the 12 605 endodontically treated teeth, 11 956 (94.8%) were treated using pulpectomy and 649 teeth (5.2%) were treated using the pulpotomy method (Paper I Table 3). Root canal filling (RCF) length and homogeneity were measured only in pulpectomy group while coronal restoration was assessed in all endodontically treated teeth. Of the pulpectomy teeth, 48.6% teeth were without AP while of the pulpotomy teeth, only 13.5% teeth were without AP. In total, 51.9% of endodontically treated teeth presented with AP. This coincides with previous knowledge that root canal filling *per se* is an important risk indicator for AP (Kirkevang & Wenzel, 2003).

However, root canal treatment quality is considered a key factor for the health of periradicular tissues (Strindberg, 1956; Sjögren *et al.,* 1990) and radiographic evidence of lower quality RCF's generally contributes to an increase in AP (Khabbaz *et al.,* 2010). In our study, shorter RCF than adequate was associated with 1.76 times higher risk for AP; while overfilled root canals were associated with 2.51 times higher risk for AP (Table 10). There was 1.61 times higher risk for AP if the RCF was not dense and there were visible voids. There was 2.22 times lower risk for AP in the presence of coronal direct filling compared to no restoration at all (OR = 0.45). There was 2.94 times lower risk for AP if the tooth was restored with crown compared to no restoration at all (OR = 0.34) and 3.03 times lower risk for AP if the tooth was restored with bridge compared to no restoration at all (OR = 0.33). In crowned and bridge retainer teeth also the quality of restoration in terms of gaps or overhangs was evaluated. Inadequate prosthetic restorations increased the risk for AP by 1.63 times while presence of a post in root canal was not a risk factor for AP.

7. Microbiota of root canal in patients with apical periodontitis

We evaluated the microbiological profile in twelve AP and periapical abscess (PA) patients using the next-generation sequencing Illumina platform. Clinical data of the patients are presented in Paper II Table 1. The study revealed highly polymicrobial communities in the root canal samples of all diagnoses groups. One sample contained 30–70 different OTU's, the mean (±SD) was lower in pAP group (36±4) than in PA group (45±4) and sAP group (43±13) ($p<0.05$). Paper II Figure 2 illustrates the clustering of microbial community data in different patient groups revealing the most remarkable differences between the communities with pAP diagnosis while sAP and abscess communities were more uniform. Some previous studies have revealed that the bacterial communities in primary endodontic infections are more diverse than those in persistent infections (Siqueira & Rocas, 2009; Chugal *et al.,* 2011), but our study did not confirm aforenamed association.

One sample contained 5–8 (mean 6.5) phyla of bacteria most prevalent being *Firmicutes and Bacteroidetes* that was similar to data described by Nobrega *et al.* (2016). In addition, *Actinobacteria, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes* and *Synergistetes* were also present in most of the samples (Figure 8). This coincides with previous data (Li *et al.,* 2010; Gomez *et al.,* 2015; Yun *et al.,* 2017).

The detected genera and species are presented in Table 11. The communities were individually different but anaerobic bacteria predominated as a rule. We reported many of the known root canal pathogens (Nobrega *et al.,* 2016; Rôças *et al.,* 2011), among others Gram negative anaerobes *Prevotella* sp., *Porphyromonas* sp., *Fusobacterium* sp., *Tannerella* sp. and *Pyramidobacter piscolens* as well as Gram positive *Dialister* sp., oral spirochete *Treponema socranskii, Solobacterium moorei* and many others. As expected from other studies (Pereira *et al.,* 2017) our study revealed known periapical pathogens like *Dialister pneumosinthes* and *Fusobacterium* spp in all samples.

We also noted increased species count as well as appearance of *Enterococcus faecalis* after treatment failure – this species was found only from sAP patients. The latter coincides with the data of Henriques *et al.* (2016) where they found *E. faecalis* only in residual population. *E. faecalis* is Gram positive cocci that normally inhabits intestinal tract but also oral cavity and cause several opportunistic infections (Penas *et al.,* 2013). Being very resistant to environmental factors, this microorganism is hard to eliminate (Pileggi *et al.,* 2013).

It has been also shown that several difficult-to-culture or nonculturable bacteria may be involved in treatment failure – like *Solobacterium moorei,* which in our study was found in all study groups, being present in 8 samples out of 12. The same implies for *Oribacterium* sp. which was present in 11 samples out of 12 being absent only in one pAP sample, and several others like

Olsenella sp and *Renibacterium* sp (Sakamoto *et al.,* 2008), which were also found from our samples.

At the same time we also revealed some novel OTUs that have not been associated with apical periodontitis like TG5, *Gardnerella vaginalis,* and *Janthinobacterium lividum*. TG5 is a genus from the phylum Synergistes belonging to cluster A group which is previously described as a potential pathogen of primary apical periodontitis (Fernandes *et al.,* 2014). *Gardnerella vaginalis* is typically found from genital samples and associated with bacterial vaginosis, however, it has been also found in case of osteomyelitis, retinal vasculitis, acute hip arthritis and other infections. Like *G. vaginalis,* also the *Janthinobacterium lividum* was found from the samples of all patient groups. One abscess sample displayed significantly high proportion (40%) of this Gram negative aerobic rod that is a major constituent of the human skin microbiota and is able to produce anti-bacterial, anti-viral, and anti-fungal compound violacein (Marton & Kiss, 2014). This may help to explain low proportions of other bacteria in this sample.

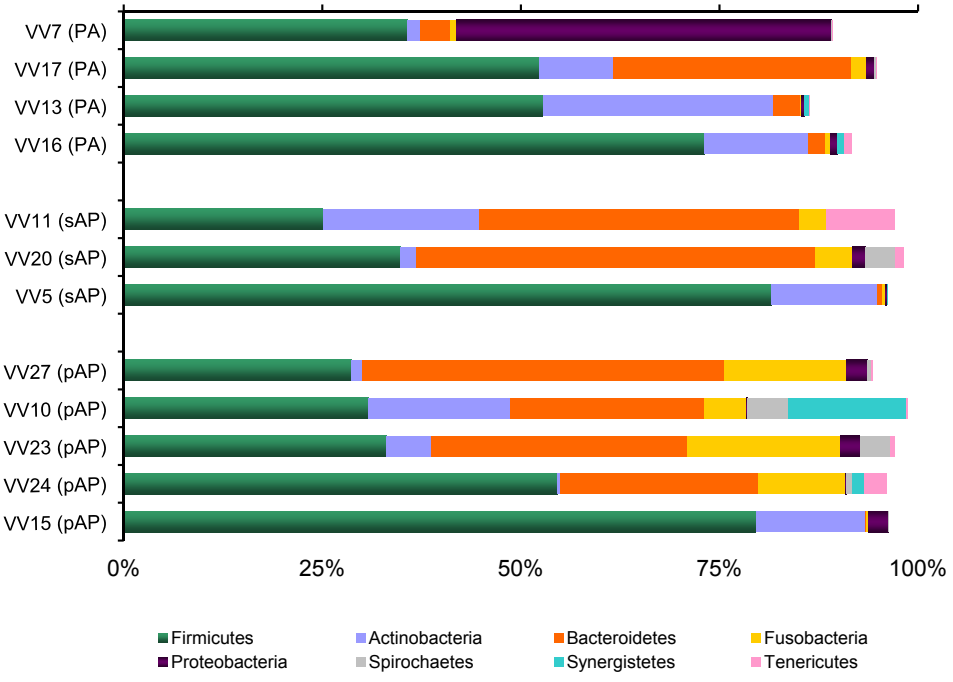

Figure 8. Microbial communities in root canals in case of AP and periapical abscess. Relative abundances of different phyla in particular teeth are presented. Each column represents one root canal of one tooth.

pAP, primary apical periodontitis; sAP, secondary apical periodontitis; PA, periapical abscess.

Table 11. Bacteria in root canals in case of pAP, sAP and periapical abscess

Phylum	Genus	Species	pAP (n=5)	sAP (n=3)	Periapical abscess (n=4)
Actino-bacteria	*Atopobium*		3	3	4
	Atopobium	*A. vaginae*	1	1	1
	Bifidobacterium		2	2	4
	Corynebacterium	*C. matruchotii*	3	1	3
	Corynebacterium	*C. amycolatum*	1	0	1
	Gardnerella	*G. vaginalis*	1	1	2
	Kocuria	*K. palustris*	1	0	1
	Olsenella		2	3	4
	Renibacterium		3	1	1
	Scardovia	*S. inopinata*	1	0	3
	Fam *Coriobacteriaceae*		1	0	2
Bacte-roidetes	*Flavobacterium*	*F. succinicans*	1	1	1
	Porphyromonas	*P. endodontalis*	2	1	3
	Porphyromonas	*P. gingivalis*	1	1	2
	Prevotella	*P. intermedia*	2	1	2
	Prevotella	*P. oris*	5	3	2
	Prevotella	*P. baroniae*	2	1	1
	Prevotella		4	3	4
	Prevotella	*P. nigrescens*	2	3	1
	Prevotella	*P. multiformis*	2	3	3
	Prevotella	*P. tannerae*	2	2	4
	Tannerella	*T. forsythia*	1	2	1
Firmi-cutes	*Anaeroglobus*		4	3	3
	Dialister	*D. invisus*	5	3	4
	Dialister	*D. pneumosintes*	5	3	4
	Enterococcus	*E. faecalis*	0	1	0
	Filifactor		3	3	3
	Lactobacillus		2	2	3
	Lactobacillus	*L. crispatus*	2	3	3
	Lactobacillus	*L. iners*	4	2	3
	Lactobacillus	*L. zeae*	3	1	2
	Lactococcus	*L. lactis*	2	0	1
	Mogibacterium		4	3	3
	Moryella		2	1	1
	Moryella	*M. indoligenes*	1	2	1
	Oribacterium		4	3	4

Phylum	Genus	Species	pAP (n=5)	sAP (n=3)	Periapical abscess (n=4)
	Peptostreptococcus		2	2	1
	Pseudoramibacter	*P. alactolyticus*	5	3	4
	Selenomonas	*S. noxia*	3	2	3
	Shuttleworthia	*S. satelles*	4	3	2
	Staphylococcus	*S. epidermidis*	1	1	2
	Streptococcus	*S. infantis*	5	3	4
	Streptococcus		2	2	4
	Veillonella	*V. parvula*	4	1	4
	Fam *Veillonellaceae*		5	3	4
Fuso-bacteria	*Fusobacterium*		5	3	4
Proteo-bacteria	*Campylobacter*	*C. rectus*	2	2	3
	Desulfobulbus		2	0	3
	Janthinobacterium	*J. lividum*	3	1	1
	Fam *Enterobacteriaceae*		1	2	1
Spiro-chaetes	*Treponema*	*T. socranskii*	5	2	3
Syner-gistetes	*Pyramidobacter*	*P. piscolens*	2	0	2
	TG5 group		2	2	1
Teneri-cutes	*Bulleidia*	*B. extructa*	0	2	2
	Solobacterium	*S. moorei*	4	2	3

8. Oxidative stress in patients with apical periodontitis

The study included a total of 86 subjects (50 female, 36 male) with mean age 38.7±14.0 years (youngest 18, oldest 83 years). Of the 86 subjects, 17 were controls while 22 subjects presented with pAP, 26 with sAP, 8 with periapical abscess and 13 with pulpitis (Paper III Table 1).

From all subjects in the study different clinical and radiological studies were obtained including pain anamnesis and periapical index (PAI). Oxidative stress (OxS) levels in the endodontium (local OxS) and saliva (systemic OxS) were assessed using three different biochemical markers: myeloperoxidase (MPO), 8-isoprostanes (8-EPI), and oxidative stress index (OSI).

Significant differences between the groups were observed (Figure 9). The highest MPO and 8-EPI levels were detected in case of pAP and pulpitis, both in the endodontium and saliva, while both markers tended to display lower values in sAP and abscess patients. The highest levels of OSI in root canal samples were seen in pAP and abscess patients; in saliva it was highest in both AP groups. Free lipid concentrations are low in saliva because lipids are bound with proteins, and they are even lower in the endodontium, making them the probable cause of the highly variable OSI values (Tomita *et al*., 2008). In addition, the TAC value may be compensatorily higher in case of pulpitis (Tothova *et al*., 2015), leading to the lower OSI index values in these patients. Differentially from all other groups, the control group showed the lowest levels of all OxS markers, both in endodontium and saliva.

Inflammation is always associated with the generation of ROS at the cellular level as well as in biofluids. OxS levels depend on one hand on the bacterial infection-related inflammation and on the other hand, the protective power of the antioxidative system. Former studies have revealed that elevated OxS levels accompany many different inflammatory conditions (Lugrin *et al.,* 2014). At the same time the OxS levels have been seldom investigated in case of oral diseases, especially AP. Dezerega *et al*. (2012) have described the pro-oxidant status in apical lesions of AP patients while Inchingolo *et al.* (2014) have investigated the influence of endodontic treatment on systemic OxS. Both studies indicated association between AP and OxS. At the same time there is no other study investigating the OxS in case of AP both at local and systemic levels.

The study also revealed that OxS was significantly associated with some important clinical markers, such as pain and periapical index (PAI) (Paper II Figure 2). Patients with pain had significantly higher OxS levels in both the endodontium and saliva in comparison with pain-free subjects (Table 12).

8-isoprostanes (8-EPI) are the major relatively stable end-products of ROS-mediated prostanoid oxidation. They are released in response to cellular activation, circulate as a free form or as esters in phospholipids in plasma and excreted in urine. 8-EPI is acknowledged by the Society of Free Radical Research round-table sessions as the best marker for the assessment of systemic

oxidative stress, allowing one to establish oxidative stress-driven lipid peroxidation as well as nucleic acid damage (Musiek *et al.,* 2006).

Isoprostanes are relevant for pain due to their ability to upregulate nociceptive pathways by stimulating prostanoid receptors (Morrow, 2006). Imbalance between the production and elimination of oxygen radicals in periapical lesions has been proposed as a factor in periapical damage and bone loss in AP (Graunaite *et al.,* 2011) as revealed by PAI in our study.

Hence, OxS is an important pathogenetic mechanism in several endodontic pathologies being significantly associated with essential clinical markers like pain and periapical index.

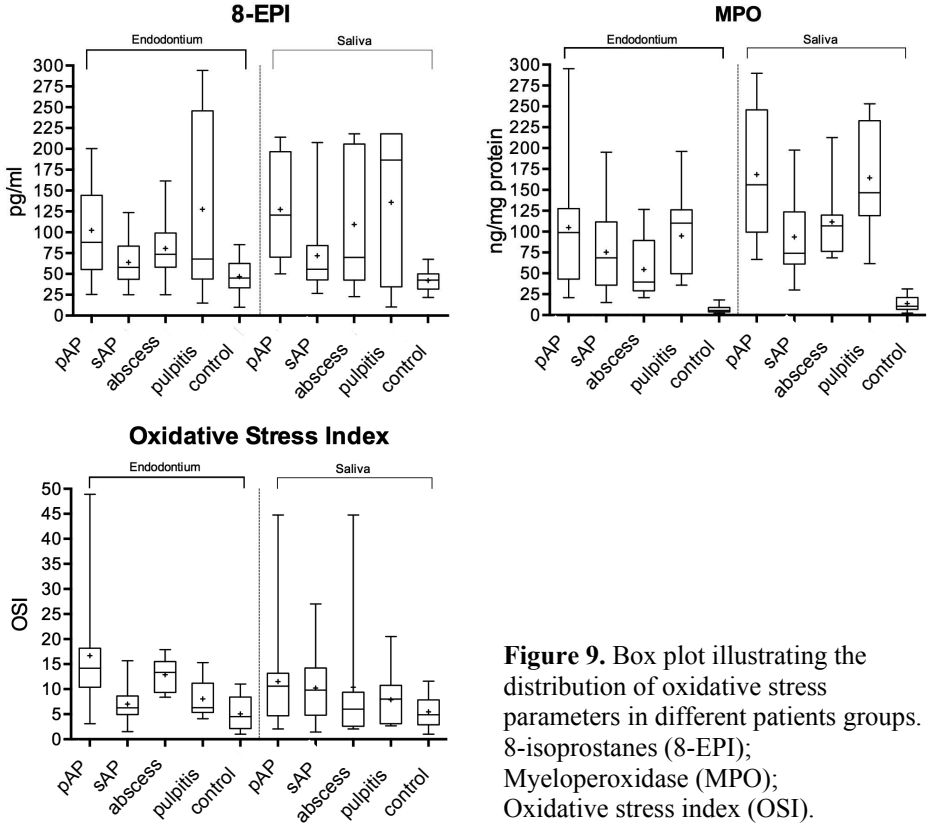

Figure 9. Box plot illustrating the distribution of oxidative stress parameters in different patients groups. 8-isoprostanes (8-EPI); Myeloperoxidase (MPO); Oxidative stress index (OSI).

Box plot explanation: upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper and lower horizontal bars outside box, range. Asterisks represent means.

Table 12. Oxidative stress parameters and PAI in presence and absence of pain

	No pain	**Pain #**	***P* value ***
Endodontium			
PAI	2.0 (1.0–4.0)	4.0 (2.0–5.0)	P<0.001
MPO (ng/mg protein)	27.9 (5.6–100.9)	72.6 (39.2–111.2)	P=0.004
OSI	6.0 (3.6–8.9)	10.4 (6.6–14.9)	P<0.001
8-EPI (pg/ml)	50.0 (36.0–67.5)	75.0 (56.1–122.4)	P<0.001
Saliva			
MPO (ng/mg protein)	34.2 (11.1–126.0	117.5 (76.0–190.9)	P<0.001
OSI	7.2 (4.1–12.0)	8.0 (3.6–10.6)	NS
8-EPI (pg/ml)	50.0 (35.0–55.0)	112.8 (60.3–210.0)	P<0.001

MPO – myeloperoxidase; OSI – oxidative stress index; PAI – periapical index; 8-EPI – 8-isoprostanes; NS – no significant.
* Mann-Whitney Rank Sum Test was used.
# Current pain or pain in recent anamnesis.

GENERAL DISCUSSION

1. Prevalence of apical periodontitis in Estonia

The prevalence of AP was evaluated for the first time in Estonia by applying a cross-sectional study. Our study revealed that about half (54.7%) of the Southern Estonian population had at least one tooth with AP. Out of all teeth evaluated, 6.3% had signs of AP. These figures are higher than in most Western European countries but lower than some other Former Soviet Union countries (Sidaravicius *et al.,* 1999; Kabak & Abott, 2005; Jersa & Kundzina, 2013,). Prevalence of subjects with RCT (58.2%) and teeth with RCT (6.9%) was nearly similar to Western European countries (De Moor *et al.,* 2000; Kirkevang *et al.,* 2006,; Lopez- Lopez *et al.,* 2012; Huumonen *et al.,* 2017). At the same time there was quite a high rate of AP in the RCT teeth (51.9%).

All these rates are related to socioeconomic status. On one hand, in better-developed countries the retaining of teeth and dental health are considered more important, therefore more RCTs are done there. Hence, to a certain point people living in more developed countries have fewer signs of AP. More retained teeth may at the same time show more signs of AP since individuals with medium income may often start but not complete the endodontic treatment. This seems to be a common case also in Estonia. On the other hand, among lower-income population the tooth extraction is more frequent option that decreases the number of teeth affected by AP. Our study group included 27.7 teeth (including wisdom teeth) as the mean. Though in Scandinavian countries the number of extracted teeth has decreased during the last decades (Bjørndal & Reit, 2004; Eckerbom *et al.,* 2007), the extraction of molars in Estonia is still quite frequent – in our study group the lower mandibular first molar was missing in 29% of patients.

Our study population was relatively young – mean age 35.5 years and the largest age groups in the study were 10–19 and 20–29 years. Individuals of these age groups tend to seek dental service more frequently. In addition, this age group is more eager to visit a dentist at the Clinic of Dentristry at Tartu University Hospital, since unlike many private clinics; the university hospital has a contract with the Estonian Health Insurance Fund, as well as a separate department of pediatric dentistry.

Our prevalence study has several strengths. It included large number of patients (n=6552), making it one of the most extensive in the field of endodontic epidemiology. The only recent study to involve a sample of comparable size was carried out in Finland (Huumonen *et al*., 2017) including a considerably smaller percentage of the total population of Finland or that of the area of Helsinki. In addition, our study included analysis of both teeth and patients, the latter being considerably infrequent in previous studies (Sidaravicius *et al.,* 1999). Moreover, the study described both endodontically treated and untreated teeth, while also indicating the proportion of intact teeth and other ancillary factors.

2. Major determinants of apical periodontitis

The **age** range in our study subjects was from 3 to 93 years (mean 35.5 ± 19.2 years), hence, representing a wide age range. The prevalence of RCF teeth and the presence of AP has been shown to increase with age (Eriksen *et al.,* 2002) and this is in line with our findings: the prevalence of AP among teeth without previous endodontic treatment (pAP) was the highest in the 60–69 age group, followed by the 70+ age group. Meanwhile, the prevalence of AP in previously endodontically treated teeth (sAP) was the highest among the middle-aged. A similar biased age distribution was also reported in previous studies (DeCleen *et al.,* 1993; DeMoor *et al.,* 2000) and may be related to better financial possibilities for seeking complex treatment among the middle-aged population, while older persons, because of their lower socioeconomic status, may be more likely to undergo tooth extraction than root canal treatment.

Preceding analogous reports have explained emerged **gender** disparity with women's higher eagerness in receiving dental care, making them less predisposed to AP (Georgopoulou *et al.,* 2005; Jiménez-Pinzón *et al.,* 2004). This idea is furthermore supported by the difference in the prevalence rate of root fillings in the present study (higher in women than in men), and different prevalence of pAP (higher in men than in women). Hence, early treatment of caries and/or pulpitis is critical for preventing AP.

Molars and premolars have been shown to be the most common RCF teeth (Tsuneishi *et al.,* 2005; Georgopoulou *et al.,* 2005) with associated AP (Kirkevang *et al.,* 2001; Loftus *et al.,* 2005). Similar results were reported in the present study, with lower molars and upper premolars requiring the most frequent treatment, followed by the upper molars and incisors. On one hand, it is easier to identify AP around mandibular molars on panoramic radiographs so it can be partly a matter of radiologic diagnostic method. On the other hand, molar teeth are the first permanent teeth to erupt. They are the most important masticatory teeth and therefore endure the largest physical force. Cumulative wear with age by combination of physical/mechanical, chemical, and microbial influences can lead to caries, pulpitis continued to periodontitis and eventually to tooth loss. Also the inner anatomy of a molar tooth is the most complicated and therefore the root canal treatment outcome may be suboptimal. There is a higher possibility that part of the RC space may retain microorganisms, their biofilm and/or necrotic pulp remnants therefore being nutrient for bacteria (Ureyen *et al.,* 2013). In our study group, mandibular molars had often been extracted – lower mandibular first molar was missing in 29% of patients. Molar teeth are infrequently needed for aesthetic reasons; in addition to the reasons listed above, smaller efforts for keeping them healthy compared to anterior teeth might therefore play a role in tooth loss (Eckerbom *et al.,* 2007). Lastly, the maxillary incisors are most commonly impaired by trauma that is also associated with apical radiolucencies (Berlinck *et al.,* 2015).

Our study also indicated that AP appears more often in the teeth of the mandible than that of maxilla. On one hand, it is easier to identify AP in the

mandible where less anatomical shadows exist but on the other hand, cortical bone lamella is usually denser in the mandibular bone that also complicates the assessment; hence, periapical lesion must be more prominent to be visible on intraoral or panoramic radiograph (Estrela *et al*., 2008; Patel *et al.,* 2009). More objective reasons for the maxilla-mandible gradient remain to be clarified.

Long-term excessive **orthodontic** forces have been previously asserted to cause a predisposition for pulp inflammation and subsequent development of irreversible pulpitis and necrosis (Vandevska-Radunovic, 1999). Orthodontic movement of teeth influences the surrounding periodontal ligament and periapical bone. On one side the bone is resorbed and from the other side the bone is under formation leading to radiological differences on both sides that can be falsely considered to be AP (deSouza et al., 2006). In our study, the presence of fixed orthodontic appliance did not increase the odds ratio for AP. Presumably, orthodontic appliances are more often worn by younger people among whom AP is in generally less prevalent. Subjects up to the age of 29 years made up 45.5% of our study group, contributing therefore greatly to this finding.

Presence of dental **caries** is a high risk factor for AP as revealed in our study. Tooth and periapical area in bone are normally sterile while destruction of tooth enamel (mostly due to caries) creates a gateway for microbes from oral environment to inside of the tooth. After the enamel is penetrated it is much easier for microbes to invade dentinal tubules and pulp space, eventually leading to AP. In addition, a carious lesion can act like a nesting place/reservoir for microbes. Even if the latter in primary stages might not directly penetrate to pulp space, microbial byproducts (endo- and exotoxins, enzymes and other bioactive molecules) by themself may also cause irritation of the pulp tissue, which can lead to pulpal necrosis and eventually to AP (Riccucci *et al.,* 2017). Therefore the prevention of caries is the utmost important factor for avoiding pulpitis and AP. Also, it is very important to fill/restore the crown of the root hermetically after root canal treatment to avoid reinfection.

3. Association of apical periodontitis with quality of root canal treatment

AP is the most frequent sequel of untreated dental caries. In vast majority of cases there are visible signs of lesions in crown portion already before the development of AP. It means that usually microbial infection migrates from crown to root direction developing AP at the periradicular area. AP is only rarely present in intact teeth, and in those rare occasions it can be explained by tooth trauma, tooth developmental problems or false positive radiological readings.

However, AP can often appear in previously treated teeth. In these cases several aspects of treatment quality have been associated to AP, including treatment type, incorrect length and density of RCF as well as poor restoration of tooth.

Treatment type is a determining factor of AP as in our study; pulpotomized teeth had greater chance of getting AP compared to pulpectomized teeth. Under the current knowledge the choice of pulpotomy should be carefully considered and used only in the situation where all the anamnestic and diagnostic methods justify its use – in case of reversible or irreversible pulpitis and the inflammation situates only in the coronal aspect of the pulp space and has not yet reached into the root canals (Alqaderi *et al., 2016*). Before the era of bioceramic materials in dentistry, the partial pulp removal had higher risk of unsuccessful treatment outcome. In our study most of the pulpotomy cases were supposedly done before the bioceramics arrival to dentistry. Even if some of the cases were done with the newest products on the market the learning curve was not on the highest level leading finally to more unsuccessful outcomes than conventional orthograde root canal therapy. Even in case of the newest knowledge, materials and methods the pulpotomy method is harder to control and might be technically more sensitive compared to pulpectomy and therefore the result of treatment might not be similarly successful (Mohapatra *et al.,* 2016). Moreover, successful pulpotomy on radiographs might be not successful clinically – the tooth pulp can be necrotic and only a step away from AP (or the AP cannot be yet visible on conventional radiograph). On the other hand, over-diagnosing must be avoided as well – pulpotomized teeth cannot be automatically considered as having AP.

Concerning the **length of RCF**, a meta-analytic study by Kojima *et al.* (2004) reported a significant difference in success rates between under- and overfilled root canal fillings. They concluded that the length of the RCF should be within 2 mm of the radiographic apex. Permanent widening of PDL has been observed around extruded RCF material (Strindberg, 1956; Ricucci *et al.,* 2016), histological analyses also show formation of granulation tissue as well as bone resorption in that area (Brynolf, 1969; Mohapatra *et al.,* 2016), and extruded filling material has been considered to cause inflammatory reactions due to persistent toxicity or bacterial contamination (Pitt Ford, 1982; Ørstavik & Mjör, 1992). However, it has been also proposed that AP is present in cases where the overfilled canal lacks proper apical seal (Siqueira *et al.,* 2014). Our study confirmed that the apical level of the RCF is strongly associated with periradicular status: in cases of correct length, 61% of the teeth showed no periradicular lesion, whereas in case of under- or overfilling, the success rate of the treatment was only 39 to 44%. Concerning **density** of RCF, we found significantly higher risk for AP if the RCF was not dense and there were visible voids.

At the same time, 40% of the teeth with technically adequate RCF also showed periradicular radiolucencies. Even though some of these lesions might have been actually healing at the time of evaluation, this result still indicates that the quality of RCF is not the only determinant for periradicular status.

The presence and quality of the **coronal restoration** are among the factors clearly correlating with the periradicular radiolucencies (Tronstad *et al.,* 2000; Matijeviæ *et al*., 2011; Gomes *et al.,* 2015). Coronal restoration together with

the RCF has been suggested to serve as a barrier against bacterial penetration into the periapical area. Moreover, some studies showed the quality of coronal restoration to be even more important for periapical health than the quality of RCT (Ray & Trope, 1995; Gillen *et al.,* 2011). However, when there is inadequate RCF the quality of coronal restoration is not so important in the success of endodontic treatment (Tronstad *et al*., 2000; Gomes *et al*., 2015). Our study confirmed that the type of restoration is a relating factor for periapical lesions. At the same time, in our study population most of the endodontically treated teeth were restored with a filling, which is not the best method for preventing AP since the teeth with indirect coronal restorations (crown, bridge etc) demonstrated significantly lower rates of AP compared to direct filling type of restorations. The latter can be explained by the fact that those teeth had been observed for longer time and were already healed and/or treated with better overall quality because of the total treatment cost and patient care. Also the prosthodontist might have used better prognosis teeth without AP to start with. However, absence of any type of restoration was most commonly associated with AP. To conclude, the best result can be expected in cases where there is high quality of both RCF and coronal restoration.

In prosthetic restorations we evaluated also the quality of **marginal seal**. Inadequate prosthetic restorations (marginal gap, overhang) significantly increased the risk for AP that is in line with previous data (Siquiera & Rocas, 2005). Alike Moreno *et al.* (2013) we found no significant associations between presence of root canal posts and AP. However, this finding is inconsistent with some other studies (Boucher *et al.,* 2002; Eckerbom *et al.,* 1991; Kayahan *et al.,* 2008) that indicated a strong correlation between root canal posts and greater incidence of AP. We have investigated the possible difference between the different materials of the posts but did not find statistical difference (data not shown). We have also hypothesized that the gender of dentists (more males in western countries, more females in post-Soviet countries) may cause the difference but it needs to be clarified in further studies.

The **overall** high prevalence of AP in Estonia is certainly substantially associated with the high frequency of inadequate endodontic treatments since only a fifth of the root canal filled teeth had acceptable RCF (concerning both length and density). These poor rates indicate an urgent need for improvement of the quality of endodontic treatment in the study region. This can be done with the help of refreshment courses and additional hands-on trainings but the most effective measure could be the referral of the patients with complicated teeth (pulpitis, pAP) at early stages to the endodontist. It means that more endodontists should be trained, and more investments are needed for their education.

4. Etiopathogenetic aspects of apical periodontitis

Dental pulp infections occur as a sequel to dental caries, trauma and dental operative procedures. Infection of the coronal pulp may spread apically, usually causing necrosis of the pulpal tissues, and may reach the apical part of the root canal (Ozok *et al.,* 2012). The infection then invades the periapical area, resulting in local bone destruction (Kirkevang & Hørsted-Bindslev, 2002). The causal relationship between bacteria infecting the root canal system and AP is well established (Kakehashi *et al.,* 1965; Sundqvist, 1976; Möller *et al.,* 1981).

Many reasons contribute to endodontic treatment failures, such as poor technical quality of root fillings, instrument fractures and root perforations. However, these factors will only contribute to endodontic failures if they are accompanied by microbial infection. The presence of microbes is required for maintenance and expansion of periradicular disease (Stashenko *et al.,* 1998).

The root canal system is a unique ecological niche for bacteria. The lack of oxygen and the availability of host tissues and primary nutrient sources are important factors in bacterial selection (Sundqvist, 1992). Therefore the organisms associated with infected root canals are mainly anaerobic and Gram-negative bacteria (Siqueira & Rocas, 2013; Hong *et al.,* 2013; Saber *et al*., 2012; Li *et al.,* 2010). Our study applying next generation sequencing method revealed highly polymicrobial communities in the root canal samples that where mostly dominated by anaerobes.

It has been proposed that endodontic infections are similar to several other human endogenous infections where no single pathogen but a set of species is involved. The concept of the community as pathogen is based on the principle that teamwork is what eventually counts. In mixed communities, a broad spectrum of relationships may arise between the component species. Bacterial species that individually may have low virulence and are unable to cause disease can do so when in association with others (Siqueira & Rocas, 2013).

At the same time, inflammatory conditions, including the oral cavity, are accompanied by elevated OxS level in human organism (Lugrin *et al.,* 2014). The latter depends on one hand on the generation of ROS at the cellular level and in biofluids, but on the other hand, on the protective power of the antioxidative system. In case of inflammation, this balance becomes disturbed in favor of the pro-oxidants (D'aiuto *et al.,* 2010).

To date, the OxS levels have been seldom investigated in oral diseases, especially AP. Our study revealed for the first time that OxS is an important pathogenetic mechanism in endodontic diseases both at the local (root canal contents) and systemic (saliva) levels. Low levels of ROS are needed for a number of biological functions, such as intracellular messaging, growth, cellular differentiation, and antibacterial activity. The latter is very important in case of AP where abundant microbial masses are located in root canal. At the same time, increased production and/or reduced elimination of ROS could result in increased risk of local diseases, while also having systemic implications, such as increased cardiovascular and neurodegenerative morbidity, as well as

depressive symptoms (Inchingolo *et al.,* 2014; Segura-Egea *et al.,* 2015; Comes *et al.,* 2018).

Our study also revealed that OxS is significantly associated with some important clinical markers, such as dental pain and periapical index (PAI). ROS are implicated in cell and tissue damage in various disorders by modifying and inactivating proteins, lipids, nucleic acids and inducing cellular dysfunctions. This tissue damage includes dentine, pulp tissue and periapical tissues (Hernandez-Rios *et al.,* 2017). We can assume that even during infectious carious process there will be imbalance of different regulatory molecules (including different MMPs, interleukines and OxS molecules) locally. From the local imbalance through pulp and dentine tissue which is connected to periapical bone and therefore to whole body it can lead to systematic elevation or imbalance of regulatory (including OxS) molecules (Comes *et al.,* 2013).

Injury and inflammation are associated with increased prostanoid synthesis and pain hypersensitivity. Prostanoids are considered to mediate inflammation and immune responses, as their administration produces hyperalgesia and other major signs of inflammation (Pradeep *et al.,* 2013; Materazzi *et al.,* 2008). Novel hypothesis on putative associations of OxS and pain in case of AP is presented in the next section.

5. Putative associations between oxidative stress and pain in apical periodontitis

Peripheral stimuli (initial pain) may lead to spinal and systemic OxS (Figure 10, path I). Nociceptive primary afferents are capable of inducing a state of increased excitability in the dorsal horn neurons of the spinal cord. Increased generation of superoxide is a potential common characteristic of at least three pathogenetic pathways. Superoxide anion is a highly reactive oxygen radical implicated in cell and tissue damage related to various disorders, including inflammatory diseases. While superoxide production in neutrophils and other phagocytic cells is essential for killing microbes, it also leads to tissue damage at the inflammation site. Imbalance between the production and elimination of oxygen radicals in periapical lesions has been proposed as a factor in periapical damage and bone loss in AP (Graunaite *et al.,* 2011). In our study, oxidative stress index (OSI) values were statistically significantly higher in pAP and sAP patients compared to healthy controls, both in the endodontium and saliva. OSI also had a strong correlation with inflammation/bone loss (higher PAI values) in the endodontium. Superoxide anion can be produced by osteoclasts and can play a part in bone resorption. It may also react with a precursor in plasma, resulting in the generation of a factor that is chemotactic for neutrophils (Graunaite *et al.,* 2011). In addition to host cells, superoxide anion can also be produced in bacteria. For example, the production of superoxide by *Streptococcus* spp. has been shown to be lytic for erythrocytes, and extracellular superoxide production

has been demonstrated to be a common trait for *Enterococcus faecalis* (Minczykowski *et al.,* 2001; Graunaite *et al.,* 2011). Our measurements of total lipid peroxides / total antioxidant capacity showed that in the case of pAP, the above-mentioned ratio was significantly higher both in the endodontium and saliva compared to controls.

Furthermore, spinal OxS (Figure 10, path II) has been shown to be a sufficient cause of pain (Schwartz *et al.,* 2008). Spinal OxS can be the key factor linking pain-producing pathways. Pathway II is the positive feedback that may be mediated by electrophilic LPP capable of passing through the cell membrane by passive diffusion (McGrath *et al.,* 2011) and therefore it can be a key link in pain-causing pathways. Additionally, LPP react with glutathione and if the level of the latter is low, electrophilic LPP are more toxic to proteins and nucleinic acids. Animal experiments have shown that the decrease of glutathione in spinal cord induces pain (Rossato *et al.,* 2010; McGrath *et al.,* 2011).

OxS on the level of the spinal cord contributes to systemic OxS in saliva (Figure 10, path III). Pathway III is mediated by LPP functioning as ligands of prostanoid receptors. These ligands include 8-EPI and prostaglandin F2α (PGF2α). Peripheral 8-EPI and spinal prostaglandin F2α may lead to sensitization, but prostanoid signaling is also a necessary step in converting short-term signaling to long-term changes in neuronal phenotype (Lerea *et al.,* 1997). This mechanism may provide additional support to the previously suggested idea that a sensitized state remains even after the initiating stimulus is lost (Nickel, 2002).

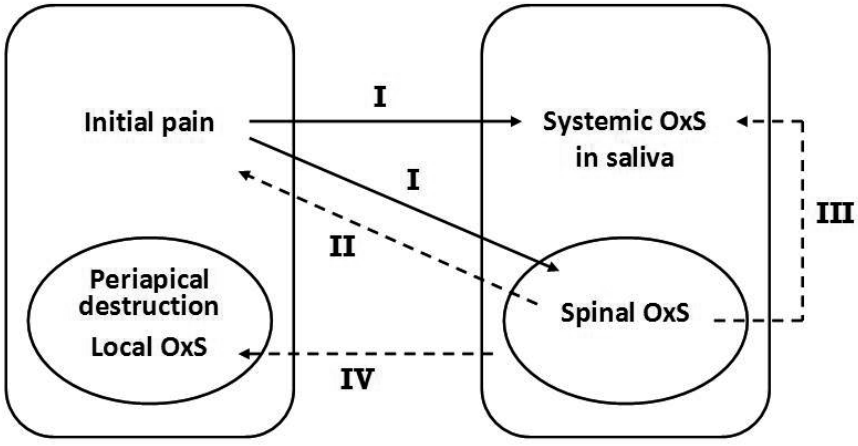

Figure 10. Putative associations between oxidative stress and pain in apical periodontitis.

I – Peripheral stimuli – initial pain may lead to the spinal and systemic OxS.
II – Spinal OxS (OxS on the level of spinal cord) is a sufficient cause of pain (Schwartz *et al.,* 2009).
III – Spinal OxS contributes to the systemic OxS.

IV – Systemically generated 8-isoprostanes could contribute to the periapical OxS as well as bone destruction and pain (Eder *et al.,* 2012).

It has been suggested by Eder *et al*. (2012) that systemically generated 8-EPI in saliva could contribute to periapical OxS, as well as bone destruction and pain (Figure 10, path IV). Therefore, the generation of 8-EPI due to the AP in saliva and OxS at the level of the spinal cord can result in positive feedback by irritating the primary afferents of the pulp. This is in line with our data where higher values of 8-EPI were seen in patients with pain and more severe bone destruction (higher PAI).

The association between pain (PAI) and AP is attributable, at least in part, to increased level of local and systemic oxidative stress in AP patients. Therefore the alleviation of local and systemic oxidative stress in AP patients may contribute to the quality of life of these patients.

6. Study limitations

The prevalence study was performed as a **cross-sectional** study that was based on panoramic radiographs. The biggest drawback was that we could not determine if the periapical radiolucency was in healing or progressing state since radiograps reflect "static" periapical state in that moment of time (Da Silva *et al*., 2009; Di Filippo *et al*., 2014). The complete quality of treatment itself is impossible to evaluate on radiograph (like disinfecting protocol, medication or used instruments), and we do not know the anamnesis of the patients. Instead, we could evaluate the radiologic aspects of RCF (length, homogeneity). At the same time it has been shown that cross-sectional studies are valid and reliable in long term as during 10years of observation the number of forming and healing periapical lesions remaines the same. (Petersson *et al.*, 1991).

Concerning the **radiographic method**, the panoramic radiographs might not be the best method to evaluate the presence of AP, compared to intraoral periapical radiographs or CBCT analysis. At the same time, panoramic radiograph is the quickest, cheapest and the most comprehensive method with relatively low radiation dose. It enables to get a lot of different information about oral health (Ridao-Sacie *et al.,* 2007; Gumru *et al.,* 2011). Panoramic radiographs are considered sufficiently specific and sensitive to evaluate endodontic status (Ahlqwist *et al.,* 1986). Our study was performed in one setting (Tartu University Hospital) with one x-ray device that produced images of equal quality.

Subjectivity is another aspect associated with observing the panoramic radiographs. On one hand, if we understand the continuity of the disease and its development we can see some additional signs on the panoramic radiographs. On the other hand, knowing "too much" could make us assume and see some things that are not really there or vice versa. For example, in case of correct RCF we tend to assume that there is no PA lesion, even though it might be

clearly visible, while more commonly, in case of bad quality dentistry we tend to see periapical radiolucency even if it is not there. Those misconceptions are more common in 2D radiology than in 3D radiology. Hence, if a clinician is in doubt concerning AP lesion they should make a definitive 3D (CBCT) analysis before the final verdict (Estrela *et al.,* 2008). In our study, the observers were calibrated and there were multiple observers simultaneously working on the same panoramic radiographs. A highly experienced dentist served as the third observer (M.S.) for cases where disagreement occurred.

Another limitation of the prevalence study was related to **study population** that originated from Southern Estonia, and herefore not necessarily representative of the entire Estonian population. People of Southern Estonia mostly attend Tartu University Hospital for radiographic analysis. Thus, comparison of current results with other populations should be made cautiously.

The main drawback of our studies concerning **microbiological and biochemical** aspects of AP is the moderate sample size. Detection of some more biochemical markers like MMP could be helpful for additional clinical considerations. In addition to saliva, also blood and urine can be used for detecting systemic OxS. Also, time-series studies would provide more information on the dynamics of oral microbiota as well as biochemical markers. Revealing of these shifts can be useful for prognosis and for design of treatment regiments. At the same time both studies were performed using strict clinical and laboratory work protocols, and comprehensive clinical anamnesis and treatment history of the patients were carefully recorded. The microbiome study was the first one investigating root canal microbiota applying Illumina sequencing, and the study of OxS was the first one describing both local and systemic OxS levels in case of different endodontic pathologies.

CONCLUSIONS

1. AP is highly prevalent in Estonia, being present in more than half of subjects and 6.3% of teeth. Primary AP can be diagnosed in nearly a third while secondary AP in nearly a half of subjects. The latter is consistent with low quality endodontic treatment in the study population. These numbers are higher than in most high-income Western European countries but lower than some other former Soviet Union countries reflecting different accessibility to dental care.

2. The main determining factors of AP are gender, age, tooth type and position, presence of caries and endodontic treatment related markers (treatment type, quality of root canal filling, presence and type of tooth restoration). Some of these factors are difficult to control, like age (elderly people have more pAP while middle-aged people have more sAP) and position of tooth (molar teeth and mandibular teeth have the highest risk for AP). At the same time most risks can be managed in collaboration with patient and dental care providers.

Caries is a major risk factor for AP, and lower prevalence of pAP in women is most probably related to their higher willingness to visit a dentist and treat carious teeth in early stage, thus avoiding the progression of caries to pulpitis and AP. Hence, prevention of dental diseases should be well covered by Health Insurance Fund so at later stages medical expenses would be less burdensome.

Previous root canal treatment tends to be highly associated with AP since most periradicular radiolucencies are associated with root canal-treated teeth, and a half of all root canal-treated teeth show radiological signs of AP. Pulpotomized teeth have greater chance of developing AP compared to pulpectomized teeth. Low quality of endodontic treatment (too short, too long or inhomogenous RCF) is significantly associated with AP, at the same time only a fifth of the investigated teeth have root canal fillings acceptable in all terms. On the other hand, high quality root canal fillings and presence of coronal restoration (crown, bridge) are significantly associated with lower risk for AP and therefore better treatment outcome.

It has been well recognized that the outcome of endodontic treatment is higher if done by endodontic specialist. At the same time this situation is far from optimal in the study region since there is lack of endodontists in Estonia. Expert pressure on the government is needed to recognize endodontics as a specialty, and to recognize the assembled curriculum for endodontic specialty. Meanwhile the level of endodontic quality among general dentists should be raised by active theoretical education and practical courses.

3. Root canal specimens in patients with AP and periapical abscess display highly polymicrobial communities with 30–70 different operational taxonomic units belonging to 5–8 different phyla. These communities contain many of the known root canal pathogens, among others Gram negative anaerobes *Prevotella*

sp., *Porphyromonas* sp., *Fusobacterium* sp., *Tannerella* sp. and *Pyramidobacter piscolens* as well as Gram positive *Dialister* sp., oral spirochete *Treponema socranskii, Solobacterium moorei* and many others. Some novel AP-associated bacteria include *Gardnerella vaginalis,* TG5 and *Janthinobacterium lividum*. The communities are individually different but anaerobic bacteria predominate as a rule. Only minor differences can be seen between the study groups like slightly lower number of different OUT's in pAP patients in comparison with other groups, and presence of *Enterococcus faecalis* in root canal of sAP patients only. The latter can be associated with root canal treatment failure.

Therefore the microbiological analysis of the root canal content in case of endodontic pathologies is meaningless, and the treatment measures must be directed against a wide spectrum of aerobic and anaerobic bacteria.

4. OxS is an important pathomechanism in endodontic pathologies (pAP, sAP, periapical abscess, pulpitis) that is evident both at the local (root canal contents) and systemic (saliva) level. Systemic OxS may be an important coupler between oral diseases and their systemic implications (cardiovascular, neurodegenerative and other disorders).

OxS is significantly associated with important clinical markers like dental pain and bone destruction. Therefore the antioxidant therapy may be considered a possible novel component in addition to convention nonsurgical or surgical RCT in the complex treatment of severe long-lasting oral diseases.

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SUMMARY IN ESTONIAN

Apikaalne periodontiit: levimus ja etiopatogeneetilised aspektid

Apikaalne periodontiit (AP) on hamba juurekanalisüsteemis paikneva infektsiooni tagajärjel tekkinud põletik hamba juuretipu ümbruses (Kakehashi jt., 1965). AP on loomulik jätk kaariesest tingitud pulpiidile ja sellest tulenevale pulbi nekroosile. Põletikulise protsessi käigus toimub periapikaalkudede destruktsioon, mis on radioloogiliselt nähtav periapikaalse helendusalana.

AP on laialt levinud seisund, mis esineb 27%-l Soome (Huumonen jt., 2017), kuid koguni 83%-l Jordaania elanikest (Al-Omari jt., 2011). Keskmiselt on ühel inimesel AP kahjustus 1,4–8% kõigist hammastest. (Eriksen ja Bjertness, 1991). AP esinemist seostatakse vanuse, soo, teiste hambahaiguste, hambaravikvaliteedi ja indiviidi sotsiaalmajandusliku seisundiga (Kirkevang jt. 2001). AP levik ja sellega seonduvad faktorid pole seni Eestis uuritud.

AP klassifitseeritakse ja diagnoositakse spetsiifilise sümptomaatika, kliinilise pildi ja radioloogiliste uuringute abil (Gutmann ja Lovdahl, 2010). Hamba juureravi peamiseks eesmärgiks on AP ennetamine või juba tekkinud haiguse ravi. Ravi käigus juurekanalisüsteem puhastatakse põletikulisest ja nekrootilisest koest ning desinfitseeritakse. Vähendatakse mikroorganismide kui peamise etioloogilise faktori hulka, vajadusel kasutatakse vaheravimeid ja lõpuks juurekanali süsteem täidetakse ning hamba krooniosa restaureeritakse (Hargreaves jt., 2016). Endodontilist ravi jaotatakse mittekirurgiliseks ja kirurgiliseks; kirurgiline endodontia on näidustatud vaid olukorras, kus mittekirurgiline endodontiline ravi ei ole andnud soovitud tulemust (Rosenberg jt., 2009). Esmakordse juureravi edukuse määr on 73–97%, sekundaarse juureravi ravi edukuse määr on 60–95%, ja kirurgilise juureravi edukus 28–80% (Elemam ja Pretty, 2011). Kuna endodontilise ravi edukus on kõrge, peaks see olema eelistatud ravimeetod hamba eemaldamise asemel.

AP mikrobiaalset etioloogiat on uuritud aastaid; esmalt külvi- ja hiljem erinevate molekulaarsete meetoditega. Selle käigus on avastatud mitmeid arvatavaid endodontilisi patogeene, enamasti biokilede koosluses (Jhajharia jt., 2015). Nende hulgas on palju raskesti- või mittekultiveeritavaid liike (Siqueira ja Rôças, 2013). AP tavapäraseks tulemuseks on bakterpõletiku vastu tekkinud immuunreaktsiooni tagajärjel tekkiv periapikaalkudede osteolüüs (Hernández-Ríos jt., 2017). Mikroobide sissetungi vastu toodavad fagotsüüdid reaktiivseid hapnikuosakesi (ROS); kui aga pro- ja antioksüdantide tasakaal häirub, tekib ülemäärane oksüdatiivne stress (OxS), mis põhjustab nii lokaalset kudede kahjustust kui ka süsteemse OxS kujunemist (Dezerega jt., 2012). OxS taset erinevate endodontiliste diagnooside korral ei ole senini põhjalikult uuritud.

Käesoleva uuringu **eesmärgiks** oli selgitada välja AP levimus Eestis ning seda põhjustavad ja soodustavad etiopatogeneetilised faktorid.

Spetsiifilised uurimisülesanded olid järgmised:

1. Koguda andmeid AP esinemise kohta Eestis, kasutades radioloogilisi panoraamülesvõtteid.
2. Määratleda samade ülesvõtete abil AP põhjustavad faktorid, leida AP seosed erinevate hambahaiguste ja eelneva hambaravi vahel Eesti patsientide hulgas.
3. Identifitseerida AP korral esinevad peamised mikroobikooslused kui AP peamine etioloogiline faktor, kasutades uue põlvkonna sekveneerimist.
4. Võrrelda oksüdatiivse stressi taset süljes ja juurekanalis erinevate endodontiliste patoloogiatega patsientidel ja tervetel kontrolluuritavatel.

Materjal ja meetodid

AP levimuse ristlõikeline-uuring hõlmas 6552 patsiendi (vanuses 35.5±19.2 aastat) radioloogilisi uuringuid (panoraamülesvõte), mis olid teostatud Tartu Ülikooli Stomatoloogia kliiniku röntgenkabinetis ajavahemikul 2010.a. novembrist kuni 2012.a maini konsultatsiooni ja/või ravi eesmärgil. Panoraamradioloogilised uuringud teostas kogemustega hambaravi radioloog, kasutades Planmeca ProMax 3D Plus (*Planmeca OÜ,* Helsingi, *Soome)* kiirguskoormuste vahemikus 54–96 kV ja 1–14 mA sõltuvalt uuritava suurusest. Ülesvõtteid hindasid eelnevalt kalibreeritud vaatlejad, arvamuste erisuse korral kasutati kolmandat vaatlejat lahkarvamuste lahendamiseks. Hinnati kõikide hammaste olemasolu ja olukorda vastavalt FDI nomenklatuurile. Samuti hinnati erinevaid kõvakudede radioloogilisi muutusi, kaasa arvatud AP esinemist, eelnevat hambaravi ja selle kvaliteeti.

Mikrobioloogilise uuringu jaoks koguti juurekanalist uuritav materjal rangelt aseptilistes tingimustes steriilse Hedström viili ja steriilsete endodontiliste pabertihvidega. Proovid koguti kaheteistkümne hamba juurekanalitest, millest viiel oli diagnoositud AP ilma eelneva juureravita (pAP); kolmel AP eelneva juureraviga (sAP) ja neljal hambal periapikaalne abstsess. Saadud proovid sisestati Eppendorf tuubi, mis sisaldas 1ml *Brucella* puljongit (*Oxoid, Basingstoke,* Suurbritannia). Sellest materjalist eraldati DNA QIAamp DNA Mini Kit'iga (*Qiagn, GmbH,* Saksamaa) ning säilitati –80 °C juures. Mikroobikooslused iseloomustati 16S rRNA baasil, kasutades Illumina HiSeq2000 sekveneerimist. Taksonoomiliste järjestuste määramisel kasutati andmebaase *Greengenes* ja *HOMD.*

Oksüdatiivse stressi uuringus osales 86 patsienti vanuses 38,7±14.0 aastat, neist 22 olid pAP-ga, 26 sAP-ga, 8 ägeda periapikaalabstsessiga, 13 sümptomaatilise pöördumatu pulpiidiga ja 17 olid terved kontrolluuritavad. Süsteemse OxS hindamiseks koguti patsientidelt mittestimuleeritud sülge enne ravi alustamist. Lokaalse OxS hindamiseks koguti patsientidelt endodontsiumi proovid rangelt aseptilistes tingimustes, kasutades Hedström viili. Proovid külmutati –80 °C kuni analüüsimiseni. OxS markerid (müeloperoksidaas [MPO], oksüdatiivse stressi indeks [OSI] ja 8-isoprostaanid [8-EPI]) määrati nii sülje kui hamba juurekanali proovidest.

Tulemused ja järeldused

AP esinemine ja seosed erinevate hambahaiguste ja eelneva hambaraviga

Panoraamülesvõtetelt diagnoositi AP esinemine "range kriteeriumi" järgi (Strindberg, 1956; Ng *et al.,* 2008) 54.7% uuritavatest. Mittekirurgiline juureravi oli teostatud 58.2% uuritavatest. Periradikulaarne radioloogiline helendusala tuvastati 44.6% endodontiliselt ravitud hammastel ja 30.8% juureravimata hammastel. Kokku uuriti 181 495 hammast, millest 52.7% olid intaktsed. AP diagnoositi 6.3% hammastel ja 6.9% hambaid oli ravitud mittekirurgilise juureravi meetodiga.

Olulisteks AP riskifaktoriteks osutusid hambakaariese esinemine (OR=2.30), mees-sugu (OR=1.44), liiga lühikesed juuretäidised (OR=1.76), üle radioloogilise juuretipu ulatuvad juuretäidised (OR=2.51), radioloogiliselt hõredad juuretäidised (OR=1.61) ja ebakvaliteetne krooniosa proteetiline restauratsioon (OR=1.63). Vanuse kasvades AP esinemissagedus kasvas. Juurekanali tihvti ja ortodontilise aparaadi paiknemine suus ei kuulunud AP riskifaktorite hulka. Madalam AP risk seostus hamba krooniosa restauratsiooniga, seda nii täidiste (OR=0.45), kroonitud hammaste (OR=0.34) kui sildproteesi tugihammaste puhul (OR=0.33).

Seega on AP Lõuna-Eesti elanike hulgas laialt levinud. Enamus juhtudest on seotud eelnevalt juureravitud hammastega. Üldine juureravi kvaliteet on madal ja AP on tugevalt seotud madalakvaliteediliste juuretäidistega. Endodontilise ravi kvaliteet vajab olulist parandamist, et saavutada AP vähenemine elanikkonnas.

Juurekanalite väga mitmekesine mikrobioota AP korral

Juurekanalitest saadud proovid sisaldasid väga mitmekesiseid mikroobikooslusi kõikides uuringugruppides. Üks proov sisaldas 5–8 (keskmiselt 6,5) bakteri hõimkonda. Enam esinesid *Firmicutes* ja *Bacteroidetes* hõimkonnad, aga ka *Actinobacteria, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes* ja *Synergistetes* olid esindatud suurel osal patsientidest. Üks proov sisaldas 30–70 erinevat operatiivset taksonoomilist üksust (OTU), keskmine (±SD) oli madalam eelneva juureravita (pAP) patsientide grupis (36±4) kui periapikaalabstsessi grupis (45±4) ja eelnevalt juureravitud (sAP) patsientide grupis (43±13) ($p<0.05$). Mikroobikooslused olid individuaalselt erinevad, kuid reeglina domineerisid anaeroobsed bakterid. *Enterococcus faecalis*'t, mida sageli seostakse juureravi ebaõnnestumise ja püsiva AP tekkega, leiti vaid eelnevalt juureravitud hammaste proovidest. Avastati mitmeid raskesti kultiveeritavaid baktereid, nagu *Solobacterium moorei, Oribacterium* sp., *Olsenella* sp. ja *Renibacterium* sp. Juurekanali proovides leidus mõningaid uusi liike, nagu TG5, *Gardnerella vaginalis* ja *Janthinobacterium lividum.*

Uuringust võib järeldada, et kuna periapikaalpatoloogia põhjustajaks on väga polümikroobsed kooslused, siis spetsiifiliste mikrobioloogiliste analüüside

teostamine juurekanalisüsteemist ei ole mõistlik ja ravi tuleb suunata väga laia spektriga nii aeroobsete kui anaeroobsete mikroobide ja nende koosluste vastu.

Oksüdatiivne stress endodontilise patoloogiaga patsientide hulgas

OxS esines kõikide uuritud endodontaalsete patoloogiate korral. Kõrgeimad lokaalsed MPO ja 8-EPI tasemed leiti pAP ja pulpiidi patsientidel. Kõrgeimad OSI tasemed leiti pAP ja abstsessi patsientidel ja sAP patsientidel süsteemselt. Tervetel kontrolluuritavatel olid OxS tasemed madalad nii lokaalsel kui süsteemsel tasemel.

Märkimisväärne positiivne korrelatsioon leiti OxS markerite, periapikaalindeksi (PAI) ja subjektiivse sümptomaatika (valu) vahel. Patsientidel, kellel esines valu, esinesid samal ajal ka kõrgemad OxS tasemed nii endodontsiumis (MPO mediaan 27.9 vs 72.6 ng/mg proteiin, $p=0.004$; OSI 6.0 vs 10.4, $p<0.001$; 8-EPI 50.0 vs 75.0 pg/ml, $p<0.001$) kui ka süljes (MPO 34.2 vs 117.5 ng/mg proteiin, $p<0.001$; 8-EPI 50.0 vs 112.8 pg/ml, $p<0.001$) võrreldes valu-vabade patsientidega.

Seega on OxS tähtis patoloogiline mehhanism endodontiliste patoloogiate korral, mis on nähtav nii lokaalsel (juurekanali sisaldis) kui ka süsteemsel (sülg) tasandil. Märkimisväärne on OxS seos hambavalu ja luu destruktsiooniga. Seetõttu võib lisaks korrektsele konventsionaalsele mittekirurgilisele või kirurgilisele juureravile lisameetmena kaaluda ka antioksüdant teraapiat kui võimalikku uut ravikomponenti.

ACKNOWLEDGEMENTS

I wish to express my warm and sincere gratitude to all those who made this work possible, with special attention to:

Professor Reet Mändar, my excellent supervisor, without whose contribution this work would never have been completed. For sharing her knowledge of science, for her constant support, excellent teaching and passing on the art of writing scientific papers. My gratitude for her all-around support is inexpressible;

Professor Mare Saag, my co-supervisor, for her support and interest in my work and for always having her door open and taking time whenever needed and for her constant belief in me;

Associate professor Tiiu Kullisaar, my co-supervisor, for introducing me the fascinating field of biochemistry, for all her laboratory work, interesting ideas and advice, for always being kind, patient and supportive;

Minh Son Nguyen is acknowledged for large-scale statistical support in prevalence studies;

Silvia Saukas for her valuable assistance in radiographic analyses, data collection and discussion;

Anneli Piir for the generous help in the biochemistry lab;

Katerina Špilka for the important help in microbiology studies;

Jens-Konrad Preem, Kristjan Oopkaup, Jaak Truu are acknowledged for the help in microbiota detection with next generation sequencing.

Irja Lutsar, Stanislav Liskman, Katrin Lang are acknowledged for their critical comments and for the help to improve current thesis during reviewing.

Ave Tammaru is acknowledged for the language correction of the current thesis.

And finally, I express my warmest gratitude to my wife Eliis and my children Agnes and Taavet for their support and understanding: I will always love You.

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