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INDICATORS FOR TONSILLECTOMY IN ADULTS WITH RECURRENT TONSILLITIS – CLINICAL, MICROBIOLOGICAL AND PATHOMORPHOLOGICAL INVESTIGATIONS

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ABBREVIATIONS

AAO-HNS	American Academy of Otolaryngology – Head and Neck Surgery
ASO	antistreptolysin O
AUC	area under the curve
BAO-HNS	British Association of Otolaryngologists – Head and Neck Surgery
BHS	β-hemolytic streptococci
CD	cluster of differentiation
CFU	colony forming units
CI	confidence intervals
CONS	coagulase negative staphylococci
CRP	C reactive protein
DNA	deoxyribonucleic acid
ENT	ear, nose and throat
FDC	follicular dendritic cell
IDC	interdigitating dendritic cell
Ig	immunoglobulin
IT	index of tonsillitis
MALT	mucosa-associated lymphatic tissue
NPV	negative predictive value
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPV	positive predictive value
PT	palatine tonsil
ROC	receiver-operating characteristic curve
RT	recurrent tonsillitis
SIGN	Scottish Intercollegiate Guidelines Network
TE	tonsillectomy
TEM	transmission electron microscopy
WBC	white blood cells

INTRODUCTION

Recurrent tonsillitis (RT), a chronic inflammatory process in the palatine tonsils (PTs), is clinically expressed by repeated attacks of tonsillitis episodes. Tonsillectomy (TE), surgical removal of PTs, has been considered as a leading therapeutic approach for such condition (Fry and Pillsbury, 1987; Blair, 1996; Marshall, 1998; Mui *et al*., 1998; Darrow and Siemens, 2002). The frequency of tonsillitis episodes reported by the patient is the most widely used indicator for surgical therapy. Patients with at least three episodes per year, despite adequate medical therapy, may be considered as candidates for TE, and surgical treatment is definitely recommended for patients with more than four or five episodes per year (AAO-HNS; BAO-HNS; SIGN, 1999). However, there is no world wide agreement among clinicians whether a specific number of tonsillitis episodes over a certain period of time warrants TE. Adult patients often have fewer or less severe tonsillitis episodes but they are characterised by the presence of systemic effects of chronic disease, such as poor general health, tiredness, lowered resistance, tendency to catch colds, unexplained fever, comorbid diseases, carriage state of *Streptococcus pyogenes* and increased antistreptolysin O titre (Becker *et al*., 1994; Dagnelie *et al*., 1998; Mui *et al*., 1998; Bhattacharyya *et al*., 2001; Bhattacharyya *et al*., 2002). The systemic effects and comorbidity cause significant time loss from school or work, decreasing the patients' life quality, and have therefore been considered as other potential indicators for TE (Bhattacharyya *et al*., 2001; Bhattacharyya *et al*., 2002). The number of physician visits and patient's own concern have also been recommended as appropriate indicators (Mui *et al*., 1998; SIGN 1999). In general, these data suggest that there is no consensus for selection of patients for TE, pointing to the need for more precise indicators.

PTs are a part of the mucosa-associated lymphatic tissue (MALT), a specialized compartment of the immune system that serves as the first line of defence against harmful environmental factors, including pathogenic microbes (Perry and Whyte, 1998). Paradoxically, PTs themselves are quite frequently affected by bacterial and viral infections causing local inflammation and systemic reactions. Although several potentially pathogenic aerobic and anaerobic bacteria have been found in the surface and deep bacterial flora of PTs (Brook and Yocum, 1988; Kielmovitch *et al*., 1989; Brook *et al*., 1993; Mitchelmore *et al*., 1994; Kuhn *et al*., 1995; Stjernquist-Desatnik and Holst, 1999), their precise role in the development of recurrent attacks of tonsillitis has remained unclear. On the other hand, continuous inflammation in the tonsillar tissue results in specific morphological changes, including narrowing of the crypts' neck and distension of their bottom, which leads to retention of the crypts' content (Altemani *et al*. 1996; Michaels, 2001). The latter change creates ideal conditions for continuous dissemination of pathologic material (microorganisms, toxic metabolites and inflammatory mediators), setting a basis for the

development of concomitant inflammatory diseases, and endangering the patients' health (Becker *et al*., 1994). Whether such generalization of infection depends on the presence of specific pathogens in the tonsillar microflora, characteristic alterations in the microbial ecology of the tonsils, patomorphological changes or an altered immune status of PTs remains to be explored.

Several studies, including those performed at the Department of Otorhinolaryngology, University of Tartu, have shown a decreased number of Ig-producing immune cells in the tonsillar tissue and a lowered amount of protective antibodies in the saliva of children with RT (Põld, 1986; Bernstein *et al*., 1988; Koch and Brodsky, 1995). At the same time, the status of cellular immunity in recurrently inflamed PTs is controversial. There have been found both the decreased numbers and immature immune cells or hyperactive immune cells in the tonsils of RT patients (Koch and Brodsky, 1995; Olofsson *et al*., 1998; Gorfien *et al*., 2001; Ebenfelt *et al*., 2002; Fujihara *et al*., 2005). Therefore, both the functional breakdown and the hyperactive immune defence of recurrently inflamed PTs have been suggested in these studies.

The main goal of the present PhD thesis was to find evidence-based indicators for TE in adults with RT. For this purpose, the functional status of recurrently inflamed PTs was investigated by exploring the associations between the characteristics and extent of morphological alterations in the tonsillar tissue and the occurrence of bacteremia during surgery. The relevant microbiological, molecular and biochemical studies were performed at the Institute of Microbiology, University of Tartu. Collection of the clinical data and their evaluation were performed at the Department of Otorhinolaryngology, University of Tartu. The patomorphological, immunohistochemical and electron microscopic studies of PTs were performed in collaboration with the Institute of Pathological Anatomy and Forensic Medicine, and the Department of General and Molecular Pathology, University of Tartu.

LITERATURE REVIEW

1. Definitions

The inflammation of the PTs' parenchyma is called *tonsillitis*, which is usually accompanied by the inflammation of other structures in the oropharyngeal region. Regardless of the speciality (e.g. pediatrics, general medicine or ENT surgery), there is no consensus over differentiating between pharyngitis and tonsillitis as the terms *pharyngitis*, *tonsillitis*, *upper respiratory tract infection*, *throat infection* or *sore throat* are used interchangeably (Blair *et al*., 1996; Marshall, 1998; Mui *et al*., 1998; SIGN, 1999; Faulgonbridge *et al*., 2000). Continuous or chronic inflammation in PTs is clinically characterised by repeated attacks of tonsillitis episodes, and is therefore called recurrent tonsillitis (RT). Although it is often claimed that there is no such condition as *chronic tonsillitis* (Hibbert and Cownan, 1997), it has been widely treated as synonymous to RT (Becker *et al*., 1994; Mui *et al*., 1998; Faulconbridge *et al*., 2000). Some authors have defined chronic tonsillitis as persisting sore throat associated with tonsillar inflammation unresponsive to medical therapy for at least 3 months, or the condition associated with malodorous breath, tonsilloliths, and persistent tender cervical lymph nodes, when no other source can be found (Brodsky, 1993; Faulconbridge *et al*., 2000; Darrow and Siemens, 2002). This description is, in fact, very similar to the RT course in adults, who often have few or less severe tonsillitis episodes. In Estonia, most practitioners have traditionally preferred the term chronic tonsillitis instead of RT. As the term RT is more prevalent in English literature, it was still used throughout our studies.

2. Etiology of RT

Acute tonsillitis has traditionally been associated with *Streptococcus pyogenes* infection (Bisno *et al*., 2002; Lildholdt *et al*., 2003). Surprisingly, less than 10% of acute tonsillar infections in adults and 30% in children are actually caused by *S. pyogenes* (Pichichero, 1995). The other β-hemolytic streptococci, *Arcanobacterium haemolyticum*, *Neisseria gonorrhoeae*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* have been considered as facultative pathogens and account for only 0.5 to 2.5% of cases each (Meier *et al*., 1990; Seppälä *et al*., 1992; Turner *et al*., 1993; Carlson *et al*., 1994; Linder, 1997). As almost 30–50% of cases have a viral origin, the remaining acute tonsillitis complaints seem to account for cases of unknown etiology (Pichichero, 1995; Little and Williamson, 1996; White and Foshee, 2000; Chi *et al*., 2003; Kumar *et al*., 2003). The latter may be attributed to limitations of diagnostic tests, indicating the need for intensive research to develop sensitive rapid-detection assays of pathogens.

Despite the high frequency among population, the etiology of RT has remained unclear. An average isolation rate of *S. pyogenes* from RT patients by conventional culture methods has been 20–30% in children (Brodsky *et al*., 1988; Surow *et al*., 1989; Gaffney *et al*., 1991; Kuhn *et al*., 1995; Gaffney and Cafferkey, 1998; Inci *et al*., 2003), but only 6–17% in adults (Brook and Yocum, 1984; Stjernquist-Desatnik *et al*., 1990; Mitchelmore *et al*., 1994; Lildholdt *et al*., 2003; Podbielski *et al*., 2003). The surface and the deep bacterial flora of recurrently inflamed PTs consist of many potentially pathogenic aerobic and anaerobic bacteria both in children and adults (Brook and Yocum, 1984; Brodsky *et al*., 1988; Brook and Yocum, 1988; Kielmovitch *et al*., 1989; Brook and Foote, 1990; Brook *et al*., 1993; Mitchelmore *et al*., 1994; Kuhn *et al*., 1995). The composition of the surface tonsillar flora correlates poorly with the deep tonsillar flora, which is most likely the source of infection (Brook *et al*., 1981; Almadori *et al*., 1988; Kielmovitch *et al*., 1988; Surow *et al*., 1989; Gaffney *et al*., 1991; François *et al*., 1992). This may explain why a superficial throat culture does not usually reveal the pathogen responsible for particular tonsillitis episodes in patients with RT (McKerrow, 2002; Inci *et al*., 2003, Lildholdt *et al*., 2003; Podbielski *et al*., 2003). The microorganisms most commonly recovered from either the surface or the deep tonsillar microflora are listed in Table 1.

Most of the listed bacteria are the usual components of the oropharyngeal microflora of healthy persons (Brook and Foote, 1990; Tanaka *et al*., 1996), which explains why the bacterial flora of recurrently inflamed PTs has frequently been considered normal (Surow *et al*. 1989; Gaffney *et al*. 1991; Stjernquist-Desatnik and Holst, 1999).

More characteristic of the deep tonsillar microflora of recurrently inflamed tonsils seem to be quantitative changes. The number of aerobic bacteria is about 10 and the number of anaerobic bacteria is up to 100 times higher in diseased tonsils compared to normal tonsils (Kielmovitch *et al*., 1989; Brook and Foote, 1990; François *et al*., 1992; Kuhn *et al*., 1995). Among the aerobic bacteria the higher quantities are shown by *S. pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae* and among the anaerobes by *Peptostreptococcus*, *Prevotella*, *Bacteroides* and *Fusobacterium* species (Brodsky *et al*., 1988; Kielmovitch *et al*., 1989; Brook and Foote, 1990; Gaffney *et al*., 1991; Brook *et al*., 1993; Mitchelmore *et al*., 1994; Kuhn *et al*., 1995; Gaffney and Cafferkey, 1998). However, the absolute count of bacteria varies greatly in different individuals and their comparison has a limited value. Therefore, proportional analysis of the indigenous microflora has found to be more successful in research into microbial ecology of the gastrointestinal and genital tracts (Mikelsaar, 1992; Mändar, 1995; Sepp, 1998). Applying such approach to the tonsillar microflora could provide better opportunities to study the microbial ecology of inflamed tonsils and to understand the pathogenesis of RT.

Table 1. Microorganisms recovered from the surface and the deep tonsillar microflora of children and adults with recurrent tonsillitis.

	References	
Microorganisms	Surface	Core
Aerobic and facultative bacteria		
α-Hemolytic streptococci	3,35,69,98,	3,35,36,38,69,75,98,
Group A β-hemolytic streptococci	35,39,69,98,160,163,	35,36,38,69,75,98,160,163
β-Hemolytic streptococci	3,35,69,98,163	3,35,36,38,69,75,98,160, 163
Streptococcus pneumoniae	35,69,160,163	35,36,38,69,75,98,160,163
Staphylococcus aureus	3,35,39,69,98,160	3,35,36,38,69,75,98,160, 163
Coagulase negative staphylococci	35,98	3,35,36,38,98
Micrococcus sp	–	38
Enterococcus sp	–	38
Moraxella sp	3,98	3,98
M. catarrhalis	69,98,163	35,38,75,98,163
Neisseria sp	3	3,35,36,38
Corynebacterium sp	3,35,98	3,35,36,38,75,98
Lactobacillus sp	3	36,38,75
Haemophilus influenzae	3,35,39,69,98,160	3,35,36,38,69,75,78,98, 160,163
H. parainfluenzae	35,69,98,160	35,36,38,69,75,98,160,163
Eikenella corrodens	35,98,160	35,36,38,75,98,160
Other non-fermentatives		35
Escherichia coli	35	3,35,36,38,75
Pseudomonas aeruginosa	3,35,160	3,35,36,160
Capnocytophaga sp	3	3,38,160
Anaerobic bacteria		
Peptostreptococcus sp	3,35,98	3,35,36,38,75,98,160
Veillonella sp	3,35,98	3,35,36,38,75,98
Propionibacterium sp	–	38,75
Bifidobacterium sp	–	3,35,36,38,75
Eubacterium sp	35	35,36,38,75
Actinomyces sp	3,77	3,35,36,75,77,98
Prevotella sp	98	35,36,38,75,98,160
Porphyromonas sp	–	38
Bacteroides sp	3,35	35,36,38,75,98,160,
Fusobacterium sp	3,35,98	3,35,36,38,75,160
Leptotrichia sp	3	3,38

In studies 36, 38, 75 and 78 only the deep tonsillar flora was analysed

3. Functional morphology of PTs

3.1. Macroscopic structure

The mucous membranes have developed an integrated immune system, called the mucosa-associated-lymphatic-tissue (MALT), which populates the internal surfaces of the upper and lower respiratory tracts, the gastrointestinal tract and the urogenital tract. The main function of MALT is to collect antigens, to destroy them, and to generate effector and memory lymphocytes which migrate to the other mucosal sites and produce specific antibodies. The tonsils are the pronounced collections of MALT at the human oropharynx, forming the *Waldeyer ring*. The ring comprises the nasopharyngeal tonsil, or the adenoids, the paired tubal tonsils, the paired PTs and the lingual tonsil. The circular band at the oropharynx is completed by the tubopharyngeal plicae, called the lateral bands, and solitary lymphatic tissue collections in all parts of the mucosa.

Clinically, the most important are PTs, which possess several unique characteristics: (1) unlike the spleen or the lymph, they are not fully encapsulated and do not possess afferent lymphatics; (2) like both the spleen and the lymph nodes, they are lymphoreticular structures, but unlike them, the tonsils are also lymphoepithelial organs; and (3) the tonsillar epithelium not only provides a protective surface cover but also invaginates and lines the tonsillar crypts. The narrow, branching, anastomosing and blind-ending tonsillar crypts, running throughout the tonsillar tissue, are among the most characteristic features of PTs in humans and some animals (Abbey and Kawabata, 1988; Sato *et al*., 1990). Each PT contains 10–30 crypts, enlarging the epithelial surface of one PT to 300 cm^2, in addition to 45 cm^2 of the oropharyngeal surface epithelium, and greatly enhances the possibility for interaction between foreign antigens and the immune system (Howie, 1980; Perry, 1994; Perry and Whyte, 1998). At the same time, narrow and poorly drained tonsillar crypts are also of crucial importance in the pathogenesis of RT (Figure 1).

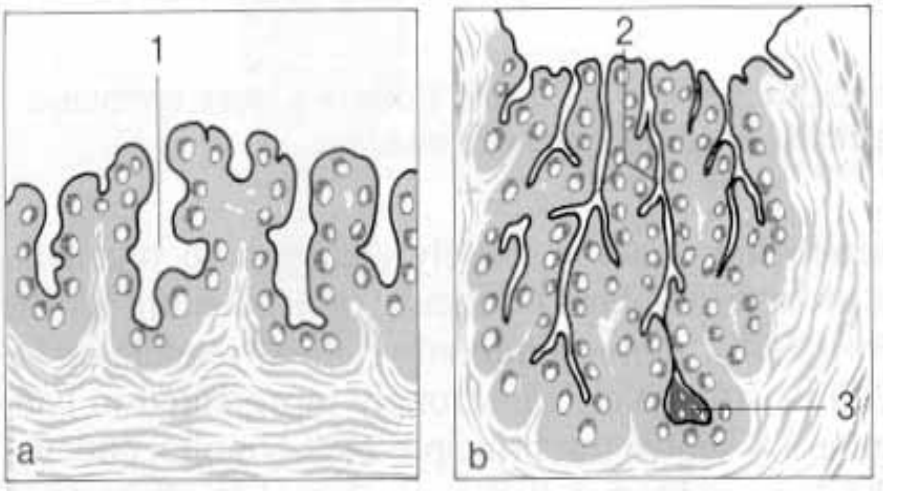

Figure 1. The structure of (a) the nasopharyngeal tonsil, the adenoids, and (b) the palatine tonsil. The broad flat niches opening into the oral cavity caused by infolding are called lacunae (1), the branching clefts running throughout the entire substance of tonsils are called crypts (2). The crypts usually contain cell debris, bacteria and fungi, but may also contain collections of pus, and encapsulated microabscesses (3). Reprinted with a permission from Becker *et al*., 1994.

3.2. Microscopic structure

The tonsillar tissue can be subdivided into several distinct, functionally interdependent microanatomical compartments: surface epithelium, crypt epithelium, lymphoid follicles and extrafollicular region.

Tonsillar surface and the crypt epithelium

The pharyngeal surface of PTs is covered by a nonkeratinized stratified squamous epithelium, which is avascular and contains very few nonepithelial cells. This epithelium is underlined by a band of thick connective tissue containing many vessels, nerves and lymphatics (Nieuwenhuis *et al*., 1992; Kelsoe, 1995; Kelsoe, 1996; Liu *et al*., 1996). The crypt epithelium is a modified form of the stratified squamous epithelium, a specialised lymphoepithelium, also called the reticulated or follicle-associated epithelium, which is underlined with disrupted basement membrane (Howie, 1980; Perry, 1994; Graeme-Cook *et al*., 1993). It contains a number of infiltrating nonepithelial cells, mainly B and T lymphocytes, neutrophils, macrophages and dendritic cells, and the network of capillaries and specialised venules, the so called high-endothelial venules, for entry of immune cells into the epithelium (Becker *et al*., 1994; Perry and Whyte, 1998; Bernstein *et al*., 1999; Výborná, 1999). The crypt epithelium is

the first tonsillar compartment that is challenged immunologically (Brandtzaeg and Halstensen, 1992; Yamanaka *et al*., 1996; Brandtzaeg *et al*., 1999). The antigens are sampled by specialized epithelial cells, which possess microvilli on their apical surface, resembling the intestinal microfold (M) cells (Neutra *et al*., 1996; Gebert, 1997; van Kempen *et al*., 2000).

Lymphoid follicles and the extrafollicular region

Shortly after birth, the germinal centres develop in primary lymphoid follicles, resulting in formation of secondary lymphoid follicles (Brandtzaeg, 1996). Germinal centres are composed of a dark zone, with large numbers of proliferating B blasts, called centroblasts, the basal and apical light zone, predominantly containing centrocytes, and the surrounding mantle zone with naive B cells (Banchereau *et al*., 1994; Brachtel *et al*., 1996). The secondary lymphoid follicles contain a network of follicular dendritic cells (FDC) and a special subset of germinal centre dendritic cells (Liu and Arpin, 1997). The extrafollicular region contains a majority of T cells (primarily of the T-helper phenotype), a network of interdigitating dendritic cells (IDC), macrophages and high-endothelial venules (Hoefakker *et al*., 1993; Perry and Whyte, 1998).

3.3. Immunologic functions of PTs

After passing the crypt epithelium, the antigens come into contact with antigen-presenting cells, IDC and macrophages, which present antigens to T-helper cells (Brandtzaeg and Halstensen, 1992; Brandtzaeg, 1995; Perry and Whyte, 1998). During T-cell-dependent antigen responses germinal centres develop, which provide a specialised microenvironment where B cells undergo extensive proliferation and differentiation into Ig-expressing memory B cells and Ig-producing plasma cells (Quidin *et al*., 1995; Camacho *et al*., 1998). The activated plasma cells in the germinal centres can produce all five Ig classes: IgG (~65%), IgA (~20%), IgM, IgD, IgE (Brandtzaeg *et al*., 1996; Boyaka *et al*., 2000; van Kempen *et al*., 2000). A substantial component of the humoral immune system of mucosal surfaces is secretory IgA (Figure 2), which is secreted by an epithelial receptor-protein complex into mucosal secretion (Morente *et al*., 1992; Quidin *et al*., 1995; Brandtzaeg, 1996; Butcher and Picker, 1996; Cebra *et al*., 1998). Normal immune function plays a central role in securing balance between the tonsillar microflora and the integrity of the mucosal membranes.

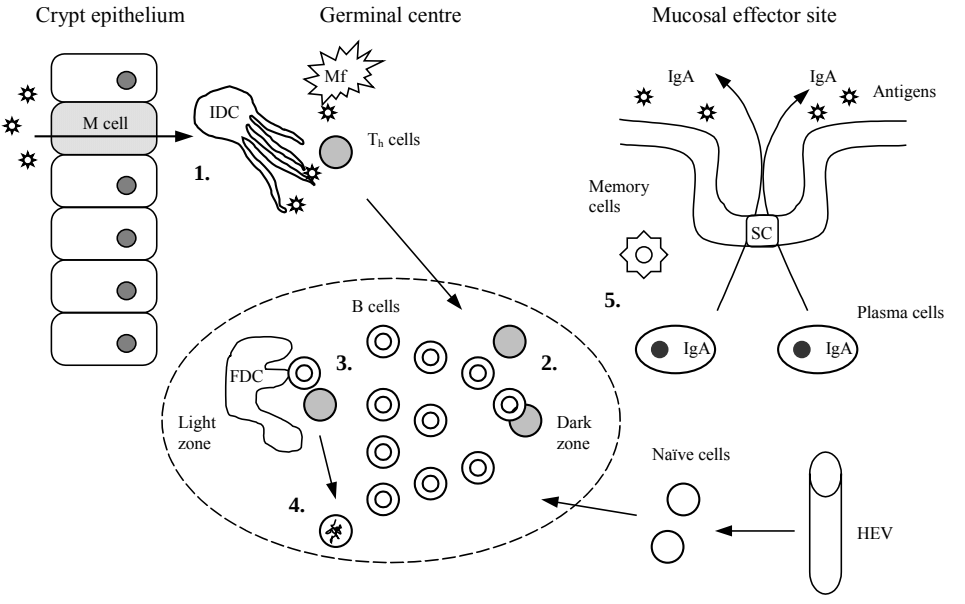

Figure 2. Schematic presentation of various important events leading to immune response in the upper respiratory tract. 1. An antigen is transported from the crypt lumen through M cells to interdigitating dendritic cells (IDC) and macrophages (Mf) and is further presented to T-helper cells. 2. Activated T-helper cells stimulate B cells (centroblasts) in the germinal centre dark zone in an antigen-specific manner. 3. Activated B cells (centrocytes) receive costimulatory signals from T cells and follicular dendritic cells (FDC) leading to their proliferation and differentiation into Ig-expressing memory B cells and Ig-producing plasma cells. 4. During appropriate B cell selection, self-reactive and unselected cells are turned to apoptosis. 5. B cells with J-chain expression differentiate into IgA-producing plasma cells which together with memory cells migrate to glandular mucosal effector sites where IgA polymers are exported by a secretory component (SC) into mucosal surfaces. Modified from Perry and Whyte, 1998 and van Kempen *et al*., 2000.

4. Pathogenesis of RT

4.1. Basic pathophysiology

The immune cells, including lymphocytes, neutrophils and macrophages, are shed in relatively large amounts from the tonsillar parenchyma and crypt epithelium into the lumen of the crypts, from where they pass further into the oral cavity. It has been estimated that one hundred million immune cells are shed by one tonsil daily into the digestive tract in this way. Besides immune

cells and cellular debris, the tonsillar crypts normally contain different bacteria and fungi some of which can be potential pathogens. As long as the crypts drain freely, the function of the tonsil is not endangered. However, even under physiologic conditions the branching crypts are poorly drained and the slightest tonsillar infection can easily cause stenosis of the crypts' neck leading to retention of cryptal content and distension of the crypts' bottom. This sets up an ideal culture medium for microorganisms, causing chronic suppuration (cryptitis), occurrence of small encapsulated abscesses in the crypts (Figure 1), and superficial ulceration of the surface of the crypts (Becker *et al*., 1994). Inflammation further extends into the tonsillar parenchyma, which in the long term undergoes more or less severe tissue fibrosis (Altemani *et al*. 1996; Michaels, 2001). In response to progression of chronic inflammation, hypertrophy of the surrounding lymphoid follicles (Eibling, 1997) and, more frequently, reduction in germinal centre size and atrophy of the tonsillar parenchyma have been described (Surjan *et al*., 1987; Zhang *et al*., 2003). Histopathologically, all this constitutes *chronic tonsillitis*.

Parenchymal fibrosis due to chronic inflammation is one of the basic alterations in diseased tonsils, which leads to many other histopathological features, such as obstruction of the crypts' neck together with distension of the crypts' bottom, and collection of cellular debris, bacteria and fungi in the crypt lumen. The latter results in changes in germinal centre size, keratinisation of the squamous epithelia lining the surface and crypts of PTs and focal destruction of the crypt epithelium (Farocki, 1967; Friedmann, 1986; Bieluch *et al*., 1989; Altemani *et al*., 1996; Zhang *et al*., 2003). However, tissue fibrosis may vary from local to generalised (Bieluch *et al*., 1989), which makes estimation of its degree difficult. Up to now, there are no widely accepted histopathological or biochemical markers serving as hallmarks of the pathogenesis of RT, which would facilitate finding of new strategies for diagnosis and treatment.

4.2. Immunopathology of recurrently inflamed PTs

Up to date, there are no systematic studies with good evidence focusing on immunopathological alterations in recurrently inflamed PTs and their impact on the patients' general health (Korsund and Brandtzaeg, 1981; Perry and Whyte, 1998; Nave *et al*., 2001). The interpretation of the immunomorphology of PTs is difficult as the concentration of lymphocytes in the tonsillar tissue varies greatly in different age groups, being usually the highest in children, and as any inflammatory process in the tonsils is superimposed onto normal cellular infiltration (Surjan, 1987; Perry, 1994). Nevertheless, many studies have found increased numbers of B and T cells, macrophages and dendritic cells in all microcompartments of recurrently inflamed PT as compared with hypertrophied or normal tonsils (Brodsky *et al*., 1988; Brodsky *et al*., 1996; Musiatowicz *et al*., 2001). There has been found a high number of activated T cells (Olofsson *et*

al., 1998) and hyperactivity of neutrophils in the epithelial layers of recurrently inflamed PTs (Ebenfeldt *et al*., 1996). In addition, an upregulation of the cytokine network with significantly increased production of IL-1α, IL-1β, IL-2, IL-6 and INF-γ has been described, indicating a persistent state of immuno-activation of such tonsils (Ågen *et al*., 1995).

In contrast, some other studies have revealed decreased numbers of B cells, macrophages and dendritic cells in the epithelial layers of PTs that express phenotypes of maturity and/or activation (Koch and Brodsky, 1993; Gorfien *et al*., 2001), and the lower ratio of CD4/CD8 cells when compared with hypertrophied or normal tonsils (Brodsky *et al*., 1988; Yamanaka *et al*., 1992; Olofsson *et al*., 1998; Musiatowicz *et al*., 2001). Decreased proliferation of CD4 cells has been associated with decreased expression rate of co-stimulatory molecules CD80 and CD86 (Yamanaka *et al*., 1992, Fujihara *et al*., 2005). Moreover, the higher number of tonsillitis episodes results in decreased Ig production by plasma cells (Bernstein *et al*., 1988; Koch and Brodsky, 1995), which may be associated with their reduced expression of the J-chain gene (Korsund and Brandtzaeg, 1981). Hence, two completely controversial understandings have been suggested: recurrently inflamed PTs have either hyperactive immune defence or breakdown of their function (Surjan *et al*., 1980; Brodsky *et al*., 1988; Hart *et al*., 1993; Koch and Brodsky, 1993; Brodsky et al., 1996; Ebenfelt *et al*., 1996; Olofsson *et al*., 1998; Gorfien *et al*., 2001). Unfortunately, the functional status of recurrently inflamed PTs, depending on the characteristics and extent of morphological alterations in their tissue, has not been studied. This could explain controversial outcomes from the aforementioned studies.

4.3. Promoting factors for post-tonsillectomy bacteremia

Up to 20–40% of surgical removals of recurrently inflamed PTs are followed by bacteremia, which is potential threat to the patient's health (Gaffney *et al*., 1992; Francois *et al*., 1992; Walsh *et al*., 1997; Anand *et al*., 1999; Kaygusuz *et al*., 2001). The process where viable bacteria of a normal indigenous microflora penetrate mucosal surfaces to reach the bloodstream and other normally sterile body sites has been defined as bacterial translocation (Wells *et al*., 1988; Berg *et al*., 1992; Deitch *et al*., 1998). Experimental and clinical studies have revealed that the factors promoting bacterial translocation include: i) disruption of the mucosal barrier; ii) compromised defence system of the host and bacterial overgrowth or alteration in the ecology of the indigenous microflora (Maddaus *et al*., 1988; Wells *et al*., 1988; Deitch, 1990; Berg *et al*., 1992; Mikelsaar *et al*., 1992; Gautreaux *et al*., 1994; Deitch *et al*., 1998).

The most important defence mechanism against bacterial translocation includes recruitment of immunocytes, particularly neutrophils and later macrophages, in response to acute injury and infection (Berg *et al*., 1992; Baran *et al*.,

1996; Fazal *et al*., 2000; Witko-Sarsat *et al*., 2000; Van der Laan *et al*., 2001). The damage of the tonsillar epithelia as a result of interactions between invading bacteria and the host immune cells may also enhance permeability of the mucous membranes. Whether the occurrence of post-tonsillectomy bacteremia depends on the increased load of invading bacteria in the deep tonsillar flora, or on the function of particular immune cells in the epithelial layers of the tonsils, which control bacterial invasion and spread, remains to be explored.

5. Clinical aspects

5.1. Surgical therapy of RT

Surgical removal of PTs, called tonsillectomy (TE), has been a leading therapeutic approach for RT both in children and adults (Younis and Lazar, 2002). Although it has been performed over 3000 years, being one of the most common operations in the history of surgery, the indications for TE have been a constant matter of debate and controversy (Curtin, 1987; Witt, 1989; Rosenfeld and Green, 1990; Bock *et al*., 1994; Blair, 1996; Mui *et al*., 1998; Darrow and Siemens, 2002; Discolo *et al*., 2003). Tonsillectomy has gone through periods of enthusiasm, as well as uncertainty, as to its overall benefit. In the early 20th century, TE was the most popular procedure for treating various respiratory and systemic diseases, with its popularity reaching a peak approximately 70 years ago (MacBeth, 1950; Kornblut, 1987). The rate of TE began to decline with the advent of antibiotics and critical assessments of the need for it (Mawson *et al*., 1967; Bluestone, 1985; Lildholdt *et al*., 2003). The growing understanding of the immunologic functions of PTs led to arguments against TE. Much of the controversy was focused on the benefits of removing chronically inflamed tissues *versus* the possible harm which TE may cause by eliminating enormous numbers of immune cells and protective antibodies from the mucosal surfaces (El-Ashmawy *et al*., 1980; Cantani *et al*., 1986). However, recent studies have found no significant long-term impairment of the immunological function and salivary defense capacity after removal of tonsils (Jung *et al*., 1996; Kirstila *et al*., 1996; Childers *et al*., 2001; İkincioğullari *et al*., 2002).

Traditionally, recommendation for use of TE has depended on the frequency of tonsillitis episodes. Patients with at least three episodes per year, despite adequate medical therapy, may be considered as candidates for TE, and surgical treatment is definitely recommended for patients with more than four or five episodes per year (AAO-HNS; BAO-HNS; SIGN, 1999). However, there is no world wide agreement among clinicians whether a specific number of tonsillitis episodes over a certain period of time warrants tonsillectomy. Many adults often have few or less severe tonsillitis episodes but they are characterised by

dominance of systemic effects of chronic disease, such as poor general health, tiredness, tendency to catch colds, unexplained fever or presence of comorbid diseases, such as cardiac valve disease, rheumatic fever, chronic glomerulonephritis or arthritis, as well as carriage state of *S. pyogenes* or increased serum concentrations of antibodies against this pathogen (Kornblut, 1987; Becker *et al*., 1994; Dagnelie *et al*., 1998; Mui *et al*., 1998; Faulconbridge *et al*., 2000; Bhattacharyya *et al*., 2001; Bhattacharyya and Kepnes, 2002, Darrow *et al*., 2002). These adults may benefit from TE due to the reduced number of days lost from school or work, number of health care visits, use of oral antibiotics and analgesics. Each of these reductions results in improved patients' well-being, educational achievements and quality of life, but also corresponding cost savings for either the medical management of the disease or for the economy as a whole (Roos *et al*., 1995; Mui *et al*., 1998; Bhattacharyya *et al*., 2001; Bhattacharyya and Kepnes, 2002). Therefore, besides the frequency of tonsillitis episodes, there could be other indicators for TE in adults suffering from chronic tonsillar disease.

Until now, no objective indicators are available for making a decision to perform TE. In older textbooks, macroscopic oropharyngeal signs, particularly the signs of sclerotic process in the tonsillar tissue, were recommended for the diagnosis of RT (Parkinson *et al*., 1951; Ballenger *et al*., 1954; Boies *et al*., 1964; Warner *et al*., 1964). Unfortunately, oropharyngeal examination is often blamed for the lack of scientific evidence and it has fallen out of favour in the past decades (Eibling, 1997; Hibbert and Cowan, 1997). We suggest that studying the pathogenesis of RT and correlating the data with the patients' anamnestic data and with the results of oropharyngeal examination could help find evidence-based indicators for TE in adults with RT.

5.2. RT and comorbid diseases

RT is of special clinical interest due to the possibility of severe accompanying comorbid diseases. An association between RT and occurrence of comorbid diseases remains a controversial issue, with opinions ranging from the rigorous denial of the possible association to the enthusiastic acceptance of such a hypothesis as a basis for treatment. Although some scepticism is justified, it must be granted that clinical experience affirms the plausibility of causal relationships between RT and concomitant inflammatory diseases of other organs and structures, and that there are at least some instances in which such relationships obviously exist. RT is known to play a role in the pathogenesis of glomerulonephritis and IgA nephropathy (Sato *et al*., 1996), arthropathy (Shido *et al*., 1992), reactive and rheumatoid arthritis (Kobayashi *et al*., 1996; Kawano *et al*., 2003), chronic inflammatory demyelinating polyneuropathy (Harsha *et al*., 2003) and pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (Swedo *et al*., 1998; Heubi and Shott, 2003). In the

pathogenesis of many of those diseases a key role has been attributed to *S. pyogenes* (Bisno, 1995). Unfortunately, the recovery rate of this pathogen by conventional throat culture both from children and adults with RT has been low (Stjernquist-Desatnik *et al*., 1990; Gaffney *et al*., 1991; Mitchelmore *et al*., 1994; Kuhn *et al*., 1995; Gaffney and Cafferkey, 1998; Inci *et al*., 2003; Lildholdt *et al*., 2003; Podbielski *et al*., 2003). Such low occurrence may be associated with the ability of *S. pyogenes* for intracellular penetration, which makes it non-cultureable (La Penta *et al*., 1994; Österlund and Engstrand, 1997; Österlund *et a*l., 1997; Norrby-Teglund and Kotb, 2000). On the other hand, an arborizing system of narrowed crypts, crypt abscesses in recurrently inflamed PTs, spongy epithelium and relatively unprotected blood vessels creates conditions ideal for the continuous dissemination of pathologic material (micro-organisms, toxic metabolites and inflammatory mediators) into the bloodstream (Becker *et al*., 1994). Although intracellular persistence of specific pathogens or clinically subthreshold bacteremia can both promote comorbid pathology, precise pathogenic mechanisms have remained unsolved and the diagnostic tools scarce.

6. Unsolved problems in the etiology and pathogenesis of RT and in its diagnostic and therapeutic modalities

There is no consensus over the terms *recurrent* and *chronic tonsillitis*. It is not clear whether they are separate nosologic entities or one and the same disease. The etiology of recurrent attacks of tonsillitis episodes has remained unclear. There has been found several potentially pathogenic aerobic and anaerobic bacteria in the surface and deep bacterial flora of PTs, but the isolation rate of specific pathogens has been low. Although pathologic shifts in the composition of the tonsillar microflora have been described, predominating populations have not been assessed.

Pathomorphological studies of PTs are restricted prior to their surgical removal due to the scattered spread of pathomorphological alterations throughout the tonsillar tissue. At the same time, the potential of estimation of the fibrotic tissue, combined with assessment of the immune status of recurrently inflamed PTs, in order to evaluate the function of the tonsils, has not been exploited. The promoting factors for bacteremia during TE are not known. Whether it depends on the impaired immune function of the tonsils or on the increased bacterial load in the tonsillar microflora has to be explored.

The value of anamnestic data and macroscopic oropharyngeal signs in the prediction of the functional status of recurrently inflamed PTs has not been studied.

AIMS OF THE STUDY

We aimed to find the anamnestic data and the macroscopic oropharyngeal signs that could be used as the indicators for tonsillectomy (TE) in adults with recurrent tonsillitis (RT). For this purpose, the functional status of recurrently inflamed palatine tonsils (PTs) was investigated by exploring the associations between the microbial ecology of PTs, the occurrence of bacteremia during TE and the characteristics of morphological alterations in the tonsillar tissue.

The specific aims of the research were:

1. to investigate the qualitative and quantitative composition of the deep tonsillar microflora in RT patients (Paper I);
2. to investigate the occurrence of *S. pyogenes* in recurrently inflamed PTs using microbiological and molecular methods, and to explore the presence of intracellular bacteria in the crypt epithelium using electron microscopic investigation (Papers I, III);
3. to study the associations between post-tonsillectomy bacteraemia, the proportion of aerobic and anaerobic bacteria in the deep microflora and the counts of immune cells in recurrently inflamed PTs (Papers I, II);
4. to explore whether recurrently inflamed PTs could be divided into different macroscopic types on the basis of oropharyngeal examination and whether such division could be confirmed by histopathological and biochemical investigations of the tonsils (Papers II);
5. to find macroscopic oropharyngeal signs predicting the functional break-down of PTs in patients with RT (Paper II);
6. to assess whether the anamnestic data, such as index of tonsillitis (consisting both the frequency of tonsillitis episodes and the duration of morbidity period), is associated with the macroscopic signs of sclerotic process in the tonsils on oropharyngeal examination (Paper III);
7. to explore what are the most frequently used anamnestic data, macroscopic oropharyngeal signs and diagnostic laboratory tests used by Estonian ENT surgeons when recommending TE in adults with RT (Paper IV).

MATERIALS AND METHODS

1. Study population

Patients with RT

The present research is divided into four parts, each was published as a separate paper (Table 2). The research involved altogether 72 RT patients (age range 15–35, median 22 years; 47 female and 25 male) selected from among 486 adults referred for TE due to recurrent attacks of tonsillitis episodes during the periods between October to December 2000, and March to June and September to December 2001 to the Department of Otorhinolaryngology, Tartu University Hospital. Every third patient (≥15 years of age) was selected from the operation list on two particular days of the week. Each patient had a history of RT episodes for at least one year, characterized by sore throat or swollen painful tonsils with fever or symptoms of systemic illness during exacerbations. The patients had been referred for TE by an ENT surgeon from the Department of Otorhinolaryngology, Tartu University Hospital. The exclusion criteria were acute tonsillitis exacerbation, acute viral respiratory infection, and antibiotic therapy within the two previous months.

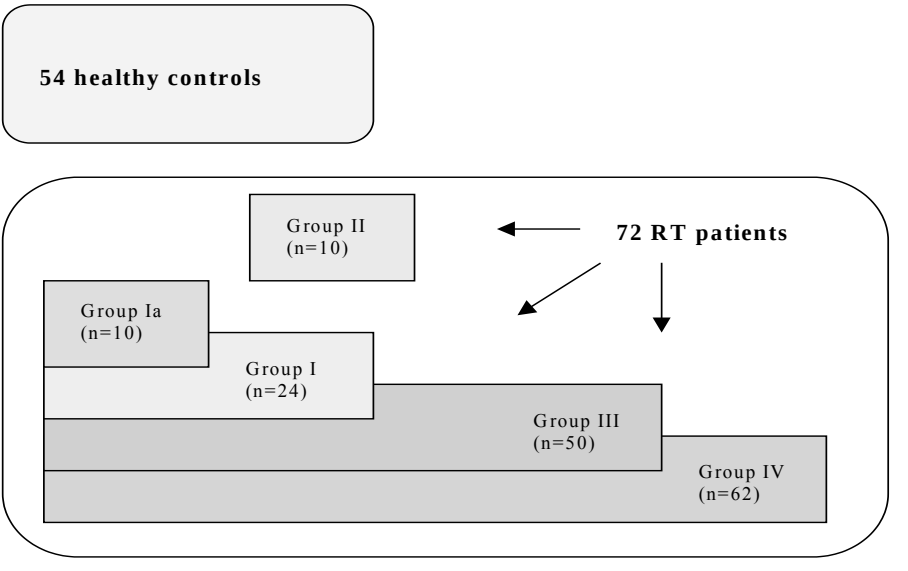

Figure 3. Schematic presentation of the division of the study groups. Group IV was formed by increasing the number of RT patients from 24 patients of group I to 50 patients of group III and further to 62 patients. From among 24 tonsils excised from the patients of group I, 10 specimens from the crypt epithelium were selected for transmission electron microscopy. In the remaining 10 out of a total of 72 patients (Group II), the occurrence of baseline bacteremia was detected. They were not included in the other patient groups or in the other studies. See Table 2 and the text for a more detailed description.

Healthy controls

The control group consisted of 54 healthy volunteer students (age range 18–24, median 20 years; 36 female and 18 male) who were not suffering from recurrent tonsillitis episodes (Table 2). The study was approved by the Tartu University Research Ethics Committee, and in each case written informed consent was obtained from each participant of the research.

Table 2. Study groups

Study subjects	Group	No of subjects	Samples	Methods	Original papers
RT patients	I	24	– clinical evaluation – tonsils – blood	– collection of anamnestic data – oropharyngeal examinations – microbial ecology of tonsils – blood cultures for aerobic/anaerobic bacteria – PCR for *S. pyogenes*	I
	Ia	10[a]	– tonsils	– TEM of the crypt epithelium	I, III
	II	10[b]	– blood	– blood cultures for aerobic/anaerobic bacteria	I
	III	50[c]	– tonsils – blood	– collection of anamnestic data – oropharyngeal examinations – histopathological investigations – immunohistochemistry – collagen content of tonsils – blood cultures for aerobic/anaerobic bacteria	II
	IV	62[d]	– clinical evaluation	– collection of anamnestic data – oropharyngeal examinations	III
Healthy controls	III	54	– clinical evaluation	– oropharyngeal examination	III
ENT surgeons	V	92	– questionnaire	– questionnaire-based survey	IV

[a] From among 24 tonsils excised from the patients of group I, 10 specimens from the crypt epithelium were selected for transmission electron microscopy (TEM)

[b] The patients of group II were not included in the other patient groups or in the other studies

[c] Group III was formed by increasing the number of patients with recurrent tonsillitis (RT) in group I from 24 to 50

[d] Group IV was formed by further increasing the number of RT patients in group III was from 50 to 62

Group I: the proportion of aerobic and anaerobic bacteria in recurrently inflamed PTs and its role in the development of post-tonsillectomy bacteremia.

The qualitative and quantitative composition of the deep tonsillar microflora was analysed and the proportion of aerobic and anaerobic bacteria was calculated in the excised PTs from 24 RT patients (15 female and 9 male, ranging from 15 to 42,

mean 24 years). Aerobic and anaerobic blood cultures were simultaneously taken from all 24 investigated patients during TE. The occurrence of *S. pyogenes* in the tonsillar tissue of the RT patients was analysed using PCR method (Paper I).

Group Ia: molecular detection of *S. pyogenes* in the tonsillar tissue and ultrastructure of the crypt epithelium.

The ultrastructure of the crypt epithelium was evaluated in randomly selected 10 PTs from among 24 patients of group I using TEM (Papers I, III).

Group II: occurrence of baseline bacteremia in RT patients.

The blood cultures were taken preoperatively from 10 patients with RT (6 female and 4 male, ranging in age from 20 to 27, mean 23 years), who served as controls for group I. These patients were not included in the patient groups or in the other studies (Paper I).

Group III: macroscopic oropharyngeal signs predicting the impaired defensive function of recurrently inflamed PTs.

The study involved 50 RT patients (31 female and 19 male, ranging from 15 to 45, mean 20 years). The study population of group III was formed by increasing the number of RT patients in group from 24 to 50. The anamnestic data and the data of oropharyngeal examinations were collected preoperatively. During operation, aerobic and anaerobic blood cultures were drawn. Immunomorphology of the excised PTs together with the measurement of their collagen content was performed postoperatively.

Group IV: association between the anamnestic data of RT patients and the macroscopic oropharyngeal sings of sclerotic process in the tonsils.

The study population was further increased from 50 to 62 RT patients and 54 healthy volunteers were included. The anamnestic data and the data of oropharyngeal examinations were collected from all 62 RT patients. In 54 healthy volunteers, oropharyngeal examinations were performed.

Group V: the questionnaire-based survey of Estonian ENT surgeons.

The survey involved all 92 ENT surgeons licensed to work in Estonia. An anonymous multiple-choice answer field questionnaire was used to explore what are the most frequently used anamnestic data, macroscopic oropharyngeal signs and diagnostic laboratory tests used by ENT surgeons in everyday practice when recommending TE in adults.

2. Clinical evaluations

Collection of anamnestic data and the data of oropharyngeal examinations

In RT patients, the collection of anamnestic data, including the number of tonsillitis episodes per year, duration of the morbidity period in years, presence of documented comorbid diseases, usage of antibiotics and changes in quality of life due to tonsillitis episodes was performed by one examiner and the oropharyngeal examinations were performed by another examiner who was

blinded to the type of the patients seen. In healthy controls, the same examiner conducted oropharyngeal examinations separately. Further, the index of tonsillitis (IT) was calculated by multiplying the number of tonsillitis episodes per year by the morbidity period in years (Fujihara *et al*., 2003).

Oropharyngeal examinations included the evaluation of the presence or absence of 6 macroscopic oropharyngeal signs: tonsillar sclerosis, scar tissue on the tonsils, obstruction of tonsillar crypts, hyperemia in the throat, cryptic debris and lymphatic tissue aggregates. Tonsillar sclerosis was defined as increased tightness of the tonsillar and peritonsillar tissues together with the fixation of PT in the tonsillar fossa. The scar tissue on the tonsils was defined as white tissue spots or streaks on the tonsillar surface. Obstruction of the tonsillar crypts was documented when a narrowing of the crypts' mouth, resulting in loss of clear cryptic pattern of the tonsillar surface, was observed. Cryptic debris was described as any white or yellow matter in the tonsillar crypts or in the supratonsillar cleft. Multiple round or elongated yellow-coloured patches on the retropharyngeal mucosa were described as lymphatic tissue aggregates, which are supposedly caused by enlargement of normal lymphatic structures in the throat (Parkinson *et al*., 1951; Ballenger *et al*., 1954; Boies *et al*., 1964; Warner *et al*., 1964; Eibling, 1997; Hibbert and Cowan, 1997).

Survey of Estonian ENT surgeons

An anonymous multiple-choice answer field questionnaire was sent to all 92 ENT surgeons licensed to work in Estonia. The list of questions is provided in Table 3.

Table 3. List of anamnestic data, macroscopic oropharyngeal signs and diagnostic laboratory tests from which the ear, nose and throat surgeons were asked to select their preferences, if any, when recommending tonsillectomy for recurrent tonsillitis in adults. Modified from Capper and Canter, 2001.

Anamnestic data	Oropharyngeal signs	Laboratory tests
A. number of tonsillitis episodes per year	A. mild hyperemia in the throat	A. isolation of *S. pyogenes* from a throat culture
B. number of health care visits due to tonsillitis per year	B. severe hyperemia in the throat	B. isolation of other groups of BHS from a throat culture
C. number of workdays missed per year	C. mild cryptic debris	C. isolation of any pathogenic bacteria from throat culture
D. frequent need for antibiotics due to tonsillitis	D. severe cryptic debris	D. elevated WBC count and CRP
E. frequent upper respiratory tract viral infections	E. tonsillar sclerosis	E. elevated ASO titre
F. unexplained high fever	F. obstruction of tonsillar crypts	
G. documented history of peritonsillar abscess	G. scar tissue on the tonsils	
	I. hypertrophic lymphatic tissue aggregates in the throat	
	J. enlarged jugulodigastric lymph nodes	

Anamnestic data	Oropharyngeal signs	Laboratory tests
H. frequent headache		
I. poor appetite		
J. snoring		
K. chronic fatigue and tiredness		
L. bad breath		
M. patient's concern about operation		
N. documented chronic glomerulonephritis		
O. documented rheumatic fever		
P. documented rheumatic heart disease		
Q. documented rheumatic or reactive arthritis		
R. asthma		

ASO – anti-streptolysin O; BHS – β-hemolytic streptococci; WBC – white blood cells; CRP – C reactive protein

3. Microbiological investigations

Blood culture sampling

Blood cultures from the RT patients, who were subjected to TE were drawn aseptically into BACTEC Plus Aerobic/F and a BACTEC Plus Anaerobic/F blood culture bottles (Becton Dickinson, USA) during the removal of the second tonsil (approximately five minutes after the removal of the first one). The blood culture bottles were promptly taken to the laboratory and incubated at 36°C in a fully automated blood culture instrument (Bactec 9050™, Becton Dickinson). All tonsillectomies were carried out under general anaesthesia with the use of orotracheal intubation by a standard dissection technique. All operated patients were followed up for postoperative infectious complications for 1 week.

The blood cultures from 10 RT patients of group II were drawn one day before the operation and before any oropharyngeal manipulation, administration of oral and parenteral drugs or having a meal. They served as controls to detect the occurrence of baseline bacteremia in RT patients.

The aerobic and anaerobic blood culture bottles were incubated for a total of 7 days. When an evidence of growth was noted, Gram staining and subculture on relevant plates under aerobic or anaerobic conditions were performed for the further identification of isolated strains (Murray *et al*., 1999). Aerobic and anaerobic bacteria were identified on the genus or group level. Isolated streptococci were identified by hemolysis on a blood agar plate and by the latex

agglutination test (Oxoid Ltd., UK). Among the gram-negative anaerobic blood culture isolates, the indole positive colistine sensitive isolate was identified as *Prevotella intermedia*, and the indole negative colistine resistant isolate, as *Prevotella melaninogenica*.

Qualitative and quantitative composition of the deep bacterial flora and proportion of bacteria in recurrently inflamed PTs

Immediately after excision, one of the tonsils was placed in a sterile Petri dish and taken on ice to the laboratory for microbiological analyses. One side of the tonsil was cauterised with a heated scalpel, and an incision was made through that area cutting the tonsil in half. For a tonsillar core culture (representing the deep microbial flora of the tonsillar crypts) approximately 0.2 g of tissue was aseptically excised and homogenised in a sterile mortar with a known amount of pre-reduced phosphate-buffered saline (PBS; pH 7.2) in an anaerobic glove box (Sheldon Manufacturing Inc., USA, with a gas mixture: 5% CO_2, 5% H_2, 90% N_2) and was further serially diluted (10^{-2}–10^{-7}). Serial dilutions of the tonsillar tissue were seeded on 8 freshly prepared media: horse blood, chocolate, Columbia, Endo, McConkey and de Man-Rogosa-Sharpe (MRS) agar for aerobic bacteria; and the Wilkins-Chalgren agar with vancomycin and a nalidixic acid supplement for gram-negative anaerobes; and the Wilkins-Chalgren agar with colistin sulphate and a nalidixic acid supplement for gram-positive anaerobes. The anaerobic plates were incubated for 5–6 days at 36˚C in an anaerobic glove box; the blood, chocolate, Columbia and MRS agar plates were incubated for 48 hours at 36˚C in an atmosphere enriched with 10% CO_2 in the Jouan IG150 incubator (Jouan, France), and the McConkey and Endo agar plates were incubated for 48 hours at 36˚C in an ambient atmosphere.

Colonies with different morphology, growing on the plates with the highest dilutions of bacteria, were Gram stained and subjected to microscopy and were further identified mostly on the genus or species level with the use of conventional methods (Murray *et al*., 1999). According to the growth results in serial dilutions, the count of microorganisms ($\log_{10}$ CFU/g – colony forming units per gram of the tonsillar tissue) from various genera and species were calculated for each patient. The detection level for bacteria was 3 $\log_{10}$ CFU/g. Further, the proportion of each isolated microorganism in the total count of microorganisms (%) was calculated (Mikelsaar, 1992).

4. Molecular methods

Total genomic DNA was extracted from tonsillar tissue samples by the method described previously (Louie *et al*. 1998). For the amplification of the specific *S. pyogenes* mitogenic factor gene (Iwasaki *et al*., 1993), the following primers were used: forward, 5´-CTA CTT GGA TCA AGA CGG-3´, and reverse, 5´-

TTA GGG TTT CCA GTC CAT CC-3′. The expected size of the amplified product was 419 base pairs. The PCR was performed in a 25 µl volume with a ~ 10 ng DNA sample by using a Ready-To-Go PCR Bead (Amersham Pharmacia Biotech Inc., USA). The extracted DNA of *S. pyogenes* ATCC 19615 served as a positive control. The PCR was performed for 35 cycles, with profiles of 95˚C for 30 s, 53˚C for 30 s, and 72˚C for 30 s, in an automated thermal cycler (Biometra, Eppendorf). The PCR products were analysed in a 2% ethidium bromide-stained agarose gel under ultraviolet light.

5. Histopathological and immunohistochemical investigations

Histological and immunohistochemical analyses were performed in collaboration with Ingrid Mesila, Department of Pathological Anatomy and Forensic Medicine, University of Tartu. The complete methodology is described in Paper II. After excision, a ~5 mm tissue section was cut vertically from the middle of one tonsil, perpendicular to the oropharyngeal surface, and was placed in 10% neutral buffered formaldehyde for 24 hours. Thereafter the samples were routinely processed and embedded in paraffin. Histological sections (5 µm slices) were stained with hematoxylin-eosin and polychrome. For immunohistochemical staining, CD15 (1:20) and CD68 (1:40) monoclonal mouse antibodies (DAKO, Denmark) were used to detect neutrophils and macrophages, respectively, in tonsillar microcompartments. This was followed by incubation with the biotinylated goat antibody to mouse immunoglobulins and streptovidin–biotin complex (StreptABC/HRPDuet, DAKO, Denmark). A distinctive brown reaction, visible by a light microscope, was developed with 3,3`-diaminobenzidine (Sigma-Aldrich Chemicals, USA). The sections were counterstained with haematoxylin.

All histological and immunohistochemical examinations were carried out without prior knowledge about blood culture results and the outcomes of oropharyngeal examination. On histological examination, the following features were evaluated: abnormal narrowing or distension of the crypts, the degree of infiltration of the crypt and the surface epithelium, keratinization of the crypt epithelium, interstitial fibrosis. All features were evaluated visually on a four-point scale where – represents the absence of changes, + mild, ++ moderate and +++ severe changes. The number of neutrophils and macrophages was quantitated for four different tonsillar microcompartments: the surface epithelium, the crypt epithelium, the follicular germinal centre and the extrafollicular area. The cells were counted per each microcompartment using an ocular square lattice grid, superimposed on the tonsillar sections under a light microscope (400x). For each individual the number of cells was recorded per 100-grid field in each of the 10 randomly selected microcompartments, which made an average of 0.625 mm² of each microcompartment per specimen for each cell type.

6. Electron microscopic investigations

Transmission electron microscopy (TEM) of the tonsillar tissue specimens was performed in collaboration with Andres Piirsoo, Department of General and Molecular Pathology, University of Tartu. Approximately 1 mm^3 samples from PTs were fixed with 2.5% glutaraldehyde (0.1M cacodylate buffer, pH 7.4) at 4ºC for 2.5h and postfixed with 1% osmium tetraoxide. After dehydration through an ethanol series and acetone, the samples were embedded in epoxy resin. Sections were cut with the ultratome MT-LX (RMC, USA). Semithin sections (1 μm) were stained with methylene blue, azure II eosin and basic fuchsin for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined by TEM using a Tecnai 10 electron microscope (FEI, Netherlands).

7. Measurement of collagen content

To determine the degree of sclerotic process in recurrently inflamed PTs, the biochemical detection of collagen content in the tonsillar tissue was introduced. The collagen content was derived from its hydroxyproline concentration in the tonsillar tissue, since this amino acid represents 13.4% of collagen (Medugorac, 1982). Hydroxyproline was measured by modifications of the previously described methods (Underwood *et al*., 2000). Briefly, freeze-dried tonsillar samples were hydrolysed in HCl, neutralised, freeze dried and reconstituted in citrate-acetate buffer. The diluted samples were mixed and incubated with chloramine T (Sigma-Aldrich, USA) Ehrlich's solution. The samples were cooled and then read at 560 nm on a Jenway 6400 (UK) spectrophotometer. Concentrations were calculated against a hydroxyproline standard curve with the GraphPat Prism software. The data were expressed as mg of collagen/g dry tissue weight.

8. Statistical methods

Statistical analyses were performed in cooperation with Krista Fischer, Department of Public Health, University of Tartu. Using 'Excel' (Microsoft Corp., USA), 'Statgraphics' (Statistical Graphics Corp., USA) and 'R' (The R Development Core Team) software, the Chi-square and the Mann-Whitney rank sum tests were employed for unpaired data and Student's *t*-test was used for paired data. Pearson's rank correlation test was used for correlation analyses. The specificity, sensitivity, positive (PPV) and negative predictive values (NPV) of macroscopic oropharyngeal signs were calculated. To find the macroscopic oropharyngeal signs predicting the impaired defensive function of

recurrently inflamed PTs, a logistic regression model was developed. In this model, the adjusted odds ratio (OR) with 95% confidence intervals (95% CI) were calculated to identify the variables that have either positive or negative association with the occurrence of post-tonsillectomy bacteremia. Based on the presence or absence of the two most common sclerotic signs on oropharyngeal examination, the receiver-operating characteristic curve (ROC) and the area under the curve (AUC) were constructed to ascertain the optimum cut-off score of IT for prediction of sclerotic tonsils (Van der Schouw *et al*., 1992). All differences were considered statistically significant for P-values less than 0.05.

RESULTS AND DISCUSSION

1. Microbial ecology of recurrently inflamed PTs (Papers I, III)

1.1. Occurrence of post-tonsillectomy bacteremia in adults with RT

Post-tonsillectomy bacteremia was found in 22 (44%) out of 50 RT patients subjected for TE. In one blood culture, the growth of two different bacteria (*S. pyogenes* and α-hemolytic streptococcus) was found (Table 4). None of the blood cultures from 10 control patients with RT showed any microbial growth.

Table 4. Aerobic and anaerobic bacteria isolated from positive blood cultures of 50 patients with recurrent tonsillitis during tonsillectomy

Aerobes	
α-Hemolytic streptococci	5
Group C β-hemolytic streptococci	4
Streptococcus pyogenes	3
Haemophilus influenzae	3
Moraxella catarrhalis	2
Anaerobes	
Peptostreptococcus sp	2
Bacteroides non-fragilis group	1
Prevotella sp	3
Total	23*

* In one patient *S. pyogenes* and α-hemolytic streptococcus were simultaneously recovered

The rate of post-tonsillectomy bacteremia was in general comparable with that found in children in other studies, although the recovery of fastidious anaerobes was higher than previously reported (François *et al*., 1992; Gaffney *et al*., 1992; Walsh *et al*., 1997; Anand *et al*., 1999; Kaygusuz *et al*., 2001). Such high incidence (>30%) has also been reported in cases when blood sampling was performed immediately after dental extractions in patients with periodontal disease (Mikelsaar and Türi, 1990). It is possible that there is no major difference between translocation rates of aerobes and anaerobes in children and adults, but that aerobes survive better than anaerobes in the bloodstream (Wells *et al*., 1988; Berg, 1992). Hence, the high rate of anaerobic bacteremia suggests that the blood sampling and culture technique used in the present research were well established.

1.2. Qualitative and quantitative composition of the deep tonsillar microflora

In all 24 investigated tonsillar core specimens, the mixed aerobic and anaerobic bacterial flora was found, yielding an average of 14.5 ± 2.5 (range 10–19) isolates per one PT including 7.5 ± 2.1 (range 4–11) aerobes or facultative anaerobes and 7.1 ± 1.7 (range 5–11) anaerobes (Table 5). The most frequently isolated aerobic bacteria were α- and β-hemolytic streptococci, *Staphylococcus aureus*, coagulase-negative staphylococci and *Corynebacterium* species. The prevailing anaerobes were *Peptostreptococcus*, *Propionibacterium*, *Actinomyces*, *Prevotella*, *Bacteroides* and *Fusobacterium* species. The mean count of aerobes was 7.2 ± 0.9 log10 CFU/g and that of anaerobes was 8.0 ± 0.9 $\log_{10}$ CFU/g, the latter outnumbering the former approximately 7 times.

Table 5. Microorganisms recovered in tonsillar core specimens from 24 patients with recurrent tonsillitis.

Microorganisms	Number of patients harbouring the isolate	Mean counts of organisms/g ($\log_{10}$ CFU ±SD)	Mean proportion of total count (%)
Aerobic and facultative bacteria			
α-Hemolytic streptococci	24	6.3 ± 0.9	1.8
Streptococcus pyogenes	–	–	–
Group C β-hemolytic streptococci	11	6.4 ± 1.0	2.2
Group F β-hemolytic streptococci	12	6.0 ± 1.1	0.8
Group G β-hemolytic streptococci	2	5.2 ± 1.3	0.1
Staphylococcus aureus	15	5.9 ± 0.9	0.7
Coagulase negative staphylococci	13	6.4 ± 1.6	2.2
Stomatococcus sp	10	4.2 ± 0.6	0.01
Enterococcus sp	2	4.5	0.02
Moraxella sp	8	5.2 ± 1.1	0.1
M. catarrhalis	11	5.3 ± 1.1	0.2
Neisseria sp	10	5.4 ± 1.0	0.2
Corynebacterium sp	16	5.7 ± 1.1	0.5
Lactobacillus sp	2	6.0 ± 0.9	0.8
Haemophilus influenzae	8	6.3 ± 0.9	1.8
H. parainfluenzae	11	5.0 ± 0.6	0.1
Eikenella corrodens	9	5.6 ± 1.0	0.4
Other non-fermentative	4	4.7 ± 0.5	0.1
Escherichia coli	1	5.2	0.1
Capnocytophaga sp	6	4.9 ± 0.8	0.1

Microorganisms	Number of patients harbouring the isolate	Mean counts of organisms/g (log_{10} CFU ±SD)	Mean proportion of total count (%)
Anaerobic bacteria			
Peptostreptococcus sp	22	7.4 ± 1.1	22.4
Veillonella sp	1	3.6	0.004
Propionibacterium sp	21	6.8 ± 1.1	5.6
Bifidobacterium sp	2	6.7 ± 2.1	4.5
Eubacterium sp	6	6.6 ± 1.0	3.6
Actinomyces sp	16	6.9 ± 1.0	7.1
Prevotella sp	22	7.0 ± 1.2	8.9
Porphyromonas sp	3	6.6 ± 1.0	3.6
Bacteroides sp	21	7.0 ± 1.2	8.9
Fusobacterium sp	22	7.4 ± 1.6	22.4
Leptotrichia sp	6	5.9 ± 1.1	0.7
Total aerobic isolates	175	7.2 ± 0.9	12.3
Total anaerobic isolates	142	8.0 ± 0.9	87.7
Total isolates	317	8.1 ± 0.9	100.0
Aerobes: anaerobes			1: 7.1

The prevalence and quantity of aerobic and anaerobic bacteria in the deep tonsillar microflora was similar to that found in children and adults with RT in other studies (Brodsky *et al*., 1988; Brook and Yocum, 1988; Kielmovitch *et al*., 1989; Brook *et al*., 1993; Mitchelmore *et al*., 1994; Kuhn *et al*., 1995; Lindroos, 2000). It has been demonstrated previously that the bacteria recovered from the tonsillar surface predict poorly the content of the deep tonsillar microflora, where the quantity of anaerobes is significantly higher (Brook and Yocum, 1981; Almadori *et al*., 1988; Kielmovitch *et al*., 1988; Surow *et al*., 1989; Gaffney *et al*., 1991; François *et al*., 1992). Therefore, we analysed only the composition of the deep tonsillar microflora, which has been considered the source of tonsillar infection.

1.3. Molecular detection of *S. pyogenes* in the tonsillar tissue

While no growth of *S. pyogenes* was found in recurrently inflamed PTs by culture analysis, the PCR method was simultaneously applied for the detection of its DNA in the tonsillar tissue (Figure 4). *S. pyogenes* was found by PCR in 7 out of 24 (29%) analysed culture negative tonsillar core specimens.

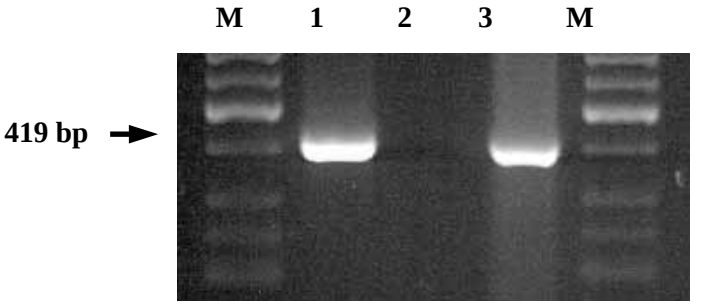

Figure 4. PCR amplification of a 419 bp fragment of the mitogenic factor of *S. pyogenes* following DNA extraction of the tonsillar core tissue. Lane M, DNA size marker; lane 1, positive sample from the tonsillar core; lane 2, negative control; lane 3, positive control (*S. pyogenes* ATCC 19615).

These data suggest that in particular patients such hidden and persistent infection by *S. pyogenes* may play an etiological role in the development of recurrent attacks of tonsillitis episodes. However, the limitation of DNA-based identification methods, including PCR, is that they do not differentiate between dead or living bacterial cells, giving the possibility of false positive cases.

1.4. Influence of bacterial proportions in the tonsils on the development of post-tonsillectomy bacteremia

The absolute count of various aerobic and anaerobic bacteria in the deep tonsillar flora of RT patients ranged from 3.0 to 9.0 $\log_{10}$ CFU/g. Further, the relative amounts of isolated microorganisms were calculated, expressed as the proportion of the total count of microorganisms (%). It was revealed that the predominating bacteria in the deep bacterial flora of recurrently inflamed PTs were *Peptostreptococcus* and *Fusobacterium* species. The proportions of *Prevotella* and *Bacteroides* species were also very close to the cut-off value, being 9%, respectively. The high proportion of anaerobic bacteria suggests that the conditions in chronically inflamed PTs facilitate first and foremost their growth. At the same time, all aerobic bacteria were found at subordinate concentrations in recurrently inflamed PTs (Figure 5). Among them, group F, C, and G β-hemolytic streptococci, coagulase-negative staphylococci and *Haemophilus influenzae* showed the highest proportions.

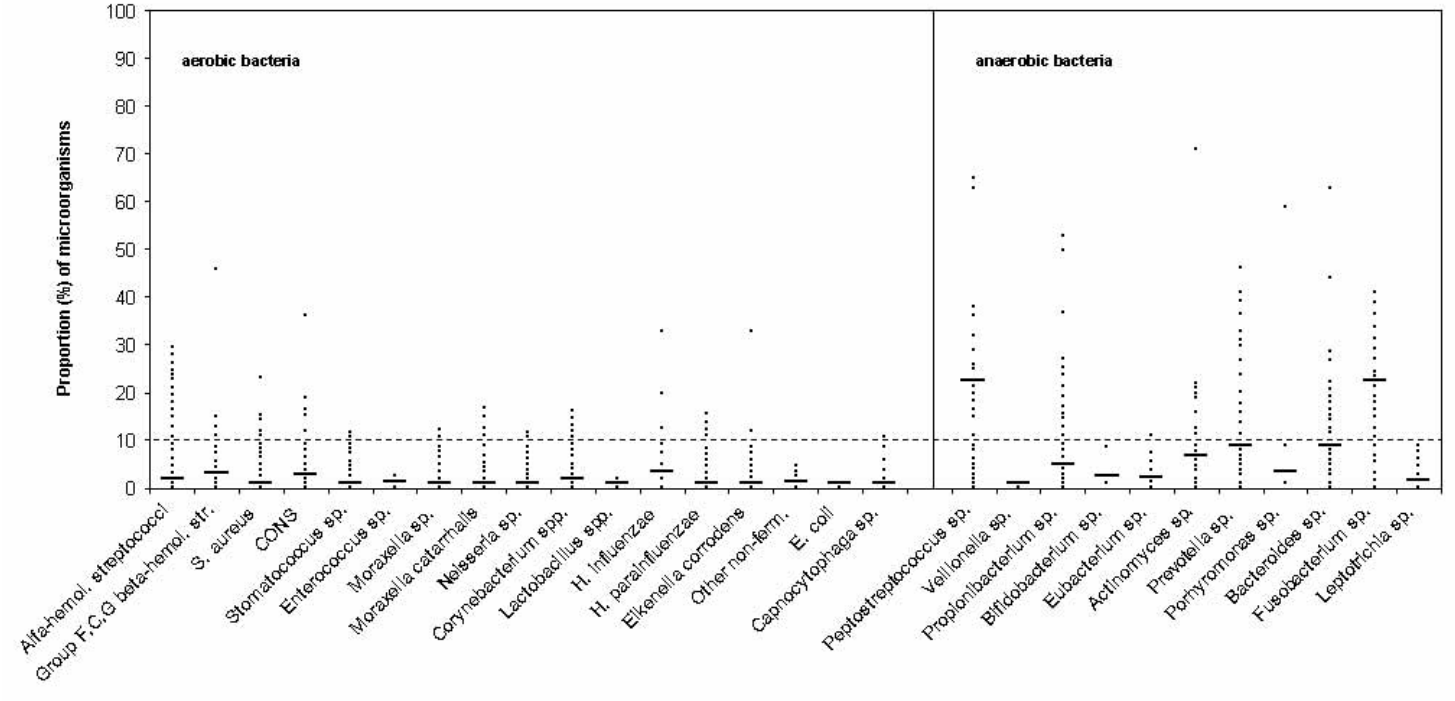

Figure 5. Proportions of aerobic and anaerobic bacteria in the deep tonsillar microflora of patients with recurrent tonsillitis. Each dot represents the proportion (%) of the bacterium in the total bacterial count in a single tonsillar core specimen. The short lines represent the mean of the proportions of the bacteria. The dotted line represents the 10% cut-off value.

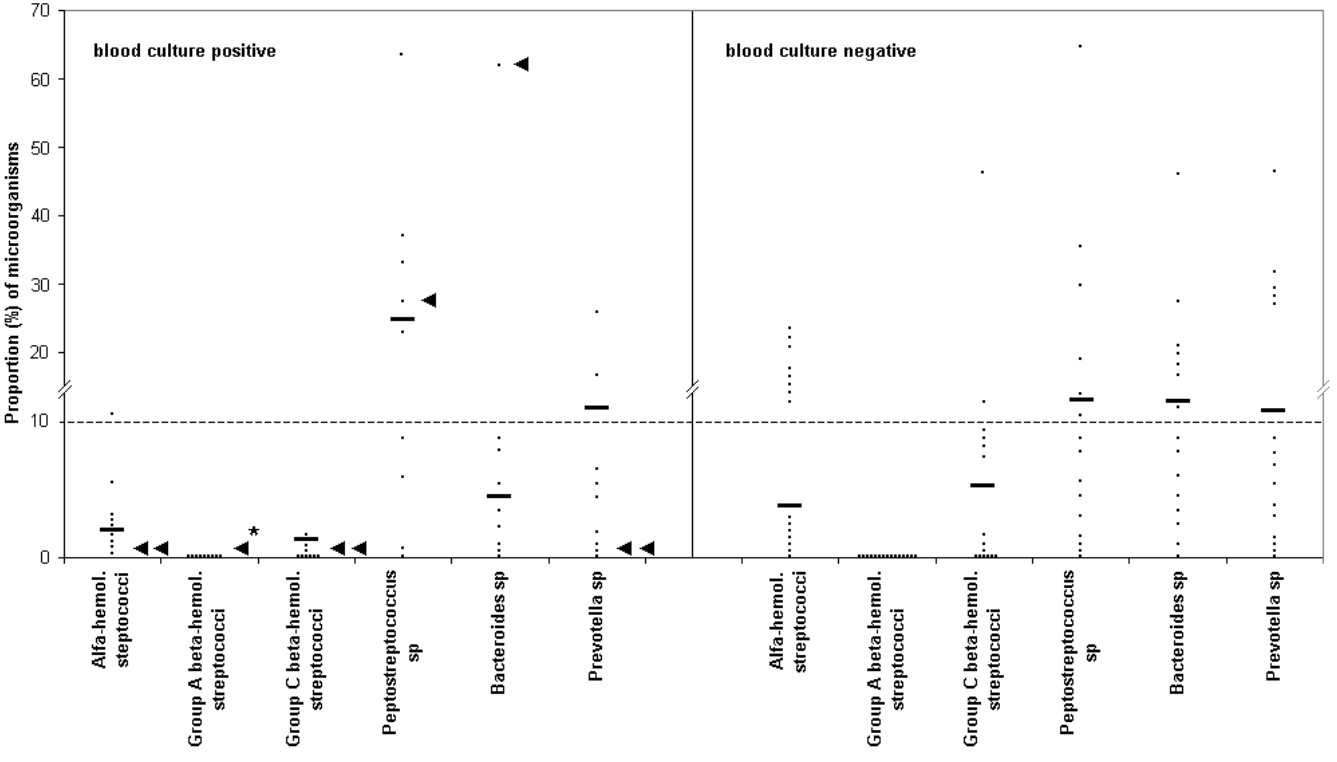

Figure 6. Predominance of blood culture isolates in the tonsils for the blood culture positive (n = 9) and negative (n = 15) groups. Each dot represents the proportion (%) of the bacterium in a single tonsillar core specimen and short lines denote the means of the proportions of the bacteria. The dotted line represents the 10% cut-off value.

In further analysis we assessed whether or not the proportion of invading bacteria in the deep tonsillar microflora could influence the development of post-tonsillectomy bacteremia. All blood culture isolates were recovered from the corresponding tonsillar tissue specimens by culture analysis, except *S. pyogenes*. Nevertheless, its presence in the tonsil of that particular patient was later established by PCR.

The most predominating bacteria in the tonsillar microflora of the blood culture positive and negative patients were *Peptostreptococcus* and *Prevotella* species whose proportions were over 10%. The blood culture negative patients had high proportions of *Bacteroides* species in their tonsils (Figure 6). However, only in cases of bacteremia caused by *Peptostreptococcus* sp. and *Bacteroides* sp. were their proportions in the corresponding tonsillar core specimen predominating (26.3% vs. 62.5%). Isolated α- and β-hemolytic streptococci and *Prevotella* species showed subordinate proportions (≤3%) in the tonsillar tissue or were below detection level as in the case of *S. pyogenes*.

These results indicate that bacterial invasion during operation may occur in spite of the very low count of the particular bacteria in the tonsillar tissue. We suggested that post-tonsillectomy bacteremia might be promoted either by the specific virulence factors of invading bacteria, which may be highly diverse (Aziz *et al*., 2004), or due to lowered host protection mechanisms.

2. Immunomorphology of recurrently inflamed PTs (Paper II)

2.1. Microscopic characteristics of recurrently inflamed PTs

Histopathological examination revealed a variable extent of morphological changes in the tonsillar tissue of RT patients. The epithelium of the pharyngeal surface of PTs consisted of the stratified squamous epithelium that was continuous with the epithelium lining of the branching crypts. The surface epithelium was infiltrated by non-epithelial cells in 46% of the cases and infiltration was mild in 29%, moderate in 13% and severe in 4% of the cases. The infiltration of the crypt epithelium was seen in all cases and it was mild in 13%, moderate in 50% and severe in 37% of the cases. Most of the infiltrating cells were lymphocytes, although neutrophils and macrophages were also seen.

Accompanying keratinization of the crypt epithelium was present in 89% of the cases and it was mild in 37%, moderate in 15% and severe in 37% of the cases. A characteristic feature was the narrowing of the crypts' neck, found in 63% of the cases, which was frequently accompanied with distension of the crypts' bottom. The lumina of the crypts were either empty, filled by intact immunocytes, degenerating cells, cellular debris, and fibrinoid or hyaline material containing bacteria (Figure 7A & B).

Interstitial fibrosis was seen in all tonsillar specimens and it was mild in 20%, moderate in 24% and severe in 56% of the cases. Unfortunately, the amount of fibrotic tissue was always unevenly distributed over the examined sections, which made the evaluation of its extent difficult. The number of germinal centres was low in 24%, moderate in 28% and high in 41% and absent in 7% of the cases.

Immunohistochemical staining of the tonsillar sections for neutrophils (CD15) and macrophages (CD68) revealed their different distribution in the tonsillar microcompartments. While macrophages were localized mainly in germinal centres (Figure 8A), the count of neutrophils was higher in the epithelial layers and in the extrafollicular areas (Figure 8B). The median count of neutrophils in the crypt epithelium was 22 (range 0–153) and in the whole tonsillar tissue 48 (range 0–318) cells per 0.625 mm^2 of the microcompartment. The median number of macrophages in the germinal centre was 187 (0–318) and in the whole tonsillar tissue 221 (range 0–599) cells per 0.625 mm^2 of the microcompartment. We found that the higher number of neutrophils in the crypt epithelium correlated with its higher numbers in the extrafollicular area ($r = 0.792$, $P = 0.001$) and in the surface epithelium ($r = 0.528$, $P = 0.001$). The location of neutrophils mainly in the crypt epithelium suggests that their most important role is to protect against bacterial invasion from the crypt's lumen into the tonsillar tissue. At the same time, the higher occurrence of macrophages in the germinal centres indicates their crucial role in activation of immune cells, their proliferation and differentiation.

2.2. Association between the counts of neutrophils and macrophages in PTs and occurrence of post-tonsillectomy bacteremia

The blood culture negative patients had significantly lower counts of neutrophils in all four microcompartments of PTs than blood culture negative patients (n=28). The median count of neutrophils in the crypt epithelium of the blood culture positive patients was 6 (range 0–49) and in the whole tonsillar tissue 36 (0–144) cells per 0.625 mm^2 of the microcompartment. At the same time, the median count of neutrophils in the crypt epithelium of the blood culture negative patients was 12 (range 0–95) and in the whole tonsillar tissue 62 (range 4–318) cells per 0.625 mm^2 of the microcompartment. These differences were statistically significant, $P = 0.028$ and $P = 0.035$, respectively (Figure 9). No differences were found in the counts of macrophages between the blood culture positive and negative patients. Nor was any statistically significant difference found between the blood culture positive and negative groups regarding the histopathological changes described above. These findings suggest that the development of post-tonsillectomy bacteremia is associated specifically with lowered count of neutrophils in the crypt epithelium of recurrently inflamed PTs.

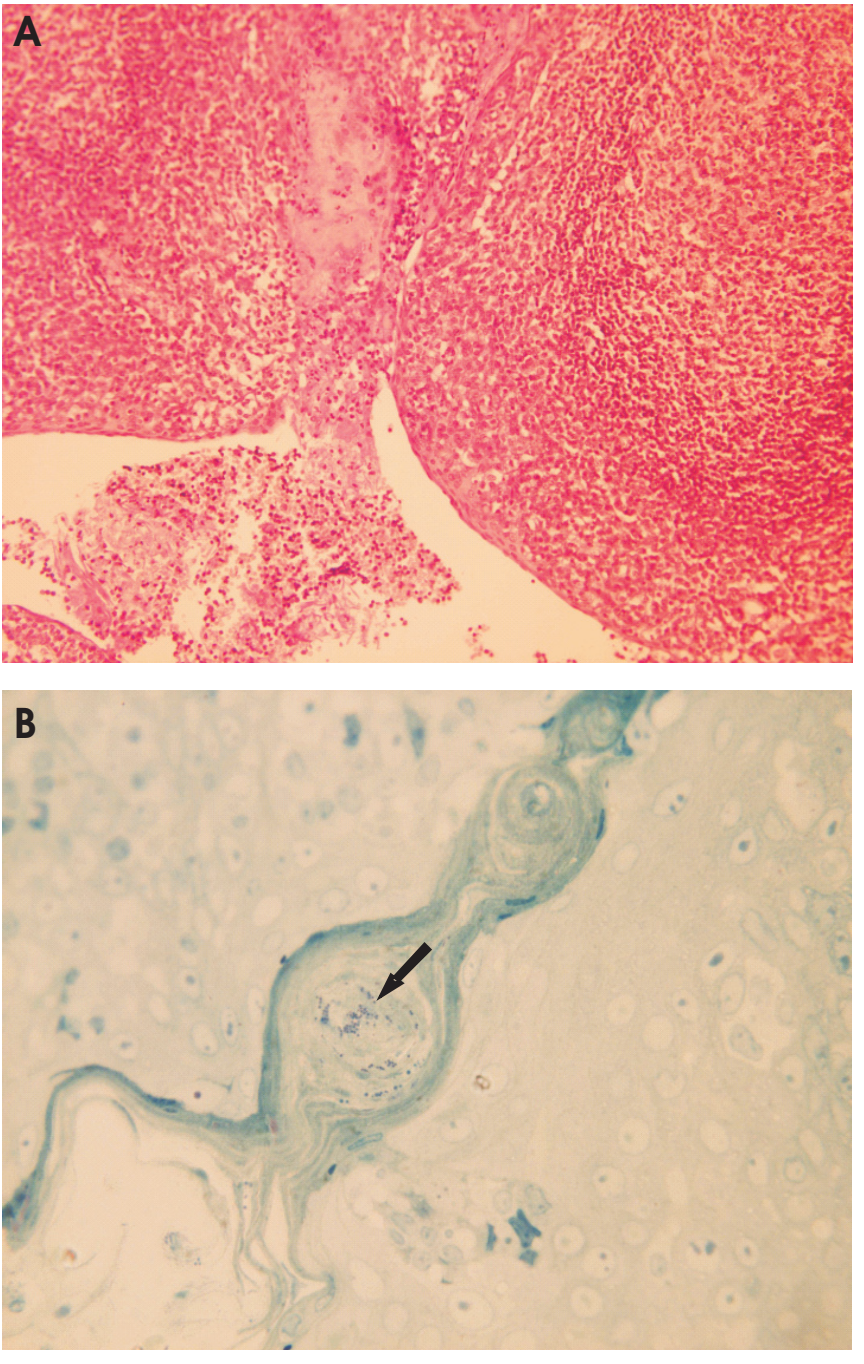

Figure 7. Histological sections of the tonsillar crypt. A) Obstruction of the crypt's lumen by cellular debris and hyaline material (H E; original x200). B) Irregular narrowing and distension of the crypt's lumen filled by hyaline material containing bacteria (arrow) (polychrome; original x1000).

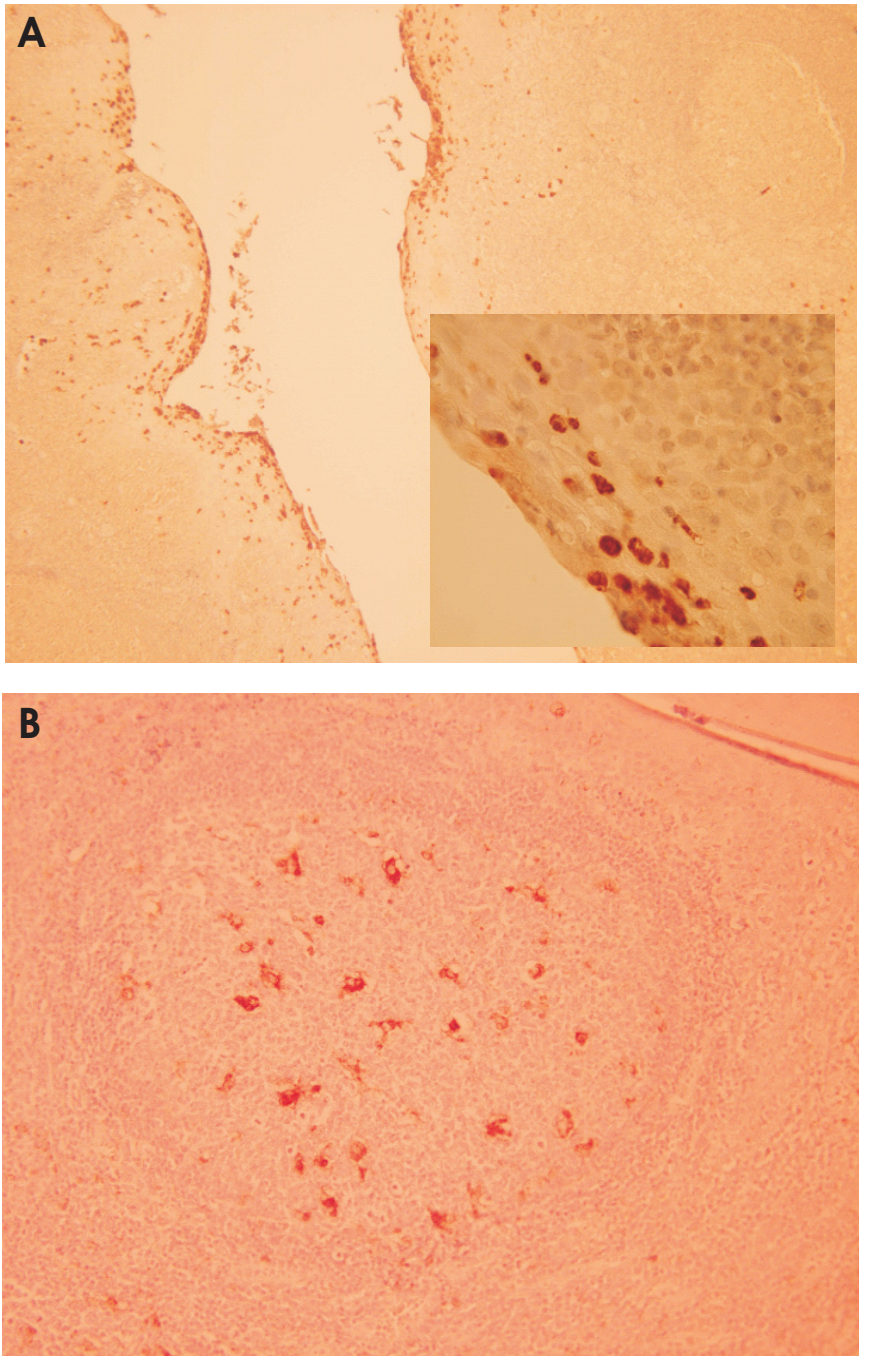

Figure 8. Immunohistochemical staining of neutrophils and macrophages in the palatine tonsils. A) CD15 staining of neutrophils in the crypt epithelium (original x100 and x400). B) CD68 staining of macrophages in the germinal centre (original x100).

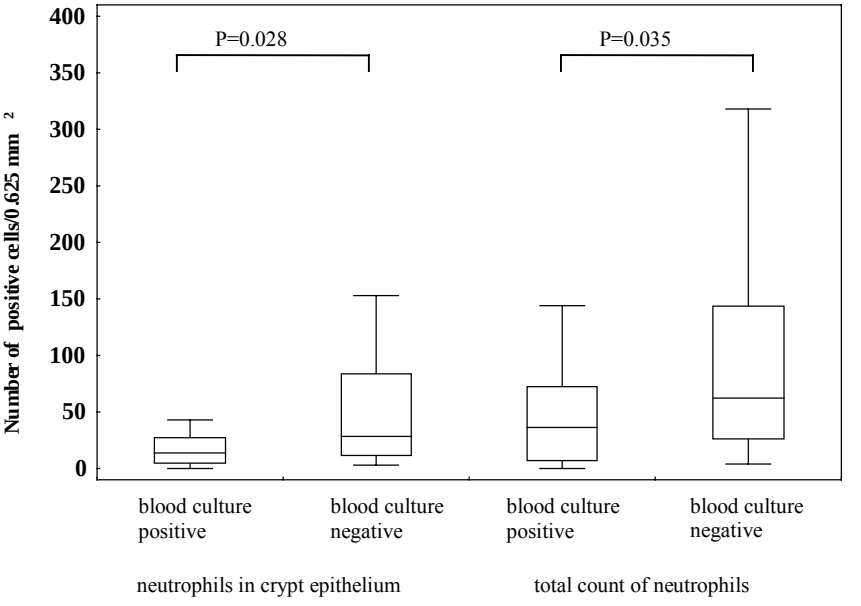

Figure 9. Box-plots of the counts of CD15 marked neutrophils in the crypt epithelium and in all tonsillar microcompartments of the blood culture positive and negative patients. Data are median counts (—) and distribution (box display 25th–75th quartile area, bars 10th–90th percentile area).

2.3. Ultrastructure of the crypt epithelium

As the PCR results indicated persistence of *S. pyogenes* in recurrently inflamed PTs, the ultrastructure of the crypt epithelium of the 10 removed PTs was investigated by TEM in order to find intracellular bacteria. We found various morphotypes of bacteria on the surfaces of epithelial cells, while many of them were in intimate contact with the cell membrane. Many coccoid forms of the bacteria were either penetrating into the cells or were located completely intracellularly (Figure 10A). The bacteria within the cells were usually intact and surrounded by cytoplasmatic tonofibrils (Figure 10B & C).

The intact crypt epithelium in most specimens was abundantly infiltrated by nonepithelial cells, including neutrophilic granulocytes, which were tightly packed between the epithelial cells (Figure 10D). However, in the case of damage of tight junctions between the epithelial cells, with the remaining desmosomes only on projections, free spaces appeared between the adjacent epithelial cells (Figure 10E). These gaps were frequently occupied by damaged or degenerating granulocytes with intact granules and bacteria (Figure 10F).

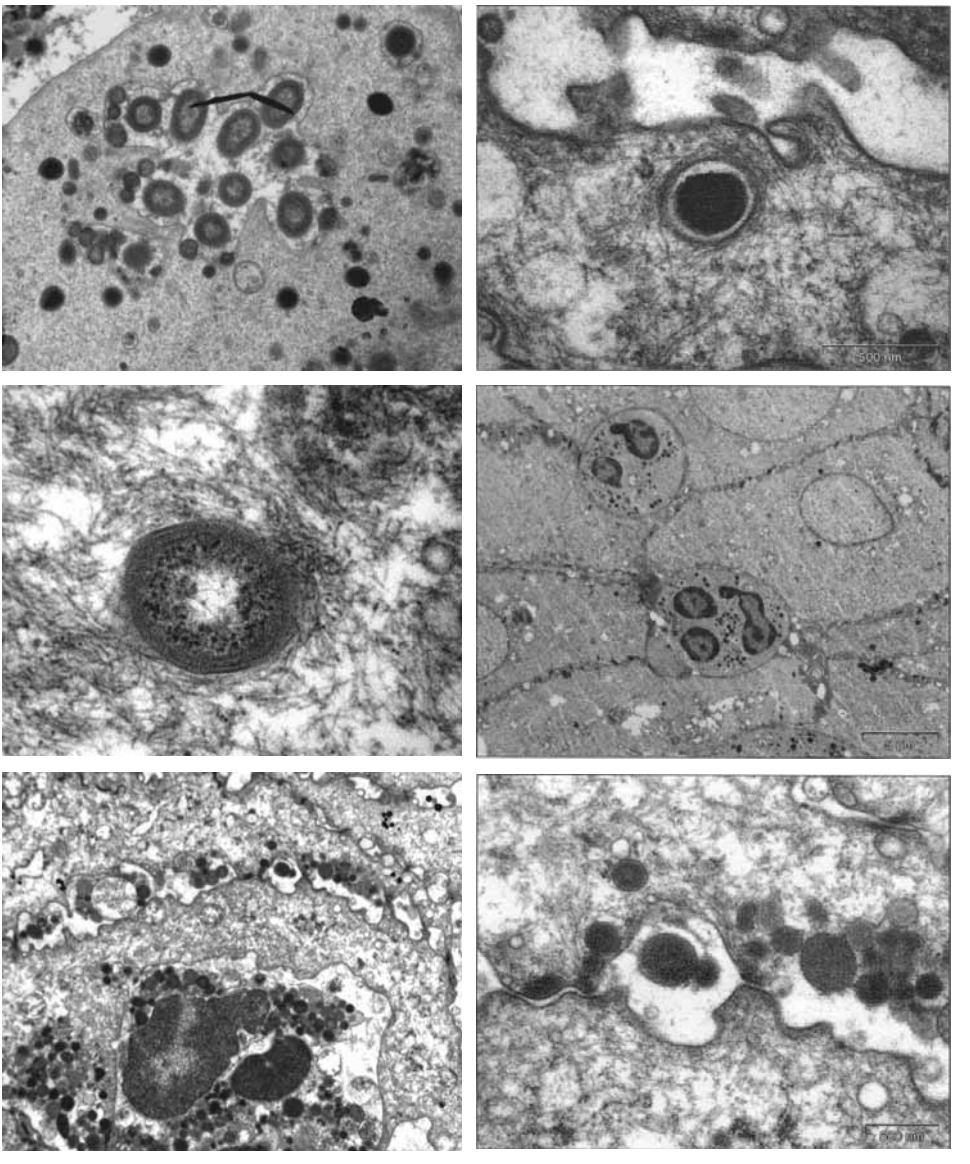

Figure 10. Transmission electron microscopy of the crypt epithelium of the palatine tonsils. A) Coccoid forms of bacteria within the epithelial cell in the crypt epithelium (original x10000). B, C) Intracellular bacteria surrounded by cytoplasmatic tonofibrils (original x44000 and x73000. D) Granulocytes between the intact epithelial cells in the crypt epithelium (original x2100). E) Damage of the tight junctions between the epithelial cells with the remaining desmosomes and free spaces between the adjacent epithelial cells (original x7000. F) The gaps between epithelial cells occupied by damaged or degenerating granulocytes with intact granules (original x27000).

Ability of *S. pyogenes* to penetrate into host cells is a well known phenomenon (La Penta *et al*., 1994; Österlund and Engstrand, 1997; Neeman *et al*., 1998; Berkower *et al*., 1999, Norrby-Teglund and Kotb, 2000). Its reservoir within epithelial cells has been associated with repeated attacks of tonsillitis episodes (Österlund *et al*., 1997). Although previous TEM studies have found morphologically different bacteria within epithelial cells during acute tonsillar infection, only intracellular *S. pyogenes* was considered to be responsible for epithelial damage (Stenfors *et al*., 2000; Stenfors *et a*l., 2001). These indirect pieces of evidence support our suggestion that persisting intracellular bacteria, specifically *S. pyogenes*, is one of the reasons for maintaining continuous inflammation in the tonsillar tissue.

3. Anamnestic data, oropharyngeal signs and diagnostic laboratory tests used most frequently by ENT surgeons in Estonia (Paper IV)

The response rate to the questionnaire was 58 out of 92 ENT surgeons. As three returned questionnaires were incomplete, 55 (60%) remained for final analysis. Among anamnestic data, the number of tonsillitis episodes and previous history of peritonsillar abscess were considered the most important indicators for TE. However, there was no agreement about a specific number of episodes that warrants surgical intervention in adult patients. Besides that, great attention is also paid to presence of systemic effects of RT, particularly comorbid diseases, when selecting candidates for TE. Less important were decreased quality of life due to missed workdays or increased number of health care visits (Table 6). Interestingly, the patients' own concern about operation influenced the decision of nearly one quarter of the practitioners. These findings are similar to previous studies where decreased quality of life due to continuous inflammation in PTs and its systemic effects has been considered appropriate indications for TE in adults (Bhattacharyya *et al*., 2001; Capper and Canter, 2002; Bhattacharyya and Kepnes, 2002; Darrow *et al*., 2002).

Among the macroscopic oropharyngeal signs, the occurrence of severe cryptic debris was considered as the most valuable sign in 80% of the cases, being closely followed by tonsillar sclerosis in 76% of the cases. The two other signs of sclerotic process, the scar tissue on the tonsils and the obstruction of tonsillar crypts, were less frequently considered, in 40% and 24% of the cases, respectively. Enlarged lymph nodes in the jugulodigastric group and severe hyperemia in the throat also seem to have a significant influence on the decision to undertake TE in adults. These data indicate that the signs of active inflammation, such as severe cryptic debris and hyperaemia in the throat or enlarged cervical lymph nodes, were considered more important by ENT

surgeons compared with the signs of sclerotic process, when recommending TE in adults.

The survey showed that diagnostic laboratory tests are performed in order to establish the occurrence of *S. pyogenes* in the throat flora of RT patients by means of culture analysis or by determining the anti-streptolysin O (ASO) titre. Isolation of group B, C, F and G β-hemolytic streptococci and of any other pathogenic bacteria from the throat culture were considered less important.

Table 6. The anamnestic data, macroscopic oropharyngeal signs and diagnostic laboratory tests used by ENT surgeons when recommending tonsillectomy for RT in adults

Disease history data	ENT (%)
A. number of tonsillitis episodes a year	100
B. documented history of peritonsillar abscess	100
C. documented rheumatic fever	89
D. documented rheumatic heart disease	80
E. documented rheumatic or reactive arthritis	78
F. documented chronic glomerulonephritis	75
G. frequent need for antibiotics due to tonsillitis	69
H. unexplained fever	66
I. chronic fatigue and tiredness	46
J. bad breath	33
K. patient's concern about operation	24
L. number of workdays missed per year	20
M. number of health care visits due to tonsillitis per year	11
Oropharyngeal signs	
A. severe cryptic debris	80
B. tonsillar sclerosis	76
C. enlarged jugulodigastric lymph nodes	69
D. severe hyperemia in the throat	49
E. scar tissue on the tonsils	40
F. obstruction of the tonsillar crypts	24
G. enlarged tonsils	13
H. hypertrophic lymphatic tissue aggregates in the throat	9
I. mild cryptic debris	8
J. mild hyperemia in the throat	7
Laboratory tests	
A. elevated ASO titre	93
B. isolation of *S. pyogenes* from the throat culture	86
C. isolation of BHS from the throat culture	46
D. elevated WBC count and CRP	24
E. isolation of any pathogenic bacteria from the throat culture	18

ASO – anti-streptolysin O; BHS – β-hemolytic streptococci; WBC – white blood cells; CRP – C reactive protein

The survey indicated that Estonian ENT surgeons take some range of the anamnestic data, the oropharyngeal signs and the results of diagnostic laboratory tests into account when recommending TE in adults. However, no uniform criteria were reported.

4. Selection of indicators for TE in adults (Papers II, III)

4.1. Collection of anamnestic data and the data of oropharyngeal examinations

Out of 62 RT-TE patients, 26 (42%) patients had six or more, 10 (16%) had four to five and 26 (42%) patients had three or less tonsillitis episodes per year. The median number of tonsillitis episodes in the whole group of RT-TE patients was 4.5 per year. The duration of morbidity ranged from 1 to 23 years; the median being 6 years. There was no difference in the length of morbidity between patients with four or more and patients with three or less tonsillitis episodes per year; the median being 7 and 5 years respectively. The comorbid disease was documented in 14 (22%) RT patients: rheumatic heart disease in 7, unspecified polyarthritis in 5, and both rheumatoid arthritis and glomerulonephritis in one patient.

The macroscopic oropharyngeal signs were classified as the signs of inflammation and the signs of sclerotic process (Table 7). The most common macroscopic oropharyngeal sign was cryptic debris, which was observed in all RT patients. Its occurrence in the healthy controls was significantly lower than hyperemia and enlarged lymphatic tissue aggregates in the throat, which were almost equally common in the RT patients and in the healthy controls. Hence, among the inflammatory signs, cryptic debris had the highest specificity and sensitivity and predictive values for the diagnosis of RT.

The most common sclerotic sign in the RT patients was the scar tissue on the tonsils, but it was also frequently found in the healthy controls. Tonsillar sclerosis and obstruction of the crypts were less frequently found in the healthy controls, but were observed in nearly half of the RT patients. Among the sclerotic signs, tonsillar sclerosis had the highest specificity and PPV, while scars on the tonsils showed the highest sensitivity and NPV.

Table 7. Prevalence of the macroscopic oropharyngeal signs in patients with recurrent tonsillitis and healthy controls together and their specificity, sensitivity and predictive values.

Groups of signs	Patients (n=62, %)	Healthy controls (n=54, %)	Speci-ficity	Sensi-tivity	PPV[a]	NPV[b]
Signs of inflammation						
cryptic debris	62 (100)	9 (17)	0.83	1.00	0.87	1.00
hyperemia in the throat	49 (79)	34 (63)	0.37	0.79	0.59	0.60
hypertrophic lymphatic tissue	38 (61)	22 (41)	0.59	0.61	0.63	0.57
Signs of sclerotic process						
tonsillar sclerosis	29 (47)	2 (4)	0.96	0.47	0.94	0.61
scar tissue on the tonsils	49 (79)	11 (20)	0.80	0.79	0.82	0.77
obstruction of the tonsillar crypts	34 (55)	8 (15)	0.85	0.55	0.81	0.62

[a]PPV – positive predictive value, [b]NPV – negative predictive value

4.2. Predictors for the development of post-tonsillectomy bacteremia

Fifty RT patients out of 62, in whom blood cultures were taken and immuno-morphology of PTs was performed, were subdivided into two separate groups: 1) those with 'sclerotic-type' tonsils (n=29, Figure 11B); and 2) and those with 'inflammatory-type' tonsils (n=33, Figure 11C & D). The sclerotic type tonsils were characterised by the presence of all three macroscopic signs of sclerotic process: tonsillar sclerosis, obstruction of the tonsillar crypts, and scar tissue on the tonsils. The patients with inflammatory type tonsils had also some random signs of sclerotic process but never all three signs at once. Their tonsils were usually soft in consistency, accompanied with hyperemia in the throat and lymphatic tissue aggregates on the retropharyngeal mucosa. The biochemical detection of collagen content in the tonsillar tissue revealed a significant difference between the macroscopically defined groups. Mean collagen content in the sclerotic-type tonsils was 137.3 ± 47.2 mg/g of dry tissue weight and in the inflammatory-type tonsils 87.9 ± 43.9 mg/g (P=0.001).

The presence of tonsillar sclerosis was in close association with the presence of the scar tissue on the tonsils (OR=16, 95% CI 2.82–303.1, P=0.01) and with the obstruction of tonsillar crypts (OR=7.67, 95% CI 2.54–26.11, P=0.0005). The signs of inflammation had no association with each other or with the sclerotic signs. The data suggest that the sclerotic signs are all the result of a continuous inflammatory process in the tonsillar tissue (Friedmann, 1986; Altemani *et al*., 1996; Michaels, 2001).

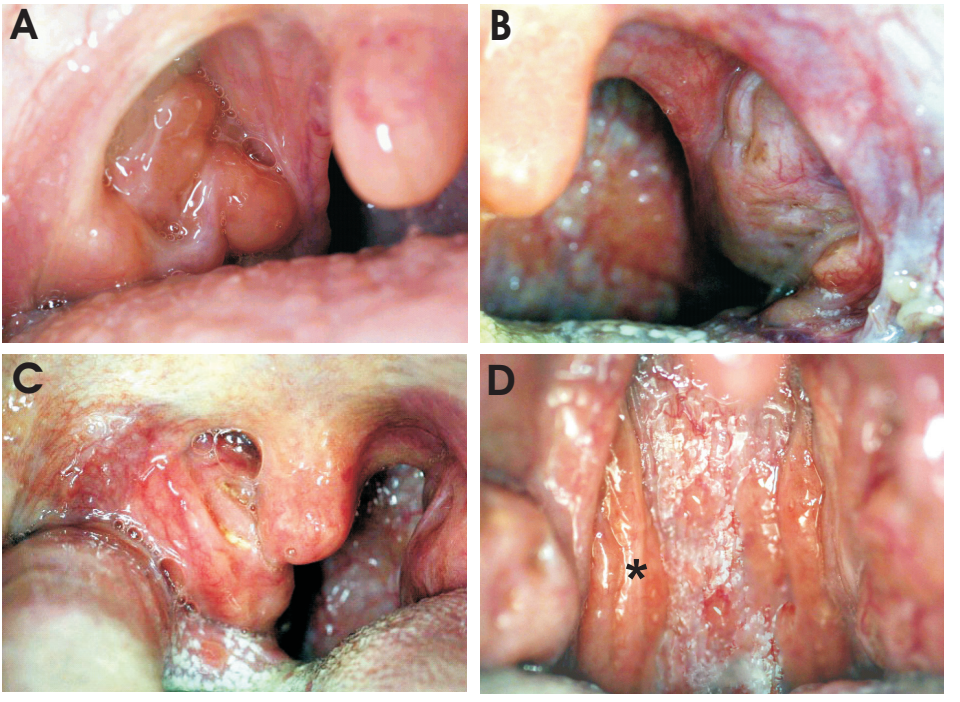

Figure 11. Oropharyngeal examination of the healthy person and of patients with recurrent tonsillitis. A) Normal palatine tonsil. B) Sclerotic type tonsil. Remarkable pallor of the tonsillar surface and narrowing of the crypts' mouth. C, D) Inflammatory-type tonsils. Severe hyperemia of the faucial arches, cryptic debris and visible hypertrophy of the lymphatic tissue on the postpharyngeal wall (asterix).

No correlation was found between the clinically established sclerotic type tonsils and histologically described tonsillar tissue fibrosis. This may be due to the irregular location of the sclerotic tissue and the limited number of the tonsillar sections investigated. At the same time, the biochemical detection of collagen content revealed marked differences between the sclerotic and the inflammatory type tonsils. Such approach has not been previously described in literature. It confirms adequacy of a macroscopic classification of tonsils and seems to be a useful tool in defining the stages of the clinical course of chronic inflammation.

In further analysis, associations between macroscopic oropharyngeal signs, the counts of neutrophils and macrophages in the tonsillar microcompartments and the occurrence of post-tonsillectomy bacteremia were studied. Sixteen out of 24 patients with the sclerotic type tonsils had a positive blood culture and 8 had a negative blood culture, while only 6 patients with the inflammatory type tonsils out of 26 had a positive blood culture and 20 had a negative blood culture (P=0.002). There were no statistically significant differences in the count of macrophages in all four tonsillar microcompartments between the sclerotic and the inflammatory type tonsils. The mean number of neutrophils was remarkably lower in all microcompartments of the sclerotic type tonsils than in the inflammatory type tonsils, but this difference was not statistically significant.

Table 8. Predictors for the development of post-tonsillectomy bacteremia in patients with recurrent tonsillitis

Predictors	Blood culture		Adjusted OR	95% CI	P-value
	Positive	Negative			
Inflammatory type tonsils	6	20	3.65	0.92–14.44	0.065
Sclerotic type tonsils	16	8	9.89	2.41–40.52	0.0015
Total count of neutrophils in tonsillar tissue	mean: 46.0[$] (SD=43.5)	mean: 93.0[$] (SD=85.9)	0.30*	0.09–0.98	0.047

[$]per 0.625 mm^2
*per 100 cell difference

To determine the predictors for post-tonsillectomy bacteremia, a logistic regression model was subsequently developed. All oropharyngeal signs, and the counts of neutrophils and macrophages in the tonsillar microcompartments were entered as the independent variables, and occurrence of post-tonsillectomy bacteremia was set as a dependent variable. After adjusting for the confounding

effects of all variables considered, it was revealed that the post-tonsillectomy bacteremia was strongly associated with presence of sclerotic type tonsils and with low count of neutrophils in the tonsillar microcompartments (Table 8). This suggests that the sclerotic process in recurrently inflamed PTs can lead to lowered counts of neutrophils in its tissue, which impairs the defensive function of such tonsils and allows generalisation of infection.

4.3. Prediction of functionally impaired tonsils on the basis of anamnestic data

In further analysis we assessed whether the anamnestic data (the frequency of tonsillitis episodes per year and length of the morbidity period) are associated with the macroscopic signs of sclerotic process in the tonsils and with the PCR results for *S. pyogenes*. The aim was to find new indicators for TE in adults with RT.

We found that higher frequency of tonsillitis episodes was in strong correlation with occurrence of obstructed tonsillar crypts and longer morbidity period was in strong correlation with tonsillar sclerosis and with presence of *S. pyogenes* in the tonsillar tissue by PCR (Table 9). The length of the morbidity period in the patients with sclerotic type tonsils was two times larger than in the patients with the inflammatory type tonsils, mean 10.33±5.96 and 5.04±2.98 years, respectively (P=0.001). These findings suggest that sclerotic process and its consequences in recurrently inflamed tonsils take a long time to develop. It is in accordance with a previous study where the evidence of tonsillar scarring was more frequently found in adults than in children with RT (Brook and Foote, 1986). The correlation between longer morbidity period and presence of *S. pyogenes* by PCR, together with the TEM results, suggests that one of the reasons for maintenance of recurrent inflammation in the tonsils may be persistent infection by intracellular bacteria.

Table 9. Correlation between the patients' disease history data, presence of sclerotic signs in the tonsils and PCR data on *Streptococcus pyogenes*.

History data	Signs of sclerotic process			
	Tonsillar sclerosis	Obstruction of crypts	All three signs	PCR for S.pyogenes
Frequency of tonsillitis episodes	NS	R_p= 0.354 P= 0.005	R_p= 0.299 P= 0.018	NS
Morbidity period	R_p= 0.437 P= 0.001	NS	R_p= 0.318 P= 0.011	R_p= 0.503 P= 0.012
Index of tonsillitis	R_p= 0.384 P= 0.002	NS	R_p= 0.325 P= 0.01	NS

R_p – Pearson correlation coefficient. NS – statistically nonsignificant correlation

In further analysis, the index of tonsillitis (IT) was calculated. The median IT for the whole group of RT patients was 30 (range 6–138). The IT values were in good correlation with number of sclerotic signs on oropharyngeal examination ($r = 0.325$, $P = 0.010$). Based on the IT values and presence or absence of tonsillar sclerosis and obstruction of tonsillar crypts, a ROC curve with AUC was constructed to ascertain the cut-off score of IT (Figure 12).

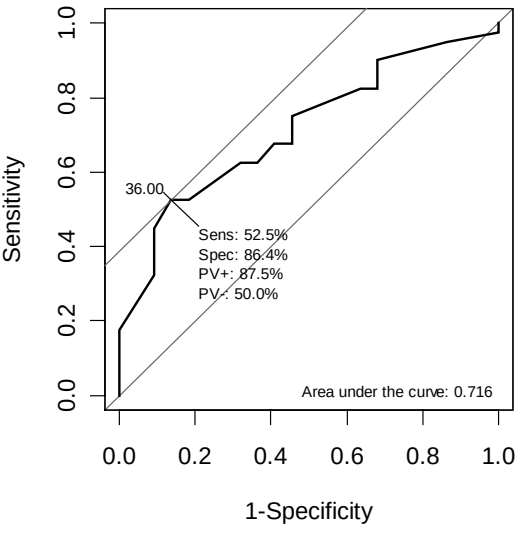

Figure 12. A receiver-operating characteristic curve (ROC) was calculated to find the index of tonsillitis (IT) predicting the sclerotic type tonsils. The optimal cut-off score of IT was 36 (the area under the curve, AUC = 0.716), with a sensitivity of 52.5%, a specificity of 86.1%, a positive predictive value of 87.5% and a negative predictive value of 50.0%.

The ROC curve revealed that an IT score of 36 is the optimal cut-off value for prediction of the sclerotic type tonsils (AUC = 0.716). This cut-off score indicates that a minimum of 36 tonsillitis episodes are required for the development of the sclerotic type tonsils. We suggest that a specificity of 86.1% and a PPV of 87.5% for IT 36 is high enough to use it for differentiating patients with advanced tonsillitis from less severe cases. As this score predicts the sclerotic process in recurrently inflamed tonsils, which have possibly lost their defensive function, high IT values could serve as an indicator for TE in adults.

GENERAL DISCUSSION

The aim of the research was to find out the anamnestic data and the macroscopic oropharyngeal signs that could be used as the indicators for tonsillectomy (TE) in adults with recurrent tonsillitis (RT). For this purpose, the functional status of recurrently inflamed palatine tonsils (PTs) was investigated by exploring the associations between the microbial ecology of PTs, the occurrence of bacteremia during TE and the characteristics of morphological alterations in the tonsillar tissue.

1. Altered microbial ecology in recurrently inflamed PTs

An abundant mixed aerobic and anaerobic bacterial flora was found in the deep bacterial flora of recurrently inflamed PTs. The most frequently isolated aerobic bacteria were α- and group β-hemolytic streptococci, *Staphylococcus aureus*, coagulase-negative staphylococci and *Corynebacterium* species. The most prevailing anaerobes were *Peptostreptococcus*, *Propionibacterium*, *Actinomyces*, *Prevotella*, *Bacteroides* and *Fusobacterium* species. The mean count of anaerobes was significantly higher than the mean count of aerobic bacteria, the first outnumbering the latter in an average 7 times. Although anaerobes outnumber aerobic bacteria also in the normal tonsils, their absolute counts and their ratio between them are significantly lower (Brook and Foote, 1990). Therefore, overgrowth of anaerobes seems to be one of the characteristic features of the tonsillar microflora of RT patients (Kielmovitch *et al*., 1989; François *et al*., 1992; Brook *et al*., 1995; Kuhn *et al*., 1995).

In further analysis, the relative amounts of isolated microorganisms were expressed as the proportion of the total count of microorganisms (%) in the deep tonsillar microflora. The bacteria found at the highest proportions were anaerobes, most often *Peptostreptococcus*, *Fusobacterium*, *Prevotella* and *Bacteroides* species. The proportional analysis of the tonsillar microflora has not previously been performed. As the absolute count of aerobic and anaerobic bacteria varies greatly from one microbial ecosystem to another, the composition of the indigenous microflora between different individuals is difficult to compare. Proportional analysis evens up such individual differences, increasing the adequacy of analysis of the microflora (Mikelsaar, 1992).

The qualitative and quantitative analysis of the tonsillar microflora, revealing the most prevailing and predominating aerobes and anaerobes, has a low value when exploring the etiology of RT. Although some isolated bacteria are potential pathogens, they are normally prevalent on the surface of the tonsils and the pharynx in healthy persons (Brook and Foote, 1990; Tanaka *et al*., 1996; Stjenquist-Desatnik and Holst, 1999; Brook, 2005). In this situation, discrimination between the etiological agents and the commensals is almost

impossible, making the interpretation of culture analyses difficult (McKerrow, 2002; Inci *et al*., 2003, Lildholdt *et al*., 2003; Podbielski *et al*., 2003). The high count of different bacteria, particularly the 'overgrowth syndrome' of anaerobes, may simply indicate that under pathologic conditions the growth of those microorganisms is facilitated first and foremost. Therefore, conventional culture analysis could only reveal randomly balanced ratios of aerobes and anaerobes in the microflora of diseased tonsils, while it is of no help when determining the need for TE in adults with RT.

2. Occurrence of intracellular bacteria in recurrently inflamed PTs

We found no growth of *S. pyogenes* in the tonsillar core specimens by culture analysis. The recovery rate of *S. pyogenes* from adults with RT has also been low in other studies when the culture method was used (Brook and Yocum, 1984; Stjernquist-Desatnik *et al*., 1990; Mitchelmore *et al*., 1994; Lildholdt *et al*., 2003; Podbielski *et al*., 2003). The low incidence of *S. pyogenes* or its absence in RT patients has been explained by its growth inhibition by oral α-hemolytic streptococci or some anaerobic bacteria, or by its misidentification as another group of β-hemolytic streptococcus (Fujimori *et al*., 1995; Brook, 1999; Brook and Gober, 1999; Hamrick and Mangum, 1999). However, simultaneously applied PCR revealed the occurrence of *S. pyogenes* in nearly one-third of the culture negative tonsillar specimens. As *S. pyogenes* is known to have great ability for intracellular penetration, being non-cultureable (La Penta *et al*., 1994; Österlund and Engstrand, 1997; Neeman *et al*., 1998; Berkower *et al*., 1999), the recovery of *S. pyogenes* from RT patients by culture analysis may be falsely low. Moreover, these data suggest that conventional culture analysis may miss several non-cultureable pathogens, which possibly play a role in the pathogenesis of RT.

Using electron microscopy, it was possible to confirm the presence of coccoid forms of intracellular bacteria in the crypt epithelium of recurrently inflamed tonsils. The presence of intracellular bacteria was in many cases accompanied with the damage of epithelial cells and the connections between them, the so called tight junctions. Formed intercellular spaces were occupied by multiple intact granules, supposedly released by phagocytes, and bacteria. The latter were freely residing in between the cells, closely adhered or penetrating into the epithelial cells. Previous electron microscopic studies have provided evidence that intracellularly residing *S. pyogenes* could be responsible for epithelial damage during acute tonsillar infection (Stenfors *et al*., 2000; Stenfors *et a*l., 2001). *S. pyogenes* can survive intracellularly during antibiotic therapy and cause recurrent attacks of tonsillitis by escaping later from epithelial cells (Österlund and Engstrand, 1995; Österlund and Engstrand, 1997;

Cunningham, 2000). We found the presence of *S. pyogenes* in the tonsillar tissue, established by PCR, was in strong correlation with longer morbidity period. Although TEM could not identify the type of intracellular bacteria, our data suggest that hidden persistence of *S. pyogenes* in the tonsils may play a role in maintenance of recurrent inflammation in the tonsillar tissue.

3. Promoting factors for the development of post-tonsillectomy bacteremia

Post-tonsillectomy bacteremia was found in 44% of the RT patients, which corresponds to the highest rates reported in literature (Gaffney *et al*., 1992; Francois et al., 1992; Walsh *et al*., 1997; Anand *et al*., 1999; Kaygusuz *et al*., 2001). Experimental and clinical studies have shown that the factors promoting bacterial translocation through the epithelial layers into the bloodstream include a disruption of the mucosal barrier, bacterial overgrowth or alteration in the ecology of the indigenous microflora and a compromised defence system of the host (Maddaus *et al*., 1988; Wells *et al*., 1988; Berg *et al*., 1992; Gautreaux *et al*., 1994; Deitch *et al*., 1998). As the physical disruption of the mucosal barriers is an essential part of surgery, we explored the influence of the proportion of invading bacteria in the deep tonsillar flora, as a promoting factor, and the number of particular immune cells in the PTs, i.e. the cells controlling bacterial invasion and spread in the host, on the development of post-tonsillectomy bacteremia.

Microecological analysis of the deep tonsillar microflora revealed that the bacteria at the highest proportions were not more prone for translocation during surgical removal of PTs. This is in accordance with a study where the absolute count of blood culture isolates in the tonsillar tissue of children with RT was not the highest (François *et al*., 1992). On the contrary, we found that bacterial invasion during TE could occur in spite of the very low proportion of a given bacterium in the tonsillar microflora.

Previous studies have found either hyperactive immune function or, on the contrary, a functional breakdown of recurrently inflamed PTs (Surjan *et al*., 1980; Brodsky *et al*., 1988; Hart *et al*., 1993; Koch and Brodsky, 1993; Onerci *et al*., 1995; Brodsky et al., 1996; Ebenfelt *et al*., 1996; Olofsson *et al*., 1998; Gorfien *et al*., 2001; Fujihara *et al*., 2005). Unfortunately, the functional status of recurrently inflamed PT,s depending on the characteristics and extent of morphological alterations in its tissue, has not been studied. As recruitment of immunocytes, particularly neutrophils and further macrophages, is one of the most important defence mechanisms in response to acute injury and infection (Berg *et al*., 1992; Baran *et al*., 1996; Fazal *et al*., 2000; Witko-Sarsat *et al*., 2000; Van der Laan *et al*., 2001), we assessed the occurrence of post-tonsillectomy bacteremia in relation to the counts of these immunocytes in

different microcompartments of recurrently inflamed PTs. We found that the number of neutrophils in all tonsillar microcompartments, particularly in the crypt epithelium, was significantly lower in the blood culture positive patients than in the blood culture negative patients. At the same time, no difference was found in the counts of macrophages in the same microcompartments between the blood culture positive and negative patients. Hence, instead of the high load of the invading bacterium in the deep tonsillar microflora, the lowered count of neutrophils in the crypt epithelium of recurrently inflamed PTs seems to be a more important factor in promoting post-tonsillectomy bacteremia.

4. Selection of indicators for TE in adults with RT

A leading therapeutic approach for RT has been TE. The most widely used indicator for surgical therapy has been the defined frequency of tonsillitis episodes per year as reported by the patient. Tonsillectomy could be considered for patients with at least three episodes per year and surgical treatment is definitely recommended for patients with more than four or five episodes per year (AAO-HNS; BAO-HNS; SIGN). However, such approach seems to be appropriate for children, but not for adults who often have fewer or less severe tonsillitis episodes, and may have plenty of other indices of chronic disease, such as poor general health, tiredness, lowered resistance, tendency to catch colds, unexplained fever, comorbid diseases, carriage state of *Streptococcus pyogenes* and increased antistreptolysin O titre (Becker *et al*., 1994; Dagnelie *et al*., 1998; Mui *et al*., 1998; Bhattacharyya *et al*., 2001; Bhattacharyya *et al*., 2002). Systemic effects and comorbidity cause significant time loss from school or work, decreasing the patients' life quality, and have therefore been considered as other potential indicators for TE (Bhattacharyya *et al*., 2001; Bhattacharyya *et al*., 2002).

The above suggestions are in accordance with the present research, where as many as 42% of the adults who were referred for TE due to recurrent attacks of tonsillitis had only three or less recurrences of inflammation per year and 22% of the patients had comorbid diseases. This indicates that in several cases the decision to undertake TE is not primarily based on the frequency of tonsillitis episodes. Moreover, the survey of ENT surgeons showed that although some range of the anamnestic data, the oropharyngeal signs and the results of diagnostic laboratory tests were taken into account when selecting adults for TE, they were all used arbitrarily and there was no consensus in specific indications. In the literature, the specific indications for TE have for a long time been under discussion and surrounded by controversy (Curtin, 1987; Fry and Pillsbury, 1987; Witt, 1989; Rosenfeld and Green, 1990; Bock *et al*., 1994; Blair, 1996; Mui *et al*., 1998; Darrow and Siemens, 2002; Discolo *et al*., 2003).

Until now, there have been provided no uniform and comprehensive indicators for surgical intervention.

Although oropharyngeal examination could serve an objective tool for quantifying the indications for TE, its reliability has not been scientifically evaluated. Patients may simultaneously have different oropharyngeal signs of inflammatory process in tonsils and surrounding oropharyngeal mucosa. There can be found signs similar to acute inflammation, like hyperemia in the throat or cryptic debris, or signs of sclerotic process, such as fixation of the tonsils combined with a scar tissue on the tonsils and obstruction of the crypts. In the present research, the immune function of recurrently inflamed tonsils was correlated with the macroscopic oropharyngeal signs in order to provide evidence-based indicators for adult tonsillectomy. We initially discriminated the signs of inflammation and sclerotic process and based on the presence or absence of the former or the latter signs, respectively, the tonsils were classified into inflammatory and sclerotic types. Biochemical investigation showed a significantly higher content of collagen in the sclerotic type tonsils as compared to the inflammatory type tonsils, which proves the accuracy of our macroscopic division method of the tonsils.

In further analysis, we demonstrated that the sclerotic type tonsils showed a markedly lower count of neutrophils in its tissue, particularly in the crypt epithelium, which may enhance the risk for bacteraemia during TE. As the crypt epithelium in recurrently inflamed PTs is continuously challenged by high numbers of bacteria, the lowered counts of neutrophils in the sclerotic type tonsils lead to their functional breakdown. Therefore, sclerotic signs, clearly visible on oropharyngeal examination, were considered appropriate indicators to select adults with RT for TE. However, despite the high occurrence of sclerotic signs in RT patients, they were also frequently encountered in a significant proportion of healthy persons. In this situation, consideration of sclerotic signs as the only indicator for TE may lead to an overestimation of the need for surgery, particularly in adults with a lower rate of tonsillitis episodes. Therefore, in further analysis we explored whether the sclerotic process in tonsillar tissue are associated with anamnestic data. We found that higher frequency of tonsillitis episodes per year had a strong correlation with the presence of obstructed tonsillar crypts, while longer disease history correlated strongly with presence of tonsillar sclerosis on oropharyngeal examination. The patients with the sclerotic type tonsils had in an average a two-fold longer disease history than the patients with the inflammatory type tonsils.

In order to combine different anamnestic data, the frequency of tonsillitis episodes per year was multiplied by the number of years during which the episodes occurred. Basically, the result represents the total number of tonsillitis episodes that the patient has ever had and was called the index of tonsillitis (IT) in an earlier study (Fujihara *et al.*, 2003). The same group of authors demonstrated that the IT equal to or more than 8 has a strong correlation with deteriorated immune function of the tonsils and this particular IT value was

therefore considered an appropriate indicator for TE in children (Fujihara *et al*., 2005). Such a specific cut-off score of IT for adults have not been provided previously. In the present research, the IT values were compared with the presence or absence of the most characteristic sclerotic signs, tonsillar sclerosis and obstruction of the tonsillar crypts, in order to construct a ROC curve for prediction of the sclerotic type tonsils. The optimal cut-off score of IT was found to be 36, which had balanced sensitivity, specificity and predictive values. This cut-off score indicates that a minimum of 36 tonsillitis episodes are required for the development of the sclerotic type tonsils. The specificity and predictive value of this score was high enough to use it for differentiating patients with advanced tonsillitis from less severe cases. The difference between cut-off values of IT found in children and in adults may arise from longer morbidity period the grownups have usually been suffered. It may also be related to different markers of the immune status of the tonsils used in the present research compared with the other study.

The present research demonstrated that the sclerotic type tonsils can be expected not only in patients with a high number of tonsillitis episodes per year but also in patients with a lower number of episodes if combined with a long morbidity period. This indicates that gradual accumulation of exacerbations over long time is also a factor for development of the sclerotic type tonsils. These findings are in accordance with the current knowledge of the pathogenesis of RT. Continuous exacerbations of chronic inflammation in the tonsillar tissue result in parenchymal fibrosis, which causes stenosis of the branched, blind-ended and narrow tonsillar crypts (Altemani *et al*., 1996, Michaels, 2001). Subsequent retention of the crypts' content sets up an ideal culture medium for microorganisms, resulting in the formation of small abscesses, sacks filled with different microorganisms. Obstruction of the tonsillar crypts and their chronic suppuration have the potential to more likely promote exacerbations of chronic inflammation compared with the widely open and freely drained crypts. At the same time, a more severe sclerotic process in recurrently inflamed PTs gradually replaces the normal lymphatic tissue and such loss of the tonsillar tissue may result in fewer or less severe tonsillitis episodes. Moreover, a decreased count of neutrophils due to sclerotic process enhances the risk for bacterial invasion and infection generalization, which may be responsible for the development of concomitant inflammatory diseases in RT patients. Such a functional breakdown of recurrently inflamed tonsils may explain the high occurrence of comorbid diseases in the studied group of patients.

In conclusion, the recommendations for TE should be based on detailed disease history, taking into account both frequency of tonsillitis episodes per year and length of morbidity period. The IT score ≥36, which is a combination of the former anamnestic data, predicts sclerotic process in recurrently inflamed tonsils. As the sclerotic type tonsils have lost their defensive function, high IT values could serve as an indicator for TE in adults.

CONCLUSIONS

1. The composition of the deep tonsil microflora of patients with recurrent tonsillitis is random, containing high quantities of different species of aerobes and anaerobes. The most predominating bacteria are anaerobes, usually *Peptostreptococcus*, *Fusobacterium*, *Prevotella* and *Bacteroides* species.

2. The presence of *Streptococcus pyogenes* in the recurrently inflamed tonsils can be confirmed mainly by sensitive molecular methods like PCR. Electron microscopic investigation showed that the presence of coccoid forms of intracellular bacteria is frequently associated with the damage of the crypt epithelium. These findings suggest that hidden pathogenic bacteria may participate in the microbial ecology of the recurrently inflamed tonsils and in maintaining continuous inflammatory process.

3. The post-tonsillectomy bacteraemia is frequent (44%) in patients with recurrent tonsillitis. The proportion of blood culture isolates in the microflora of the corresponding tonsil is usually low and plays no role in the development of post-tonsillectomy bacteremia. Instead, occurrence of bacteremia during tonsillectomy is associated with low counts of CD15 marked neutrophils in the tonsillar tissue, particularly in the crypt epithelium which is continuously challenged by pressure of aerobic and anaerobic bacteria. Hence, neutrophils in the tonsillar tissue play a crucial role in prevention of bacterial invasion through the crypt epithelium into blood.

4. Macroscopically, the inflammatory and sclerotic types of tonsils can be differentiated on oropharyngeal examination. There were found no differences in the extent of parenchymal fibrosis on histological examination. However, biochemical investigation revealed the significantly higher content of collagen in the sclerotic type tonsils as compared to the inflammatory type tonsils, proving the accuracy of macroscopic division of tonsils in RT patients.

5. The occurrence of post-tonsillectomy bacteraemia is closely associated both with lowered counts of neutrophils in the tonsillar tissue and presence of sclerotic signs on oropharyngeal examination. Hence, the sclerotic type tonsils have lost their defensive function. Therefore, the macroscopic oropharyngeal signs of sclerotic process in the tonsillar tissue can serve as an indicator to select adults with recurrent tonsillitis for tonsillectomy.

6. Among the anamnestic data, high frequency of tonsillitis episodes per year and longer disease history are strongly correlated with presence of the obstructed tonsillar crypts and with tonsillar sclerosis, respectively. On the contrary, the inflammatory type of signs has no correlation with the anamnestic data. The index of tonsillitis, obtained by multiplying frequency of tonsillitis episodes by length of the morbidity period, is in good

correlation with the number of sclerotic signs on oropharyngeal examination. The IT scores ≥36 serve as an optimal cut-off value for prediction of sclerotic process in recurrently inflamed tonsils.

7. Although some range of the anamnestic data, the oropharyngeal signs and the results of diagnostic laboratory tests are taken into account by Estonian ENT surgeons when selecting adults with RT for TE, their arbitrary use and absence of consensus in selection criteria points to the need for elaboration of evidence-based indications for surgical intervention.

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To conclude, in everyday praxis both high index of tonsillitis (≥36) and the macroscopic oropharyngeal signs of sclerotic process in the tonsillar tissue can be used as the evidence-based indicators for selection of adults with recurrent tonsillitis for tonsillectomy.

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SUMMARY IN ESTONIAN

TONSILLEKTOOMIA NÄIDUSTUSED TÄISKASVANUTE KROONILISE TONSILLIIDI KORRAL – KLIINILISED, MIKROBIOLOOGILISED JA PATOMORFOLOOGILISED UURINGUD

Krooniline tonsilliit on korduvate ägenemistega kulgev püsiv põletikuline protsess kurgumandlite koes. Peamiseks ravimeetodiks on kurgumandlite kirurgiline eemaldamine e. tonsillektoomia, mille esmaseks näidustuseks loetakse vähemalt kolme tonsilliidi ägenemist esinemist aastas, vaatamata medikamentoossele ravile, ning kindlaks näidustuseks vähemalt nelja või viie tonsilliidi ägenemise esinemist aastas. Samas on kroonilise tonsilliidi kulg täiskasvanutel sageli alaäge ja väheste ägenemistega ning kliinilises pildis on esikohal krooniliselе põletikule omased organismi üldreaktsioonid. Kuna need vähendavad oluliselt patsientide elukvaliteeti, on kaasuvaid tervisehädasid välja pakutud tonsillektoomia võimalike näidustustena. Sellele lisaks arvestatakse ka patsiendi enda soovi kurgumandlite eemaldamiseks. Eespool toodu näitab vajadust teaduslikult põhjendatud ühtsete tonsillektoomia näidustuste välja töötamiseks.

Kuigi kurgumandlite ülesandeks on pakkuda esmast kaitset väliskeskkonnast pärinevate kahjulike tegurite, peamiselt infektsioonitekitajate vastu, kujunevad neis endis sageli püsivad põletikukolded. Kurgumandlite kroonilise põletiku tekkepõhjused on jäänud ebaselgeks. *Streptococcus pyogenes*'e kui kõige tähtsama ägeda bakteriaalse kurgumandlite põletiku tekitaja esinemissagedus kroonilise tonsilliidiga lastel ja täiskasvanutel on madal kurgu limaskestalt võetud külvides. Samas on kroonilisest põletikust haaratud kurgumandlite süvafloorast leitud suurtes hulkades mitmeid teisi potentsiaalselt patogeenseid mikroobe, kuid nende etioloogiline tähtsus on jäänud ebaselgeks. Seetõttu oleks oluline rakendada uuemaid ja täpsemaid uurimismeetodeid raskesti kultiveeritavate mikroorganismide esinemissageduse ja lokalisatsiooni täpsustamiseks kurgumandlites kroonilise tonsilliidiga patsientidel.

Krooniline põletikuline protsess kurgumandlites põhjustab neis püsiva iseloomuga koemuutuste teket, nende seas krüptiavade ahenemist sidekoestumise tõttu ning krüptide distaalsete osade laienemist krüptisisaldise peetumise tagajärjel. Sellega luuakse ideaalsed võimalused mikroobide hulga oluliseks suurenemiseks, mis omakorda loob eeldused nende sattumiseks vereringesse koos toksiliste metaboliitide ja põletikumediaatoritega. Kuigi mikroobide translokatsioon võib olla aluseks patsiendil esinevate üldreaktsioonide ja kaasuvate haiguste tekkel, olles suureks ohuks patsientide tervisele, pole nimetatud protsessi täpne tekkemehhanism teada. Oluline oleks välja selgitada, kas see sõltub spetsiifiliste patogeenide või iseloomulike mikroobiökoloogiliste muutuste esinemisest kurgumandlites, patomorfoloogilistest muutustest või hoopis kurgumandlite immunoloogilisest funktsioonist.

Mitmete, seal hulgas TÜ Kõrvakliinikus eelnevalt teostatud uuringutega on näidatud laste krooniliselt põletikulistes kurgumandlites immuunoglobuliine sekreteerivate rakkude hulga vähenemist koos vastavate immuunoglobuliinide hulga vähenemisega süljes. Andmed põletikuliste kurgumandlite rakulise immuunsuse kohta on samas vasturääkivad, kuna on kirjeldatud nii kurgumandlite immunoloogilise funktsiooni võimalikku üle kui ka alatalitlust. Käesolevas uurimuses kasutasime kurgumandlite funktsiooni häirumise murdepunktina immuunotsüütide, täpsemalt neutrofiilide ja makrofaagide hulka kurgumandli koes mis ei ole enam suuteline ära hoidme tonsillektoomiajärgse batereemia teket. Kurgumandlite funktsiooni võrdlus morfoloogiliste muutuste iseloomu ja ulatusega võeti aluseks uute tonsillektoomia näidustuste leidmisel.

Uurimistöö eesmärgid ja ülesanded

Uurimuse eesmärgiks oli leida anamnestilisi andmeid ja neelupiirkonna makroskoopilisi tunnuseid, mida saaks kasutada kriteeriumitena kroonilise tonsilliidiga täiskasvanud patsientide valikul tonsillektoomiaks. Selleks hindasime krooniliselt põletikuliste kurgumandlite funktsionaalset seisundit uurides kurgumandlite mikroobiökoloogia, iseloomulike morfoloogiliste muutuste ja tonsillektoomiajärgse baktereemia omavahelisi seoseid.

Uurimustöö täpsemad ülesanded:

1. hinnata erinevate aeroobsete ja anaeroobsete mikroobide esinemist ja hulkasid krooniliselt põletikuliste kurgumandlite süvaflooras;
2. uurida *S. pyogenes*'e esinemist kroonilise tonsilliidiga patsientide kurgumandlites mikrobioloogiliste ja molekulaarsete meetodite abil, ning elektronmikroskoopilist analüüsi kasutades uurida mikroobide rakusisest paiknemist kurgumandlite krüptiepiteelis;
3. uurida tonsillektoomiajärgse baktereemia, kurgumandlite süvaflooras olevate aeroobsete ja anaeroobsete mikroobide osakaalu ning mandlikoes olevate neutrofiilide ja makrofaagide hulkade omavahelisi seoseid kroonilise tonsilliidiga patsientidel;
4. uurida milliseid krooniliselt põletikuliste kurgumandlite makroskoopilisi tüüpe saab eristada neelupiirkonna vaatluse, patomorfoloogilise uuringu ja biokeemilise analüüsi alusel;
5. leida need neelupiirkonna makroskoopilised tunnused, mis ennustavad kurgumandlite kaitsevõime langust kroonilise tonsilliidiga täiskasvanutel;
6. uurida anamnestiliste andmete, sealhulgas tonsilliidi indeksi kui kurgumanlite põletiku ägenemiste sageduse ja haiguse kestvuse korrutise seost kurgumandlite sidekoestumise tunnuste esinemisega neelupiirkonna vaatlusel;
7. vastava küsimustiku abil uurida milliseid anamnestilisi andmeid, neelupiirkonna vaatluse tunnuseid ja laboratoorsete uuringute tulemusi arvestavad kõrva-nina-kurguarstid kroonilise tonsilliidiga täiskasvanute suunamisel tonsillektoomiaks.

Uuritavad ja meetodid

Uuritavate rühma moodustasid 62 kroonilise tonsilliidi tõttu tonsillektoomiale suunatud 47 nais- ja 25 meessoost patsienti vanuses 15–35, keskmiselt 22 eluaastat. Uuritavatel patsientidel paluti operatsiooni eelselt täita ankeet anamnestiliste andmete kogumiseks, millele järgnenud neelupiirkonna vaatluse käigus registreeriti eelnevalt defineeritud põletiku ja sidekoestumise tunnuse esinemist või puudumist. Kontrollrühma moodustasid 54 tervet kurgumandlite sagedaste põletikkudeta üliõpilast (36 nais- ja 18 meessoost) vanuses 18–24, keskmiselt 20 eluaastat. Lisaks saatsime 92-le uuringu ajal Eestis töötavale kõrva-nina-kurguarstile küsimustiku eesmärgiga välja selgitada need anamnestilised andmed, neelupiirkonna vaatlusel registreeritavad tunnused ja laboratoorsed uuringud mida arvestatakse kroonilise tonsilliidiga täiskasvanud patsientide suunamisel tonsillektoomiaks.

Perifeerne veeniveri aeroobseks ja anaeroobseks külviks koguti uuringurühma kroonilise tonsilliidiga patsientidelt operatsiooni ajal ning kontrollrühma patsientidelt operatsiooni eelselt, enne neelu vaatlust või teisi manipulatsioone. Verekülvist isoleeritud mikroobide samastamiseks kasutati standardseid mikrobioloogilisi meetodeid.

Kurgumandlite süvamikrofloora kvalitatiivseks ja kvantitatiivseks analüüsiks kasutati 0.2 g kaaluvat koetükikest kurgumandli sisemusest. Koetükikesest tehtud lahjendused külvati erinevatele söötmetele, mida inkubeeriti aeroobsetes ja anaeroobsetes tingimustes. Suurimatest lahjendustest isoleeritud mikroobid samastati tavalisi mikrobioloogilisi meetodeid kasutades. Isoleeritud aeroobsete ja anaeroobsete mikroobide koguhulga alusel leiti iga üksiku mikroobi osakaal (%) kurgumandlite süvamikroflooras. Mikroobe, mille osakaal mikroflooras ületas 10%, loeti domineerivateks mikroobideks. Lisaks sellele uurisime *S. pyogenes*'e esinemissagedust kurgumandlite koes PCR analüüsil.

Kurgumandlite histoloogiliseks analüüsiks värviti koelõigud hematoksüliin-eosiiniga. Valgusmikroskoobi all hinnati mitmesuguste patohistoloogiliste muutuste esinemist 4 punkti skaalal. Immunomorfoloogilisel analüüsil CD15 ja CD68 monoklonaalsete antikehadega määrati vastavalt neutrofiilide ja makrofaagide hulk kurgumandlite erinevates struktuursetes piirkondades: pinna- ja krüptiepiteelis, lümfifolliikulites ja interfollikulaarses koes. Rakkude hulk leiti 0.625 mm^2 kohta igas kurgumandli piirkonnas eraldi. Elektronmikroskoopiliseks analüüsiks eraldati juhuslikult valitud kurgumanlitest koetükikesed koos krüptiepiteeliga, eesmärgiga uurida rakusiseste mikroobide esinemist kurgumandlite koes ja selle seost koekahjustusega.

Kurgumantlite sidekoestumise ulatuse selgitamiseks määrasime kollageeni sisaldust mandlikoes. Kollageeni sisalduse leidsime hüdroksüproliini sisalduse määramise kaudu, kuna nimetatud aminohape moodustab kollageenist 13.4%. Kollageeni sisaldus määrati kurgumandlitest eraldatud koetükikese hädrolüsaadis spektofotomeetriliselt, kasutades värvusreaktsiooni esilekutsumiseks klooramiin T ja Erlich'i lahuseid. Hüdroksüproliini hulk leiti eelnevalt

koostatud kalibratsiooni kõveralt ning arvutuslikult saadud kollageeni hulka väljendati kui mg/g kuivmassi kohta.

Statistiliseks anlüüsiks kasutati 'Excel' (Microsoft Corp.), 'Statgraphics' (Statistical Graphics Corp.) ja 'R' (The R Development Core Team) arvutiprogramme, rakendades χ^2-, Mann-Whitney Rank Sum või Student t-testi. Korrelatsioonianalüüsiks kasutati Pearson'i testi. Arvutamise iga sidekoestumise tunnuse spetsiifilisuse, tundlikkuse, positiivse (PEV) ja negatiivse eeldatava väärtuse (NEV). Vastavalt kahe kõige olulisema sidekoestumise tunnuse esinemisele või puudumisele konstrueeriti suhteliste töökarakteristikute kõver (*receiver-operating characteristic, ROC*) ja kõveraalune pindala (*area under curve, AUC*) eesmärgiga välja selgitada tonsilliidi indeksi väärtus, mis ennustab sidekoeliste kurgumandlite esinemist patsiendil.

Uurimustöö tulemused ja järeldused

1. Kroonilise tonsilliidiga täiskasvanud patsientidelt eemaldatud kurgumandlite süvafloora koostis on juhusliku iseloomuga, sisaldades suurtes hulkades erinevaid aeroobseid ja anaeroobseid mikroobe. Suurimat osakaalu omavad anaeroobid, neist sagedamini *Peptostreptococcus*, *Fusobacterium*, *Prevotella* and *Bacteroides* liigid.
2. *S. pyogenes*'e esinemist krooniliselt põletikulistes kurgumandlites saab kõige täpsemini määrata molekulaarseid meetodeid, sealhulgas polümeraasi ahelreaktsiooni (PCR) kasutades. Kurgumandlite krüptiepiteeli elektronmikroskoopiline analüüs näitas, et arvukate rakusiseste kokkoidse kujuga mikroobide esinemine on sageli seotud epiteelirakkude kahjustusega. Uuringu tulemused näitavad, et varjatud rakusisese paiknemisega haigustekitajad omavad tähtsust kurgumandlite mikroobiökoloogiste suhete kujunemises ning etendavad olulist osa kroonilise põletiku tekkes ja säilitamises.
3. Kroonilise tonsilliidiga patsientidel on tonsillektoomiajärgse baktereemia esinemissagedus kõrge (44%). Verest isoleeritud mikroobide osakaal vastavate kurgumandlite süvaflooras on ebamikel juhtudel madal, mistõttu see omab vähest mõju tonsillektoomiajärgse baktereemia tekkele. Samas on baktereemia teke tihedalt seotud CD15 markerit kandvate neutrofiilise arvu vähenemisega krooniliselt põletikuliste kurgumandlites, iseäranis krüptiepiteelis. Viimane on kurgumandlite kroonilise põletiku korral pidevas kokkupuutes suure hulga erinevate mikroobidega. Seega omab neutrofiilide piisav arv vastavas sissetungiväratis olulist osa mikroobide translokatsiooni vältimisel.
4. Makroskoopiliselt saab neelupiirkonna vaatlusel eristada põletikulist ja sidekoelist tüüpi kurgumandleid. Patohistoloogilisel analüüsil ei ilmnenud kurgumandlite parenhüümi sidekoestumise raskusastmes olulist erinevust kurgumandlite makroskoopiliste tüüpide vahel. Samas oli biokeemiliselt

määratud sidekoe hulk oluliselt suurem sidekoelist tüüpi kurgumandlites võrreldes põletikulist tüübiga, mis kinnitab makroskoopilise leiu alusel kurgumandlite tüüpide eristatavust kroonilise tonsilliidiga täiskasvanutel.

5. Tonsillektoomiajärgse baktereemia teke on tihedasti seotud neutrofiilide arvu vähenemisega kurgumandlite koes ja sidekoestumise tunnuste samaaegse esinemisega neelupiirkonna vaatlusel. Nimetatud tulemus näitab, et kroonilise põletiku tagajärjel tekkiv kurgumandlite parenhüümi järk-järguline asendumine sidekoega viib kurgumandlite kaitsevõime langusele. Seega võib makroskoopilisi sidekoestumise tunnuseid kasutada objektiivsete näitajatena täiskasvanud patsientide valikul tonsillektoomiaks.
6. Kurgumandlite tihkestumine on tugevas korrelatsioonis kroonilise tonsilliidi pikema kestvuseja ning krüptisuudmete ahenemine/sulgumine suurema tonsilliidi ägenemiste sagedusega. Seos põletikuliste tunnuste, haiguse pikkuse ja ägenemiste sageduse vahel puudus. Tonsilliidi indeks ehk kroonilise tonsilliidi kestvuse ja ägenemiste sageduse omavaheline korrutis on tihedas seoses sidekoestumise tunnuste suurema arvuga neelupiirkonna vaatlusel. Leidmsime, et tonsilliidi indeksit ≥36 saab kasutada valikukriteeriumina kroonilise tonsilliidiga täiskasvanud patsientide suunamisel tonsillektoomiaks.
7. Kuigi kõrva-nina-kurguarstid arvestavad tonsillektoomia näidustustena väga erinevaid anamnestilisi andmeid, neelupiirkonna tunnuseid ja laboratoorsete uuringute tulemusi, viitab ühtse seisukoha puudumine vajadusele välja töötada teaduslikult põhjendatud objektiivsed tonsillektoomia näidustused.

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Järeldame, et igapäevases töös saab tonsillektoomia näidustusena kroonilise tonsilliidiga täiskasvanutel kasutada nii patsiendi anamneesil baseeruvat kõrget tonsilliidi indeksit (≥36) kui mitme sidekoestumise tunnuse samaaegset esinemist neelupiirkonna vaatlusel.

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Teadustöö

Peamiseks uurimisvaldkonnaks on põletikuliste haiguste, sealhulgas kroonilise tonsilliid, mikroobiökoloogia, patogeneesi, immunomorfoloogia, patomorfoloogia ja kliinilise diagnoosiga seotud probleemid. Avaldanud 5 teaduspublikatsiooni ja 16 konverentsiettekandet.