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Virus infections, microbiome, and immune responses

Lotta Ivaska

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University of Turku

Faculty of Medicine
Department of Paediatrics and Department of Otorhinolaryngology – Head and Neck Surgery
Doctoral Programme in Clinical Research (DPCR)
Turku University Hospital, Finland

Supervised by

Professor Tuomas Jartti, MD, PhD
Department of Paediatrics and Adolescent Medicine
Turku University Hospital and University of Turku
Turku, Finland

Associate Professor Tuomo Puhakka, MD, PhD
Department of Otorhinolaryngology – Head and Neck Surgery
Turku University Hospital and University of Turku
Turku, Finland

Reviewed by

Professor Marjo Renko, MD, PhD
Department of Paediatrics
Kuopio University Hospital and University of Eastern Finland
Kuopio, Finland

Professor Anne Pitkäranta, MD, PhD
Department of Otorhinolaryngology – Head and Neck Surgery
Helsinki University Hospital and University of Helsinki
Helsinki, Finland

Opponent

Associate Professor Johanna Nokso-Koivisto, MD, PhD
Department of Otorhinolaryngology – Head and Neck Surgery
Helsinki University Hospital and University of Helsinki
Helsinki, Finland

ISBN 978-951-29-8932-4 (PRINT)
ISBN 978-951-29-8933-1 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2022

To my boys

UNIVERSITY OF TURKU
Faculty of Medicine
Department of Paediatrics and Department of Otorhinolaryngology – Head and Neck Surgery
LOTTA IVASKA: Tonsils as an *in vivo* model – virus infections, microbiome, and immune responses
Doctoral Dissertation, 109 pp.
Doctoral Programme in Clinical Research
June 2022

ABSTRACT

Tonsillectomy and adenotomy are two of the most common operations in the otorhinolaryngology field. Recurrent infections and hypertrophy are the main indications for these surgical procedures. Palatine tonsils and nasopharyngeal tonsil (adenoid) continuously interact with our environment. Tonsils consist of mucosa-associated lymphoid tissue and serve as a site for bacteria, viruses, and other antigens to be presented to the immune cells. Physiologically complex tonsil tissue is crucial in inducing and maintaining immune responses. However, the underlying mechanisms of the pathological conditions are not fully understood.

This thesis primarily aimed to investigate tonsillar virus infections, microbiome, and their association with immune responses. The diagnostics and immunomodulatory effects of human bocavirus 1 (HBoV1) were particularly emphasised.

Elective adeno-/tonsillectomy patients of all ages were recruited from Satakunta Central Hospital 2008–2009 and Turku University Hospital 2013–2015. Adeno-/tonsillectomy was performed according to routine clinical procedures; tonsil tissues, nasopharyngeal aspirate (NPA), and serum samples were collected. Virus diagnostics was done by polymerase chain reaction (PCR); the expression of cytokine and transcription factors was analysed by reverse-transcription PCR. RNA sequencing was used for microbial profiling.

Our results support earlier data that respiratory virus infections in palatine tonsil tissue, adenoid tissue, and NPA are common (with a total detection rate of 94%) in non-acutely ill adeno-/tonsillectomy patients. Additionally, in one or more of the sample sites, human herpesviruses 6 and 7 and the Epstein-Barr virus were commonly found (67%, 51%, and 35%, respectively). High HBoV1 DNA loads in NPA had no correlation with serology or messenger RNA results. Furthermore, HBoV1 infection was associated with suppressed expression of tonsillar cytokines, suggesting the immunosuppressive capacity of the virus. Our study revealed that atopy associated with lower intratonsillar bacterial diversity, suggesting differences in microbial balance between atopic and non-atopic subjects.

Palatine tonsil and adenoid tissues serve as an *in vivo* model to investigate viruses, bacteria, and their interactions with local immune responses.

KEYWORDS: adenoid, atopy, human bocavirus 1, immune responses, microbiome, nasopharyngeal aspirate, tonsil, virus

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Lastentautioppi ja Korva-, nenä- ja kurkkutaudit – pään ja kaulan kirurgia
LOTTA IVASKA: Risakudos *in vivo* -mallina – virusinfektiot, mikrobiomi ja immuunivasteet
Väitöskirja, 109 s.
Turun kliininen tohtoriohjelma (TKT)
Kesäkuu 2022

TIIVISTELMÄ

Nielurisa- ja kitarisaleikkaus ovat yleisiä toimenpiteitä korva-, nenä- ja kurkkutautien alalla. Leikkausindikaatio on useimmiten risatulehdukset ja risojen liikakasvu. Nielu- ja kitarisat ovat jatkuvasti vuorovaikutuksessa ympäristön kanssa. Risakudos koostuu limakalvon ja lymfaattisen kudoksen yhdistelmästä ja toimii antigeenin ja immuunijärjestelmän solujen kohtaamispaikkana. Fysiologisesti monimuotoinen risakudos toimii merkittävässä roolissa immuunivasteiden aktivoinnissa ja muokkaamisessa.

Tämän väitöskirjan tarkoituksena oli tutkia risakudoksen virusinfektioita, mikrobiomia ja niiden yhteyttä immuunivasteisiin. Erityisesti tutkittiin bokaviruksen (HBoV1) diagnostiikkaa ja HBoV1 infektion yhteyttä immuunivasteisiin.

Aineistoon kerättiin kaiken ikäisiä kita- ja nielurisaleikkauspotilaita Satakunnan keskussairaalasta vuosina 2008–2009 ja Turun Yliopistollisesta keskussairaalasta vuosina 2013–2015. Leikkauspotilaista otettiin verinäytteet, nenän imulimanäytteet (NPA) ja risakudosnäytteet. Virukset analysoitiin polymeraasiketjureaktio (PCR) menetelmällä. Sytokiinigeenien ilmentymistä tutkittiin käänteistranskriptaasi-polymeraariketjureaktiolla (RT-PCR) ja RNA-sekvensointia käytettiin risakudoksen mikrobiomin kartoittamiseen.

Kroonista risasairautta sairastavien leikkauspotilaiden risakudoksissa ja nenän imulimassa esiintyy runsaasti hengitystieviruksia (esiintyvyys 94 %), kuten aikaisemmin on osoitettu. Herpesvirus 6 (HHV6), herpesvirus 7 (HHV7) Epstein-Barr virus olivat yleisiä (67 %, 51 %, 35 %) yhdessä tai useammassa näytelaadussa. HBoV1 korkea virusmäärä ei korreloinut vasta-ainetasoihin tai lähetti RNA tuloksiin. HBoV1 oli yhteydessä risakudoksen sytokiinigeenien vähäisempään ilmaantuvuuteen viitaten HBoV1:n kapasiteettiin muokata immuunivastetta. Lisäksi tutkimuksessa kävi ilmi, että atooppisilla potilailla nielurisakudoksen bakteerien monimuotoisuus on alhaisempi.

Nielu- ja kitarisa soveltuvat käytettäväksi *in vivo* mallina tutkittaessa risakudoksen viruksia, bakteereja ja niiden vaikutusta immuunivasteisiin.

AVAINSANAT: atopia, human bocavirus 1, immuunivaste, kitarisa, mikrobiomi, nielurisa, nenänielun aspiraationäyte, virus

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Abbreviations

AdV	Adenovirus
APC	Antigen-presenting cell
cDNA	Complementary DNA
CMV	Cytomegalovirus
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EV	Enterovirus
FLU A	Influenza A virus
FLU B	Influenza B virus
FOXP3	Forkhead box protein 3
GATA	GATA-binding factor 3
HBoV1	Human bocavirus 1
HHV	Human herpesvirus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MPV	Metapneumovirus
mRNA	Messenger ribonucleic acid
NPA	Nasopharynx aspirate
OME	Otitis media with effusion
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PIV	Parainfluenza virus
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RORC2	Retinoic acid receptor-related orphan receptor C2

rRNA	Ribosomal ribonucleic acid
RSV	Respiratory syncytial virus
RT	Reverse transcription
RTI	Respiratory tract infection
RV	Rhinovirus
SARS	Severe acute respiratory syndrome
TGF-β	Transforming growth factor-β
Th	T helper cell
Treg	T regulatory cell
VZV	Varicella zoster virus

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Ivaska LE, Christensen A, Waris M, Puhakka T, Vuorinen T, Allander T, Söderlund-Venermo M, Jartti T. No correlation between nasopharyngeal human bocavirus 1 genome load and mRNA detection or serology in adeno-/tonsillectomy patients. *The Journal of Infectious Diseases*, 2019;4: 589–593.

II Ivaska LE, Silvoniemi A, Palomares O, Turunen R, Waris M, Mikola E, Puhakka T, Söderlund-Venermo M, Akdis M, Akdis CA, Jartti T. Persistent human bocavirus 1 infection and tonsillar immune responses. *Clinical and Translational Allergy*, 2021; 6: e12030.

III Ivaska LE, Silvoniemi A, Mikola E, Puhakka T, Waris M, Vuorinen T, Jartti T. Herpesvirus infections in adenoids in patients with chronic adenotonsillar disease. *Journal of Medical Virology, 2022;* Apr 29. Online ahead of print.

IV Ivaska LE*, Hanif T*, Ahmad F, Tan G, Altunbulakli C, Mikola E, Silvoniemi A, Puhakka T, Akdis CA, Toppila-Salmi S, Jartti T. Tonsillar microbial diversity, abundance, and interrelations in atopic and non-atopic individuals. *Allergy*, 2020; 8: 2133–2135. *Equal contribution.

1 Introduction

Palatine tonsils in the oropharynx, adenoid in the nasopharynx, lingual tonsils in the glosso-epiglottic space, and tubal tonsils at the pharyngeal site of the Eustachian tube constitute a circumferential ring of lymphoepithelial tissue in the pharynx area (Figure 1). German anatomist Wilhelm Gottfried von Waldeyer-Hartz first described this group of lymphoid tissue in 1884. Therefore, this group is named Waldeyer's ring (Waldeyer, 1884). This ring of lymphoepithelial tissue is strategically located at the entrances of the digestive and respiratory tracts (Figure 1). Furthermore, everything we breathe and ingest includes potentially dangerous antigens (Arambula et al., 2021). Consequently, the mucous membrane of the upper respiratory tract comprises mucosa-associated lymphoid tissue (MALT). This distinctive mucosal immune system specialises in sampling inhaled and ingested viruses, bacteria, and other antigens, and initiates immune responses (van Kempen et al., 2000). Adenoid and palatine tonsils are immunologically most active during childhood and adolescence, respectively (Johnston et al., 2019; Mitchell et al., 2019).

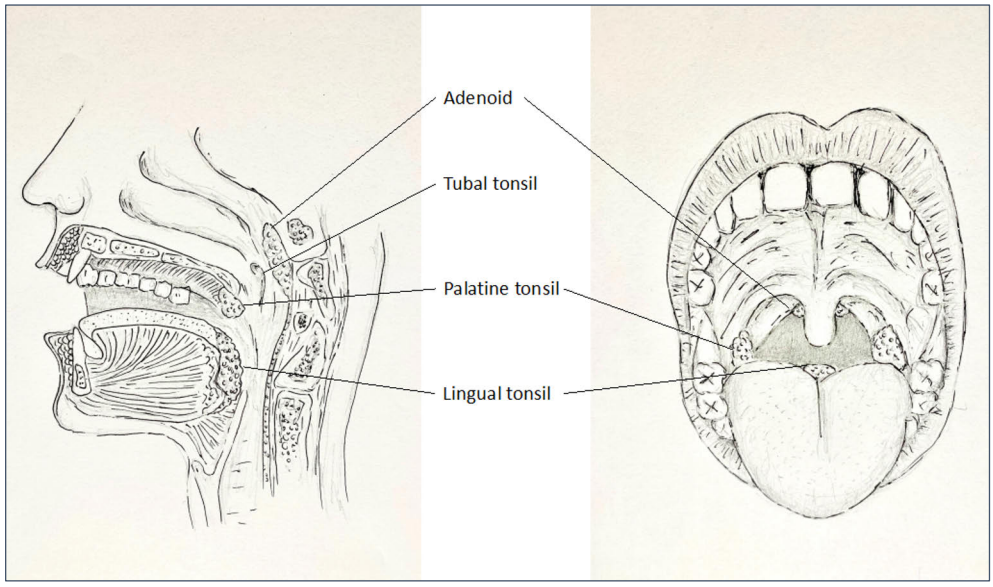

Figure 1. Waldeyer's ring. Picture by Katariina Tamminen.

Respiratory viruses and herpesviruses are often detected by a polymerase chain reaction (PCR) from adenoid, tonsil tissue, and nasopharyngeal aspirate samples of non-acutely ill adenotonsillar surgery patients (Berger et al., 2007; Kourieh et al., 2019; Proenca-Modena et al., 2012). For example, human bocavirus 1 (HBoV1) seems to persist in the respiratory tract for weeks, and the diagnosis by PCR is insufficient (Christensen et al., 2019). Moreover, it is suggested that HBoV1 might have immunomodulatory effects (Lukkarinen et al., 2014). The role of prolonged intra-adenoid and intratonsillar virus infections in the pathogenesis of chronic adenotonsillar disease and local immune responses remains unsolved.

Furthermore, next-generation sequencing techniques have revealed that the abundance and diversity of the microbiome at the tissue levels also concern tonsil tissue (Johnston & Douglas, 2018). The imbalance of the skin, respiratory tract, and the gut microbiome is shown to be associated with allergic diseases, establishing the connection between the microbiome and immunologically mediated disorders (Huang et al., 2017). However, tonsillar microbiome related to atopy was not studied earlier. Viruses and bacteria in tonsils and adenoids are in close contact with the immune system, potentially modulating immune responses and participating in the pathogenesis of diseases.

This thesis aimed to increase the knowledge of virus infections, immune responses, and the microbiome in secondary lymphoid tissue using tonsils and adenoids as an *in vivo* model.

2 Review of the Literature

2.1 Tonsils

2.1.1 Anatomy

Palatine tonsils are a paired organ of lymphoid tissue in the oropharynx – at the back of the mouth behind the oral cavity (Figure 1). Tonsillar fossae, located laterally at the oropharynx, are bordered by the palatoglossal arch anteriorly and the palatopharyngeal arch posteriorly. These mucosal folds comprising muscle fibres are also called anterior and posterior tonsil pillars (Masters et al., 2019). Laterally, the tonsil is covered by a tonsillar capsule – a compact membrane separating the tonsil tissue from the superior constrictor muscle. Five arteries provide the main blood supply: the tonsillar branch of the facial artery, dorsal lingual artery, ascending palatine artery, ascending pharyngeal artery, and lesser palatine artery. The internal carotid artery lies lateral to the superior constrictor muscle, approximately 2,5 cm posterolateral to the tonsillar fossa in adults; in children, the distance can be only 1,5 cm. Major venous drainage occurs through the paratonsillar vein connected to the pharyngeal plexus of veins and the common facial vein. Lymphatic drainage leads to the submandibular and retropharyngeal lymph nodes (Arambula et al., 2021; Nave et al., 2001; Shah et al., 2015). Sensory innervation of the tonsils and tonsillar fossae comes from two different cranial nerves: the trigeminal nerve and the glossophagyngeal nerve (Smithard et al., 2009). Tonsils reach their maximum size by puberty and reduce after that (Arambula et al., 2021; Shah et al., 2015).

2.1.2 Function

The purpose of the tonsil tissue is to interact with pathogens and immune cells. Non-keratinised stratified squamous epithelium covers the surface of tonsils. Moreover, the surface invaginates and forms crypts covered by stratified squamous epithelium and reticulated crypt epithelium (i.e. lymphoepitelium which contains epithelial and lymphoid cells). Histologically, tonsils are constructed of four lymphoid compartments: reticulated crypt epithelium, follicular germinal centre surrounded by the mantle zone, and an extrafollicular area. The antigen uptake occurs in the crypt

epithelium (Fossum et al., 2017; Nave et al., 2001; Perry et al., 1998; van Kempen et al., 2000). The crypts invaginate deep into the lymphoid tissue, increasing the surface area for the antigen uptake (Fossum et al., 2017; Nave et al., 2001). Membrane-cells (M-cells) are thought to be involved in the antigen uptake process and initiation of the immune responses. After entering the epithelium, the antigen is processed by antigen-presenting cells (APCs) such as macrophages and dendritic cells. In the extrafollicular region, the antigens are presented to T-cells. If the antigen is recognised, a secondary immune reaction is activated by T-cells and B-cells. If the antigen is new, it is identified by T helper cells (Th), and naïve T-cells are activated and differentiated into antigen-specific T-cells. These T-cells stimulate B lymphocytes to proliferate and migrate from the mantle zone (i.e. dark zone) of the lymphoid follicle to the follicular germinal centre (i.e. light zone). The B-cells differentiate into memory cells and immunoglobulin-producing plasma cells during the migration. This process is mediated by numerous cytokines and chemokines produced by T-cells (Arambula et al., 2021; Brandtzaeg, 2011, 2003; Nave et al., 2001). Immunoglobulins (IgA, IgG, IgM, IgE, IgD) extravasate to and between tonsillar epithelial cells and participate in immune responses (Arambula et al., 2021; Fossum et al., 2017). An increase of poorly functioning macrophages and a decrease of B-cells differentiating into plasma cells in tonsil tissue have been associated with tonsil infections (Brandtzaeg, 2003; Gorfien et al., 2001). Since tonsils harbour precursor B-cells responsibly for IgA production in oral mucosa, tonsillectomy in small children is suggested to influence mucosal immunity (Brandtzaeg, 2003). Interestingly, IgA nephropathy is associated with an excessive amount of IgA partly produced by tonsillar plasma cells. In Japan, tonsillectomy is a choice for treating IgA nephropathy (Barratt et al., 2018; Gesualdo et al., 2021). It has been shown *in vivo* that tonsil tissue contains allergen-specific forkhead box protein 3 (FOXP3) T regulatory cells (Treg), which participate actively in the oral tolerance for allergens. Furthermore, the tolerance can be disrupted by Th2 and Th17 type cytokines; in atopic patients, the tonsillar immune profile is shifted towards type 2 response (Kücüksezer et al., 2013; Palomares et al., 2011).

2.1.3 Disease

In several studies, the terminology concerning infection of the tonsils is unclear. “Sore throat” is a term that means any painful condition in the oropharynx area. Typically, an acute sore throat is a symptom of an inflammatory process in the pharynx, tonsils, or nasopharynx during the common cold (Pelucchi et al., 2012; Richardson, 1999). Viral or bacterial infection of the pharynx or palatine tonsils or both is also called strep throat, acute tonsillitis, pharyngitis, or tonsillopharyngitis (Mitchell et al., 2019). One of the most common causes of sore throat is acute

tonsillitis, defined as swelling and redness of the palatine tonsils, possible exudate on the tonsils, cervical lymphadenopathy, and fever >38.3ºC (Shulman et al., 2012). Respiratory viruses such as rhinovirus (RV), HBoV, coronavirus (CoV), adenovirus (AdV), parainfluenza viruses (PIV), influenza viruses (FluV), respiratory syncytial virus (RSV), coxsackie virus, and metapneumovirus (MPV) can cause acute tonsillitis. Other viruses, namely the herpes simplex virus (HSV), Epstein–Barr virus (EBV), and human immunodeficiency virus (HIV), can also act as causative agents (Bathala et al., 2013; Richardson, 1999). Group A beeta-hemolytic *Streptococcus* causes 15%–30% of the acute tonsillitis cases in children and 5%–10% in adults, respectively. Other bacteria that can cause acute tonsillitis are group C and G *Streptococci*, *Corynebacterium diphtheriae, Neisseria gonorrhoeae, Chlamydia pneumoniae, and Mycoplasma pneumoniae* (Bathala et al., 2013; Chan, 2010; Windfuhr et al., 2016).

The most common complication of acute tonsillitis is a peritonsillar abscess, where pus accumulates between the superior constrictor muscle and the tonsillar capsule (Galioto, 2017). Parapharyngeal and retropharyngeal abscesses are rare complications and may be associated with acute tonsillitis (Chang et al., 2010; Friedrichs et al., 2004). *Fusobacterium necrophorum* is the etiologic agent of Lemierre's syndrome, which begins with tonsillitis or peritonsillar infection. Other symptoms include unilateral neck swelling due to thrombophlebitis of the internal jugular vein (Jensen et al., 2013).

Recurrent acute tonsillitis refers to repeated tonsillar infection episodes and may indicate the need for a tonsillectomy. The term "chronic tonsillitis" is also used in the literature and refers to prolonged sore throat symptoms and halitosis. Chronic tonsillitis often presents the retention and secretion of a cheese-like crypt material. Calcification of this crypt material may lead to tonsilloliths forming (Bamgbose et al., 2014; Ferguson et al., 2014; Richardson, 1999). PFAPA (periodic fever, aphthous stomatitis, pharyngitis and adenitis syndrome) is an inflammatory process that usually begins in early childhood (11 months–4 years), often causing infection of the tonsils and pharynx (Marshall et al., 1987; Renko et al., 2019). The aetiology and pathogenesis of this syndrome are not completely known, but alterations in the serum cytokine levels are observed compared to healthy controls (Stojanov et al., 2011). At the tissue level, the tonsils of PFAPA patients contain fewer B-lymphocytes and more naïve T-cells than the tonsils of other tonsillectomy patients (Petra et al., 2015). In a culture-based study, *Candida albicans* was found more often and *Staphylococcus aureus* less often in the tonsils of PFAPA patients (Lantto et al., 2015). Interestingly, tonsillectomy is the cure for PFAPA, even if all the criteria for the syndrome are not fulfilled (Renko et al., 2019).

Palatine tonsils reach their maximum size by puberty (Arambula et al., 2021; Perry et al., 1998). Tonsillar hypertrophy can cause sleeping disorders such as snoring, mouth breathing, and even obstructive sleep apnea (Marcus et al., 2013; Mitchell et al., 2019). The mechanisms of tonsillar hypertrophy are not completely understood. Recent studies suggest that increased cellular senescence (cessation of cell division) in the germinal centres is associated with hypertrophic tonsils. Furthermore, the increased number of the lymphoid cells may lead to enlargement of the tonsillar follicles and tonsillar hypertrophy (Chen et al., 2020; Önal et al., 2015; Zhang et al., 2003).

Most tonsil malignancies are squamous cell carcinoma. Other malignancies found are minor salivary gland tumours and lymphomas (Guay et al., 1995). The human papillomavirus (HPV) is one risk factor for tonsillar squamous cell carcinoma, and the incidence of HPV-positive tonsil carcinomas has increased during the past years (Näsman et al., 2020).

2.2 Adenoid

2.2.1 Anatomy

Adenoid (i.e. pharyngeal tonsil) comprise a pyramidal mass of lymphoid tissue in the midline of the nasopharynx (Figure 1) and is situated at the level of the occipital and sphenoid bones in the upper and posterior wall of the nasopharynx. Adenoid is not covered by a capsule like tonsils. However, there is a capsule superiorly, separating the adenoid from the sphenoid bone periosteum. This capsule extends into the adenoid tissue, dividing it into 4 to 6 segments. Deep into the inferior edge of the adenoid lies the pharyngobasilar fascia. The blood supply to the adenoid is provided by the ascending pharyngeal artery, facial artery, and maxillar artery. Venous drainage passes from the pharyngeal plexus into the facial and internal jugular veins. Lymphatic drainage runs through pharyngomaxillary and retropharyngeal lymph nodes (Arambula et al., 2021; Mnatsakanian et al., 2021). The innervation of the adenoid originates from two cranial nerves: the vagus and the glossopharyngeal nerves (Geiger et al., 2021; Marseglia et al., 2009; Mnatsakanian et al., 2021).

2.2.2 Function

The main function of the adenoid is similar to the tonsils: to entrap antigens and initiate airway mucosal immune responses especially. Adenoid is exposed to antigens from the outside air and the alimentary tract (Brambilla et al., 2014; Ogasawara et al., 2010). The surface of the adenoid is covered by respiratory type

ciliated epithelium. Adenoid tissue is structured of mucosal folds mainly composed of pseudostratified ciliated columnar epithelium and the crypts have patches of lymphoepithelium. The functional histological structure of the adenoid is similar to the tonsils and composed of four lymphoid compartments: reticulated crypt epithelium (i.e. lymphoepithelium), follicular germinal centre surrounded by the mantle zone, and the extrafollicular area (Brandtzaeg, 2003; Marseglia et al., 2009; van Kempen et al., 2000). After the antigen uptake into the crypt epithelium, the APCs interact with the antigen and extrafollicular T-cells. In the case of a recognised antigen, a secondary immune response is activated by T-cells and the antibody production of B-cells. In the case of a novel antigen, T-cell activation, proliferation, and differentiation occur. Naïve B-cells, stimulated by the specific T-cells, differentiate into plasma cells and memory B-cells. The most common antibodies produced in adenoid tissue are secretory IgA and IgG. IgA is associated with protecting mucosal membrane surfaces (Arambula et al., 2021; Brambilla et al., 2014; B. Wang et al., 2012). Infections may influence the immune responses at the tissue level. *Streptococcus pneumoniae* swabbed from adenoid tissue is associated with higher intra-adenoid IL (interleukin) -17 levels than controls without the bacteria in the nasopharynx (Huang et al., 2019).

Elevated specific IgE levels and high amounts of eosinophils can be detected in the adenoid tissue of some patients with adenoid hypertrophy (Cho et al., 2018; Ekici et al., 2018). Furthermore, Th1 type cytokine response was detected in the messenger ribonucleic acid (mRNA) obtained from the adenoid tissue of non-allergic children compared to ones with a clinical allergy (Masieri et al., 2014). These results suggest that local atopy exists in the adenoid tissue of allergic patients undergoing an adenoidectomy.

2.2.3 Diseases

Adenoid hypertrophy is a common cause of upper airway obstruction in children and can lead to nasal obstruction, mouth breathing, snoring, recurrent rhinosinusitis, obstruction of the Eustachian tube, and sleep apnea. The prevalence of adenoid hypertrophy in children is estimated to be around 35% (Dogru et al., 2017; Patel et al., 2020; Pereira et al., 2017). The aetiology of adenoid hypertrophy is unknown. However, infections, environmental exposures such as parental smoking, and immune alterations such as allergic diseases are thought to play a role (Brambilla et al., 2014; B. Wang et al., 2012). Adenoid tissue usually increases during childhood, reaching its maximum size by 6–8 years, and then decreases during puberty (Geiger et al., 2021; Pereira et al., 2017). A recent review suggests an association between allergy and hypertrophic adenoids (De Corso et al., 2021). Adenoid hypertrophy is

relatively rare in adults; the underlying cause can be HIV, lymphoma, or sinonasal malignancy (Rout et al., 2013).

Adenoiditis is an inflammation process of the adenoid tissue caused by viruses, bacteria, or both. Moreover, evidence of an association between laryngopharyngeal reflux and adenoid hypertrophy and inflammation exists (Niu et al., 2018). Adenoiditis can occur during or after an upper respiratory virus infection and is usually seen as part of pharyngitis or rhinosinusitis. Symptoms of adenoiditis include rhinorrhea, nasal obstruction, post-nasal drip, halitosis, and fever (Bowers et al., 2021; H. Wang, 2020).

The virus detection rate in adenoid tissue in chronic adenotonsillar disease is 85%–100% (Proenca-Modena et al., 2012; Sato et al., 2009). The most abundant bacterial genera found from adenoid tissue by culture-based studies are *Streptococcus*, *Haemophilus,* and *Staphylococcus* (Subtil et al., 2017). Using fluorescence in situ hybridisation (FISH), *Haemophilus influenzae, Streptococcus,* and *Bacteroidetes* were commonly detected from the adenoid and tonsil tissue samples of chronic adenotonsillar disease patients (Swidsinski et al., 2007). Adenoids may serve as a reservoir for bacteria, affecting the middle ear. An adenoidectomy reduces the bacterial load in the nasopharynx and is recommended in certain cases adjunct to tympanostomy in treating otitis media with effusion (OME) (Rosenfeld et al., 2022).

More than 300 distinct operational taxonomic units (OTUs) have been detected from adenoid tissue using 16S rRNA (ribosomal ribonucleic acid) gene sequencing. The most abundant genera found were *Haemophilus, Moraxella, Fusobacterium, Streptococcus,* and *Porphyromonas* (Johnston et al., 2018a; Johnston & Douglas, 2018). Biofilm of adenoid tissue has been associated with OME (Nistico et al., 2011). However, recent studies suggest that the microenvironment of the adenoids differs from that of the middle ear (Johnston et al., 2019). However, Fagö-Olsen compared the microbiome between tonsils and adenoids using 16S rRNA gene sequencing. Classical pathogens causing otitis media, *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* were found almost solely in adenoids rather than in tonsils. This finding suggests that adenoid tissue harbours potential pathogens of OME (Fagö-Olsen et al., 2019).

2.3 Microbiology and immune functions of tonsils

2.3.1 Human Bocavirus 1 (HBoV1)

2.3.1.1 Clinical presentation

In 2005, Allander et al. discovered a new single-stranded DNA virus belonging to the *Parvoviridae* family. It was originally found in samples from the respiratory tracts of children with respiratory tract infections (RTIs). The virus was named human bocavirus 1 (HBoV1) (Allander et al., 2005). HBoV1 is transmitted primarily by respiratory tract droplets and can cause RTIs such as the common cold, acute otitis media, bronchiolitis, wheezing, and pneumonia, typically in small children younger than two years (Allander et al., 2007; Christensen et al., 2019; Norja et al., 2012; Qiu et al., 2017; Xu et al., 2017). In a hospital-based study, sole HBoV1 infections were characterised by asthma exacerbation and pneumonia. However, coinfections with other viruses are common (up to 75%), making knowing which symptoms are due to HBoV1 and which result from a coinfection difficult (Calvo et al., 2016; Christensen et al., 2010; Fry et al., 2007). HBoV types 2–3 are often found in stool samples, but clinical relevance is controversial. Paloniemi et al. did not find a causal connection between HBoV in hospitalised patients with gastroenteritis (Paloniemi et al., 2014). However, a study of Latvian children showed that HBoV1 and HBoV2 as a single pathogen could cause acute gastroenteritis (Nora-Krukle et al., 2018).

The prevalence of HBoV in certain lung and colorectal carcinomas was approximately 20% using PCR and fluorescence in situ (Schildgen et al., 2013). Another study found a significantly higher prevalence of HBoV DNA in tonsil squamous carcinoma than in chronically infected tonsils (Höpken et al., 2018). An *in vitro* study using RNA sequencing showed that the pro-cancerogenic cytokine expression was increased in HBoV1 infection (Schildgen et al., 2018), leading to speculations on whether HBoV is associated with tumour genesis or if the virus has tumour tissue tropism.

2.3.1.2 Diagnosis of HBoV1

HBoV1 can be detected by qualitative PCR from a nasopharyngeal aspirate in up to 25% of children suffering from an RTI. However, HBoV1 is also a common finding in asymptomatic patients, the prevalence rate being similar to symptomatic patients, especially in children under five. HBoV1 DNA can persist for months in the respiratory tract after a symptomatic infection (Blessing et al., 2009; Byington et al.,

2015; Christensen et al., 2010, 2019; Martin et al., 2010; Petrarca et al., 2020) (Figure 2). Because of its long persistence, qualitative PCR is not recommended for detecting HBoV1 in acute respiratory infection (Christensen et al., 2013, 2019; Wagner et al., 2016; Xu et al., 2017). Concerning serology, positive HBoV1-IgM detection with low IgG or a 4-fold increase in IgG titre in the paired serum samples is considered the gold standard for diagnosing acute HBoV1 infection (Söderlund-Venermo et al., 2009; Xu et al., 2017). However, the downside of serodiagnosis is the limitations in obtaining serum samples, HBoV1-4 cross-reactivity, and the phenomenon of original antigen sin (Kantola et al., 2015; Xu et al., 2017). HBoV1 DNA load measured from respiratory tract specimens by quantitative PCR has been studied. However, the results are controversial. The clinical sensitivity of HBoV1 DNA load measurement is thought to be moderate (Christensen et al., 2010, 2019; Xu et al., 2017). Furthermore, HBoV1 mRNA detection by reverse transcription PCR (RT-PCR) has shown high specificity in diagnosing symptomatic infections since mRNA is a marker for viral replication (Christensen et al., 2013; Schlaberg et al., 2017; Xu et al., 2017). A rapid HBoV1 antigen test based on immunodetection was released in 2014. Generally, antigen tests have lower sensitivity than tests based on nucleic acid detection (Bruning et al., 2016).

The mechanism of the HBoV1 persistency is not fully understood. HBoV1 has been detected in elective adeno-/tonsillectomy patients suffering from chronic adenotonsillar disease. The intratonsillar HBoV1 prevalence in most studies was 4%–20%, and intra-adenoid prevalence was approximately 25% when detected (Günel et al., 2015; Jartti et al., 2014; Mikola et al., 2019; Norja et al., 2012; Proenca-Modena et al., 2012, 2014; Silvoniemi et al., 2020). These studies concerning adeno-/tonsillectomy patients clearly show how HBoV1 is found in patients without acute infection.

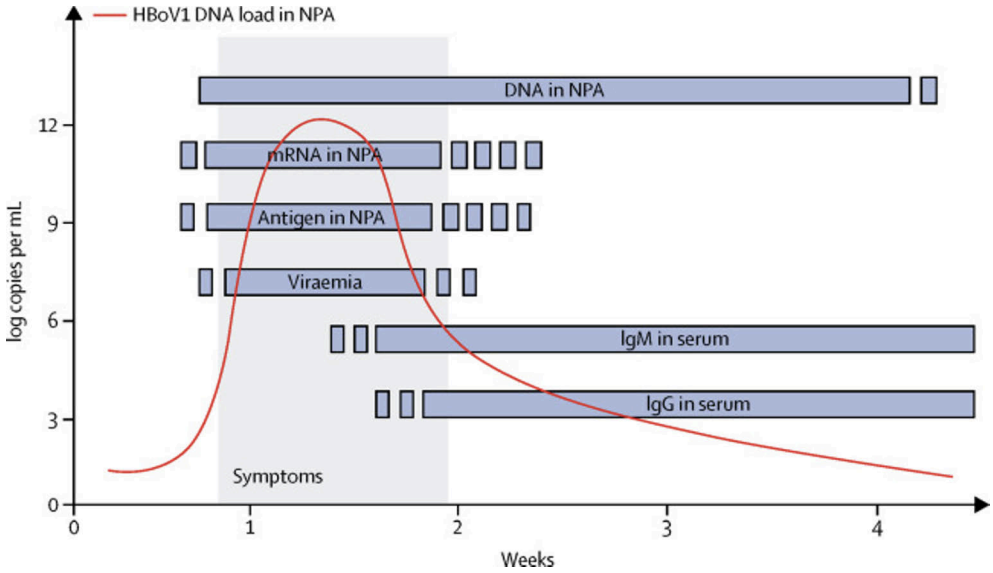

Figure 2. Clinically relevant variables during an acute primary HBoV1 infection in temporal order. The course of HBoV1 DNA load in NPA is shown by the red curve. All other parameters are represented by blue bars. The dotted ends of the blue bars indicate variation or uncertainty. Modified from Christensen et al. (2019); used with permission of Elsevier.

2.3.1.3 Immune responses

There are studies concerning the possible immunomodulatory effects of HBoV1. In young children with HBoV1 bronchiolitis, cytokines IL-4, IL-2, and IFN (interferon)-γ were elevated in the nasopharynx aspirate (NPA) samples compared to control patients without HBoV1 infection (Chung et al., 2008). *In vitro* studies have shown that HBoV affects the balance of IFN-β regulators (Luo et al., 2013; Zhang et al., 2012). Interestingly, co-infection of HBoV1 and RV caused a modified non-Th2 type cytokine response. Th1, Th2, and proinflammatory cytokine levels in serum were elevated in the RV group compared to the RV and HBoV1 co-infection group (Lukkarinen et al., 2014). In our earlier study, HBoV1 was associated with tonsillar hypertrophy compared to other indications. Furthermore, the expression of IL-37 was elevated in the tonsillar hypertrophy group (Mikola et al., 2018).

2.3.2 Other respiratory viruses

As well as HBoV1, other respiratory viruses are often found in the tonsil tissue of elective adeno-/tonsillectomy patients. The virus rates are quite high, and co-infections are especially common in children and adolescents under 18 (Proenca-Modena et al., 2012). AdV (3.3%–8%) and enterovirus (EV) (8%–17.7%) were the two most prevalent intra-tonsillar respiratory viruses in two studies, including adult

patients. However, the virus's prevalence decreases by age (Jartti et al., 2014; Silvoniemi et al., 2020). In children, intra-tonsillar AdV detection rates have been up to 27% and EV rates over 30% in elective tonsillectomy patients (Faden et al., 2016; Proenca-Modena et al., 2012, 2019; Proença-Módena et al., 2014). AdV causes acute respiratory infections and febrile exudative tonsillitis clinically similar to *Streptococcous* disease (Proenca-Modena et al., 2019). In one study, EV was associated with tonsillar hypertrophy, with the prevalence rate at 47% (Proenca-Modena et al., 2012).

The most common cause of RTI in children is RV (Toivonen et al., 2016), often found in the nasopharynx secretions, although the intratonsillar rates are low. In children, intratonsillar RV prevalence is around 12%–13% (Faden et al., 2016; Proenca-Modena et al., 2012). In the few studies including adults, the intratonsillar RV prevalence had been only 2%–4% (Jartti et al., 2014; Silvoniemi et al., 2020). Seasonal variations of RV infections in Finland are well-known (Mäkelä et al., 1998). Furthermore, the detection rate of RV in the tonsils of children who had had a tonsillectomy from February to March (Finland) using in situ hybridisation (RV-ISH) was as high as 62% (Suvilehto et al., 2006). RSV, MPV, Flu A and B, PIV 1–4, and seasonal CoV 229E, OC43, NL63, and HKU1 are detected in tonsil samples by PCR 0–10% each (Jartti et al., 2014; Mikola et al., 2019; Proenca-Modena et al., 2012; Silvoniemi et al., 2020).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been detected from tonsil tissue. Kadriyan et al. reported two cases in which the patients had recovered from coronavirus disease 2019 (COVID-19). Preoperatively the nasal swab tests using PCR were negative, serum IgM antibody for COVID-19 was negative and IgG was positive in both cases indicating a past infection. However, SARS-CoV-2 was detected by RT-PCR from tonsil tissue samples of both patients (Kadriyan et al., 2021). COVID-19 mainly causes respiratory tract symptoms, but the clinical manifestation and the disease burden in children are especially obscure. Considering this, patient selection for adenotonsillectomy becomes difficult concerning not only the patients but all the medical workers as well (Torretta et al., 2020).

Usually, tonsil samples are obtained from elective adeno-/tonsillectomy patients suffering from chronic adenotonsillar diseases. Therefore, only a few studies have studied the tonsil tissue of healthy controls due to ethical aspects. Proenca-Modena et al. took small punch biopsies from the tonsils of control patients (n=12) without adenotonsillar disease. The intra-tonsillar adenovirus rate was 17% compared to the disease group at 27% (Proenca-Modena et al., 2019). These studies show that tonsil

tissue harbours multiple respiratory viruses, but the function of virus persistence or prolonged virus infections remains unclear.

2.3.3 Herpesviruses

Herpesviruses are a group of enveloped, double-stranded DNA viruses. Nine of the known herpesviruses infect humans. Human herpesviruses (HHV) include herpes simplex 1 (HSV1), herpes simplex 2 (HSV2), varicella-zoster virus (VZV), cytomegalovirus (CMV), human herpesvirus 6A and 6B (HHV6A, HHV6B), human herpesvirus 7 (HHV7), EBV, and Kaposi's sarcoma-associated herpesvirus (KSHV). Ubiquitous HHV infections are usually acquired via respiratory tract secretions or saliva droplets during childhood. Although the primary infections are symptomless or cause only mild symptoms, the viruses persist for the rest of the host's life (Kourieh et al., 2019). EBV and KSHV persist in B-cells, whereas HHV6, HHV7, and CMV persist in T-cells. HSV1 and HSV2 affect cells of the nervous system (Berger et al., 2007). HHV6, HHV7, and EBV are commonly found in the tonsil tissue of chronic adenotonsillar disease patients, the prevalence being around 70%, 54%–70%, and 70%, respectively. Other human herpesviruses are rarely found in tonsils, the prevalence being 1%–2% (Kourieh et al., 2019; Silvoniemi et al., 2020).

Primary EBV infection often occurs in childhood without symptoms or with mild symptoms. In adolescents and young adults, EBV can cause acute mononucleosis. Typical signs and findings include fever, sore throat, and swollen and exudative tonsils. CMV can paint a similar clinical picture, especially in immunocompromised patients (Ebell et al., 2016; Thorley-Lawson et al., 2009). EBV is associated with certain lymphomas, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorder, which shares similarities with non-Hodgkin's lymphoma (Shannon-Lowe et al., 2019; Thorley-Lawson et al., 2009). A large cohort study published in February 2022 suggests that EBV has a causal effect on multiple sclerosis (MS) (Bjornevik et al., 2022).

Primary HHV6 and HHV7 infections cause childhood *exanthema subitum* (roseola infantum); in adults, a connection to autoimmune diseases such as Sjögren's disease has been suggested (Caserta et al., 1993; Comar et al., 2010). HHVs can reactivate in an immunocompromised host and cause severe morbidity. For example, immunosuppressive medication after organ transplantation predisposes to clinically significant herpesvirus infections. The symptoms range from mild fever to severe infections such as hepatitis and encephalitis (Bai et al., 2000; Sánchez-Ponce et al., 2018).

2.3.4 Microbiome

The surface of the tonsils is composed of crypts and folds. Thus, this surface is colonised by bacteria. In the cultivation studies or using PCR, the most common pathogenic bacteria have been *Streptococcus* spp., *Haemophilus* spp., *Staphylococcous aureus, Fusobacterium necrophorum, Helicobacter pylori* and *Actinomyces.* Moreover, *Corynebacterium diphtheriae, Neisseria gonorrhoeae, Chlamydia pneumoniae,* and *Mycoplasma pneumoniae* have also been associated with acute tonsillitis (Chan, 2010; Johnston et al., 2018a; Johnston & Douglas, 2018).

Currently, next-generation techniques utilising RNA or DNA sequencing are used to define microbial communities in certain tissues or specific body sites. Data analysis tools enable evaluating microbial richness (the number of species or taxa found in a sample), evenness (relative distribution of species or taxa in a sample), and diversity. Diversity is a calculated index that considers richness and species distribution. Alfa diversity measures the richness and species distribution in a sample and beta diversity between samples (Huang et al., 2017).

Recently, a method based on 16S ribosomal RNA (16S rRNA) gene sequencing has been used to discover the microbiome of tonsil swabs or tonsil tissue. This method is based on PCR amplification and searches 16S rRNA genes – present in all bacteria and it enables identifying bacteria to the genus or even the species level (Jensen et al., 2013; Johnston & Douglas, 2018). Using the 16S rRNA sequencing method to identify pathogenic bacteria has shown the complexity of the tonsil microbiome. Children suffering recurrent tonsillitis had a higher relative abundance of genera *Parvimonas, Prevotella,* and *Treponema* in tonsil tissue than children with obstructive sleep apnea, who had a higher relative abundance of *Haemophilus* and *Capnocytophaga* (Johnston et al., 2018b). Another study showed a significant difference between the tonsil microbiome of adults with recurrent tonsillitis and healthy adults. *Prevotella melaninogenica/histicola, Streptococcus intermedius,* and *Fusobacterium necrophorum* were associated with recurrent tonsillitis. Interestingly, *Streptococcus pyogenes* and *Staphylococcus aureus* were not commonly found. The same study compared the tonsil microbiome of adults and children. *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria* species were almost solely detected in children (Jensen et al., 2013). Moreover, the tonsil microbiome differs depending on the sample site. A higher relative abundance of genera *Streptococcus, Actinobacillus,* and *Neisseria* were found in the tonsil crypts than in the swab and tissue samples (Johnston et al., 2018a). A recent study compared the tonsil tissue microbiota of chronic tonsillitis patients and tonsillar hypertrophy patients. Tonsillar hypertrophy patients had a higher relative abundance of bacterial genera

Haemophilus, Streptococcus, Neisseria, Capnocytophaga, Kingella, Moraxella, and *Lachnospiraceae,* whereas chronic tonsillitis patients had a higher relative abundance of *Dialister, Parvimonas, Bacteroidales, Aggregatibacter,* and *Atopobium* (S. Wu et al., 2021).

Non-communicable diseases such as asthma and atopic dermatitis have been associated with the imbalance of the gut, respiratory, and skin microbiome (Huang et al., 2017). Tonsil tissue sample studies of healthy individuals are rare. One study used 16S rRNA gene sequencing to study the tonsil crypt microbiome of adults and children with recurrent tonsillitis, children with tonsillar hyperplasia, and healthy adults. Twelve abundant genera – *Actinomyces, Rothia, Streptococcus, Gemella, Granulicatella, Johnsonella, Prevotella, Porphyromonas, Fusobacterium Veillonella, Neisseria,* and *Haemophilus* – were found in all samples despite age and health, supporting the ideas of a core microbiome and the abundance of bacteria closely connected to the immune system (Jensen et al., 2013).

2.4 Viruses in adenoid tissue

2.4.1 Respiratory viruses

Non-acutely ill adenotonsillectomy patients are known to harbour respiratory viruses in adenoid tissue. According to literature, the detection rates using PCR of the most common intra-adenoid respiratory viruses are Adv 14%–70%, EV 0–31%, RV 25%–54% and HBoV 24%–28% (Faden et al., 2016; Proenca-Modena et al., 2012, 2019; Vinícius et al., 2014). In-situ hybridisation was positive for RV in 45% of the adenoid samples collected from children with adenoid hypertrophy, recurrent otitis media or both (Rihkanen et al., 2004). Other viruses detected by PCR (prevalence rate < 10 %) are seasonal CoV, PIV1–4, RSV, MPV, and influenza viruses (Proenca-Modena et al., 2012). A few studies indicate an association between intra-adenoid AdV infection and airway obstruction. However, the meaning of latent and persistent adenoid virus infections remains unclear, and an association or causal relationship to adenotonsillar diseases has not been made (Faden et al., 2016; Proenca-Modena et al., 2019).

2.4.2 Herpesviruses

According to a few studies, herpesviruses persist for life and are found in adenoid tissue. All the studies concern children with chronic adenotonsillar disease. EBV is the most common herpesvirus found in adenoid tissue, the prevalence being 40%–80% (Berger et al., 2007; Günel et al., 2015; Sato et al., 2009). CMV, HHV6, and HHV7 prevalence rates vary from 3% to 50%, 3% to 67%, and 51% to 66%,

respectively. Other herpesviruses have not been found in adenoids (Berger et al., 2007; Comar et al., 2010; Sato et al., 2009). The morbidity of herpesviruses is discussed earlier (tonsil herpesviruses). The significance of intra-adenoid herpesviruses is unclear. Moreover, there are similarities in the prevalence of herpesviruses in the tonsil and adenoid tissues (Berger et al., 2007).

2.5 Atopy

2.5.1 General aspects of atopy

Atopy is the genetic predisposition to produce an abnormal immunoglobulin E (IgE) dependent reaction to harmless environmental substances: allergens. Common allergens are grass and tree pollen, animals, and certain foods (e.g. codfish, cow's milk, egg, peanuts, wheat, and soybeans). Re-exposure to the specific allergen triggers an allergic reaction in a sensitised subject (Galli et al., 2013). Testing for IgE sensitisation is the basis for diagnosing a suspected allergic disease. Skin prick tests and serum-specific IgE assays are the most commonly used tools for diagnosing allergic sensitisation (Ansotegui et al., 2019). Allergic diseases are a growing problem worldwide. The risk of allergic disease depends on genetic and environmental factors. Exposure to animals, rural living, and infections to parasites such as helminths may reduce the risk.

Conversely, high hygiene standards, low environmental biodiversity, and an urban lifestyle increase the risk for allergic diseases (Cruz et al., 2017; Pawankar et al., 2011). The loss of environmental biodiversity is suggested to influence the human microbiome, leading to dysbiosis at the tissue level. Furthermore, dysbiosis affects immune responses and the occurrence of allergic diseases (Haahtela, 2019; Haahtela et al., 2013). Under normal conditions, type 2 immune response is responsible for the defence against helminths and other parasites. However, due to the hosts' genotype and certain environmental risk factors, the same mechanisms produce an abnormal type 2 immune response to a specific allergen – clinically seen as an allergic inflammation (Bohnacker et al., 2020; Galli et al., 2013). The association between helminths and allergic disorders is under investigation. An inverse correlation between parasitic infections and allergic diseases has been suggested, but the results remain controversial. A helminth molecule may someday help treat allergic diseases (Bohnacker et al., 2020; Cruz et al., 2017).

2.5.2 Local immune functions

In asthma and allergic diseases, the local immune functions are disturbed, and the balance is shifted towards a type 2 response (Galli et al., 2013). At the tissue level, allergens are encountered and processed by dendritic cells and further transported to regional lymph nodes. Processed allergens are presented to naïve T-cells, in which the presence of co-stimulatory molecules differentiate into Th2 cells (Figure 3). Th2 cells produce IL-4 and IL-13, stimulating B-cells to produce antigen-specific IgE. IgE is then diffused systemically and binds to high-affinity immunoglobulin E receptors on the mast cells and basophils (i.e. effector cells). This event is called sensitisation. Re-exposure to the specific allergen produces symptoms by triggering effector cells to release preformed biogenic mediators such as the proteases, histamine, and leukotrienes responsible for allergic symptoms. Other Th2 cell produced cytokines are IL-5, IL-9, and IL-31. IL-5 activates eosinophils, IL-9 contributes to mucus production and eosinophilia, and IL-31 impacts itchiness.

However, the interleukins mentioned above are not only produced by Th2 cells. Recently discovered retinoic acid receptor-related orphan receptor alpha (RORα) and GATA-binding factor 3 (GATA3) dependent type 2 innate lymphoid cells (ILC2) produce IL-13 to help dendritic cell migration to lymph nodes and boost the Th2 cell reactions. Furthermore, eosinophils, ILC2, basophils, and mast cells participate in IL-4 production during sensitisation. Activated ILC2 by epithelium-derived cytokines can also produce IL-5 and IL-13, contributing to airway inflammation (Alvaro-Lozano et al., 2020; Galli et al., 2013; Leung et al., 2020). The expression of IL-22 is conducted by activated Th22 and Th17 cells, NK cells, and ILCs. The role of IL-22 is controversial, and proinflammatory effects are seen in allergic skin diseases. Conversely, IL-22 may have immunomodulatory effects on allergic airway inflammation (Akdis et al., 2016; Fang et al., 2014).

Th1 and Treg (Figure 3) cell-derived cytokines counteract a type 2 response. Th1- and Treg-derived suppressor cytokines maintain the peripheral tissue balance in a healthy individual. The most important T cells controlling the immune responses of the oral mucosa are FOXP3+ Treg cells. These cells produce cytokines such as IL-10, transforming growth factor-β (TGF-β) and IL-35, which participate in the immune suppression and homeostasis of oral mucosa and associated secondary lymphoid tissue (Pelaez-Prestel et al., 2021).

In conclusion, the pathogenesis of allergy at the tissue level is a complex process orchestrated by multiple immune cells, cytokines, and other immune-related molecules. Whether the subject develops an allergic reaction to the allergen encountered depends on the balance of type 2, Treg, and Th1 responses.

The major cytokines expressed by Th17 cells are IL-17A, IL-17F, and IL-22. Differentiation of naïve T-cells into activated Th17 cells is mediated by a retinoic acid receptor-related orphan receptor C2 (RORC2) transcription factor. The fundamental task of Th17 cells is to defend against bacterial and fungal infections and also their role in autoimmune diseases is well-known. IL-17A and IL-17F are structurally similar and broadly involved in stimulating proinflammatory cytokines. However, in the presence of TGF-ß, the T-cell differentiation results in Th17 cells, which have more regulatory effects, including IL-10 expression. At the disease level, IL-17A and IL-17F are connected to, for example, allergic asthma, allergic skin diseases, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, contact hypersensitivity, and graft-versus-host-disease (Akdis et al., 2012, 2016).

By studying *in vivo* cytokine and transcription factor expression levels in tonsil tissue, atopic dermatitis was associated with higher expression levels of intratonsillar IL-37. Moreover, asthma was associated with lower IL-28 and IFN-α and allergic rhinitis with lower IL-13 expression levels in tonsil tissue compared to non-allergic study patients (Jartti et al., 2014). It has been shown *in vivo* that allergen-specific suppressive FOXP3+ Treg cells exist in pharyngeal and lingual tonsils and that the proportions were higher than in the peripheral blood (Palomares et al., 2011). Furthermore, a distinctive cytokine pattern, including lower expression of IL-10, IFN-λ, and T-bet, was seen in the tonsil tissue of atopic tonsillectomy patients compared to non-atopic (Kücüksezer et al., 2013). Interestingly, immune reactions were studied in the adenoids of children without allergy, those with allergic disease, and those whose allergy was treated with sublingual immunotherapy (SLIT). A Th1 pattern was seen in the adenoid tissue of non-allergic children, and SLIT induced a down-regulated Th2 response in the allergy group (Masieri et al., 2014). When considering the complexity of the mechanisms behind allergy and the recent results concerning local secondary lymphoid tissue, the adenoids and tonsils offer multiple routes to be studied.

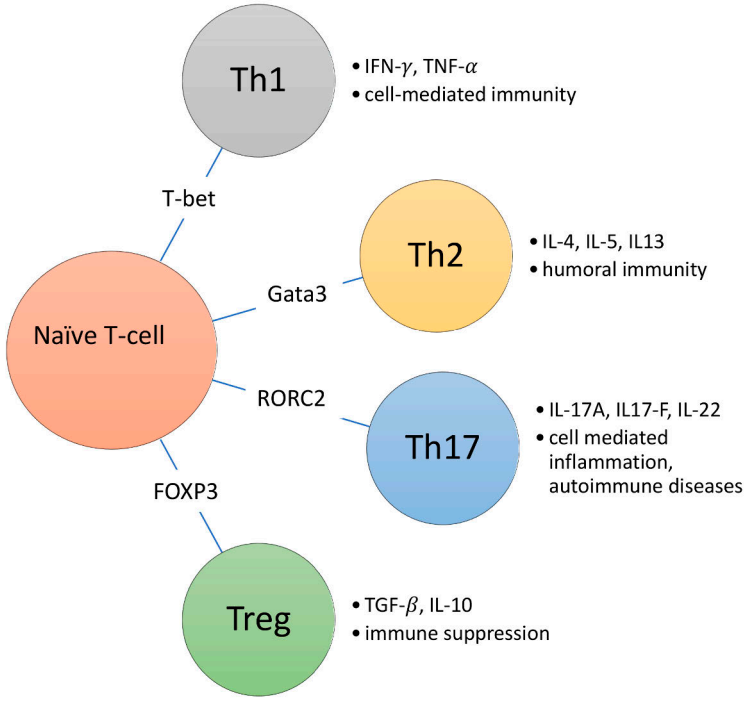

Figure 3. Differentiation of T-cells showing the main transcription factors and cytokines and functions of T-cell subpopulations. (Figure by Lotta Ivaska, modified from Leung et al., 2010).

3 Aims

This study aimed to define the ability of tonsil and adenoid tissues to serve as *in vivo* models for investigating tonsillar/adenoid virus infections, the microbiome, and their association with immune responses.

The specific objectives were as follows:

1. To evaluate whether an association between a high HBoV1 genome load and mRNA and serology findings in elective adenotonsillar surgery patients exists (I).
2. To compare the expression of tonsillar cytokines and transcription factors in HBoV1-positive and -negative adenotonsillar surgery patients (II).
3. To study and compare herpesvirus and respiratory virus findings in the adenoid and tonsil tissues of chronic adenotonsillar disease patients (III).
4. To compare the tonsillar microbiome between atopic and non-atopic adeno-/tonsillectomy patients using RNA sequencing (IV).

4 Materials and Methods

4.1 Study patients

Data for all studies I–IV was collected from the Boka-study carried out in the Satakunta Central Hospital, Pori, Finland, between April 2008 and March 2009 (Jartti et al., 2014). The inclusion criteria were adenoidectomy, adenotonsillectomy, or tonsillectomy due to chronic adenotonsillar disease and written informed approval from the study subject or his/her guardians. Exclusion criteria were suspicion of malignancy in adenoid or tonsil tissue. In total, 200 patients of all ages were recruited.

Also, in study IV, the data of two patients from the Tons2-study was used. The Tons2-study was carried out in the Turku University Hospital, Turku, Finland, and Salo Regional Hospital, Salo, Finland, between October 2013 and December 2015 (Silvoniemi et al., 2020). The inclusion criteria were elective tonsillectomy, according to chronic tonsillar disease, and written informed consent from the study subject or his/her guardians. The exclusion criteria were suspicion of tonsil or adenoid malignancy, systemic use of anti-inflammatory medication within four weeks before surgery, or a chronic systemic disease affecting the immune system. In total, 351 patients were recruited.

4.2 Study design and protocol

The Boka-study and Tons2-study were prospective, predefined, and observational clinical studies using standard protocols. Concerning the Boka-study, adenoidectomy, adenotonsillectomy, or tonsillectomy was performed by otorhinolaryngologists according to routine clinical procedure. A part of internal tonsil tissue, adenoid tissue or both was sterilely cut into 3–4 mm cubes by the surgeon. For analysis of the cytokine and transcription factor expression levels and RNA sequencing, the samples were stored in RNAlater – an RNA stabilisation reagent (Qiagen, Hilden, Germany), incubated at +2–8ºC until the next working day and finally stored at -80ºC (Jartti et al., 2014; Palomares et al., 2011). During general anaesthesia, NPA samples were collected through a nostril using a standardised

procedure (Osterback et al., 2013). For viral analyses, a piece of the removed tonsils and the NPA sample were frozen in dry tubes at -80°C. Also, the first sample of the paired serum samples was collected during the adeno-/tonsillectomy. The follow-up sample was taken after a median of 58 days (range 36–104). In the Tons2-study, a tonsillectomy was performed according to routine clinical practice. After tonsil removal, the internal part was cut into pieces, put into dry tubes, and stored at -70°C.

A standard questionnaire was used to obtain information about the general health condition, medication, allergic diseases, smoking habits, and respiratory symptoms before surgery (Jartti et al., 2014).

4.3 Laboratory methods

4.3.1 PCR for respiratory- and herpesviruses

Regarding all studies (I–IV), viruses in tonsil tissue, NPA, and adenoid tissue were detected by PCRs (including the reverse transcription step when applicable) on nucleic acid extracts (Jartti et al., 2004). For respiratory virus detection in all sample types, laboratory design real-time PCRs were used for HBoV1, EV, RV, and RSV, including quantitative PCR (qPCR) to measure HBoV1 DNA load, as described earlier (Koskenvuo et al., 2008; McLeish et al., 2012; Peltola et al., 2008). In tonsil tissue and NPA samples, Seeplex RV12 ACE Detection (Seegene, Seoul, Korea) multiplex PCR kit was used to detect respiratory viruses, including AdV, CoV 229E/NL63, and OC43/HKU1, Flu A and Flu B, MPV, PIV types 1–3, RSV group A and B, and RV.

In adenoid tissue samples, Allplex respiratory panels I-III (Seegene, Seoul, South Korea) were used for multiplex PCR detection of AdV, HBoV1–4, CoV 229E, NL63, and OC43, EV, FluA and FluB, MPV, PIV types 1–4, RSV, and RV (Jartti et al., 2004; Koskenvuo et al., 2008; McLeish et al., 2012; Peltola et al., 2008; Tiveljung-Lindell et al., 2009). GeneProof PCR kits (GeneProof, Brno, Czech Republic) were used for CMV and EBV. Laboratory design real-time quantitative PCRs were used for HSV1, HSV2, VZV, HHV6, and HHV7 (Mannonen et al., 2012; Wada et al., 2009). All commercial tests were used according to the manufacturer's instructions. PCR tests were done at the Department of Virology, University of Turku, Finland. Also, HBoV1 qPCR was done at Karolinska University Hospital, Stockholm, Sweden.

4.3.2 Serology for HBoV1

Serology data were used in studies I–II. Serum samples were collected from 123 adeno-/tonsillectomy patients, and HBoV1-specific IgM and IgG were analysed. HBoV1-specific serology was not analysed from sole adenoidectomy patients. The serum samples analysed were blocked with HBoV2 and HBoV3 antigens to confirm that the IgG result was specific to HboV1. Serology was analysed at the Department of Virology, University of Helsinki (Kantola et al., 2011; Söderlund-Venermo et al., 2009).

4.3.3 Messenger RNA (mRNA) for HBoV1

Reverse transcription PCR (RT-PCR) was used to analyse the expression levels of HBoV1 in tonsil and NPA samples in study I (Christensen et al., 2013). An RT-PCR detecting human beta-actin mRNA was used to control the intactness of mRNA in the samples (Nyström et al., 2004). HBoV1 mRNA RT-PCR was done at the Norwegian University of Science and Technology, Trondheim, Norway.

4.3.4 Reverse-transcription PCR for cytokines and transcription factors

To detect cytokines and transcription factor expressions (study II), total RNA was directly isolated from tonsils by homogenising tissue (previously stabilised in RNAlater) in grinding tubes containing ceramic beads. RNeasy mini kit (Qiagen, Hilden, Germany) was used to isolate RNA from cell samples and Revert Aid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) to perform reverse transcription. Commercial tests and kits were used according to the manufacturers' protocol. The mRNA expression levels of IFN-α, IFN-β, IFN-ɣ, IL-10, IL-13, IL-17, IL-28, IL-29, IL-37, TGF-β, FOXP3, GATA3, RORC2, and Tbet (T-box transcription factor) were analysed in quantitative real-time PCR (Jartti et al., 2014). The detection of cytokines and transcription factors was done at the Swiss Institute of Allergy and Asthma Research, Davos, Switzerland.

4.3.5 RNA sequencing

Details of RNA sequencing are presented in the original publication: study IV. Here, the principles of RNA sequencing are briefly illustrated (Koch et al., 2018) (Figure 4). After isolating RNA from cell samples, the isolated RNA was converted into complementary DNA (cDNA), which was additionally fragmented. Fragmented cDNA was sequenced ("read") by a high-throughput platform (Illumina HiSeq 2500). Sequencing resulted in raw read data that was demultiplexed, aligned, and mapped into genes to generate a raw counts table. Raw reads were assessed using FASTQC

(Andrews, 2019) to filter low-quality reads. Trimmomatic (Bolger et al., 2014) was used for removing low-quality reads, Illumina adapters, and short read-length. The quality-filtered reads were aligned to the human genome (GRCh38-v93) using Hisat (Kim et al., 2015). Human and non-human reads were separated. The non-human reads were used as microbial reads for taxonomic identification of microbial communities. Uniquely mapped reads differed between 41 and 50 million per sample. After filtering out reads mapped to the human genome, the rest were considered microbial reads (~1 million reads on average, 6% of total). A taxonomic sequence classification system, Kraken2, was used to get more functional units and increase the accuracy of the results (Wood et al., 2014). Therefore, the reads were mapped into nucleotide databases using Kraken2, and the outputs were converted into BIOM format using the Kraken-BIOM tool. The BIOM file contained the read counts for every microbial species found in any sample. RNA sequencing via Illumina HiSeq 2500 and subsequent transcriptomics analysis for differential gene expression was performed at the Functional Genomics Center Zurich, Switzerland, and the following bioinformatic analysis of RNA sequencing at the University of Turku, Finland.

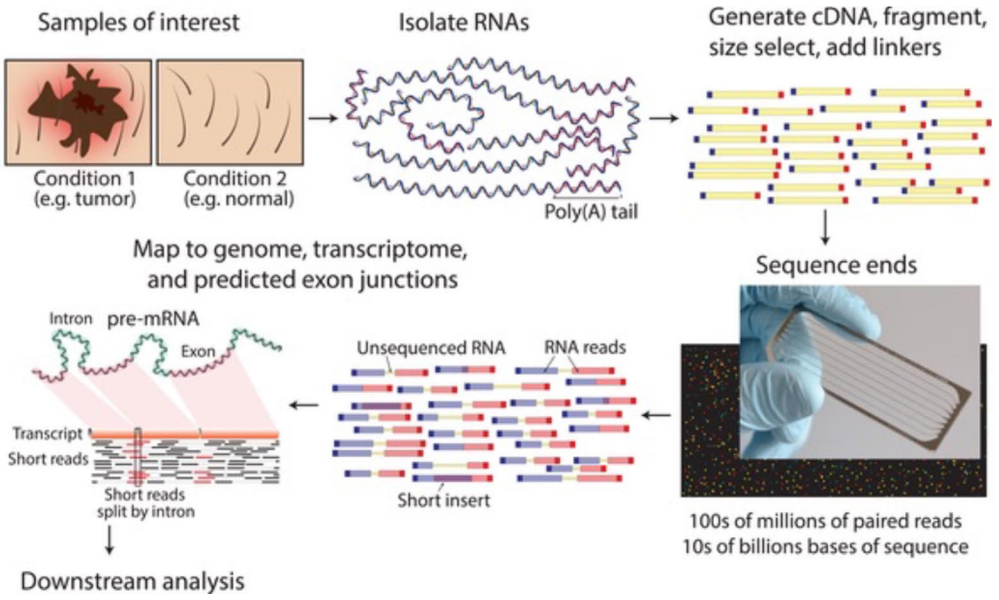

Figure 4. RNA seq data generation. A typical RNA seq experimental workflow involves isolating RNA from samples of interest, generating sequencing libraries, using a high-throughput sequencer to produce hundreds of millions of short paired-end reads, and aligning reads against a reference genome or transcriptome, as well as downstream analysis for expression estimation, differential expression, transcript isoform discovery, and other applications. Reprinted from Griffith M, Walker JR, Spies NC, Ainscough BJ, Griffith OL (2015) Informatics for RNA Sequencing: A Web Resource for Analysis on the Cloud. PLOS Computational Biology 11(8): e1004393. (Griffith et al., 2015). Used with the permission of Creative Commons Attribution License.

4.3.6 Atopy diagnostics

Atopy diagnostics were used in study IV. Allergic sensitisation was analysed by detecting allergen-specific immunoglobulin IgE antibodies in any of the common aeroallergens (cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum,* and *Dermatophagoides pteronyssinus*) or food (codfish, cow's milk, egg, peanuts, wheat, and soybeans) with a cut-off level of 0.35 > kU/L (Phadiatop Combi®, Phadia, Uppsala, Sweden). Birch, mugwort, timothy, and *Cladosporium herbarum* were considered pollen aeroallergens. Animal sensitisation was defined as positive IgE antibodies to dogs, cats, horses, or *Dermatophagoides pteronyssinus* (Elenius et al., 2017).

The clinical definition of allergy was based on the standard questionnaire. The following questions were asked from the study patients or their guardians: any allergy, yes/no; if yes, which/what; allergic rhinitis, yes/no; doctor-diagnosed atopic eczema, yes/no; doctor-diagnosed asthma, yes/no.

4.4 Statistical analysis

In all studies, where applicable, conventional statistics comparing patient groups was carried out using SPSS software Statistics for Windows, version 25.0–27.0 (IBM SPSS Statistics for Macintosh, Armonk, NY). For continuous variables, the statistical significances of the differences among the study groups were tested with a student's T-test, Mann–Whitney U test, or ANOVA. For categorical variables, the statistical significance of the differences among frequencies and percentages were tested with the Chi-square test or Fisher's exact test when cell counts were <5. Statistical significance was established at the level of a two-sided p-value < 0.05.

In study I, the mean age was calculated. The rest of the data, such as indication for operation, symptoms on operation day, HBoV1 positivity, HBoV1 mRNA results, and the number of cases with a high HBoV1 viral load were described using percentages. HBoV1 serology results were described as positive or negative.

In study II, we compared the cytokine and transcription values between HBoV1-positive and -negative patients. Cytokine and transcription factor values were log-transformed because of positively skewed distributions of the data, and clinical, viral, and immunological differences between study groups were analysed using unadjusted and multivariable linear model analyses. The analyses were adjusted for virus infections and clinical factors – which significantly varied between the groups – and age. The backward stepwise method was used for the final adjustment model separately for each cytokine and transcription factor, keeping only significant factors

in the final model. The relationships between virus load and cytokine/transcription factor levels were computed using a Spearman correlation.

In study III, the primary aim was to analyse the presence of herpes and respiratory viruses in adenoid tissue. The secondary aim was to compare the virus findings among sample types of the same patient. Cohen's Kappa was used to determine the agreement regarding the detection of respiratory and herpesviruses among tonsil, adenoid, and NPA samples. Agreement statistics between virus diagnostics of sample types were calculated with kappa coefficients and could be interpreted as follows: < 0.40 =poor, ≥ 0.40 to < 0.75 = fair to good, and ≥ 0.75 = excellent (Landis et al., 1977). Virus DNA levels were tested for equality between adenoids and tonsils using the Wilcoxon signed-rank test.

In study IV, the primary purpose was to compare the intratonsillar microbiome between atopic and non-atopic study patients. After processing the tonsil RNA and read mapping, bioinformatic analysis was done. Briefly, Phyloseq was used to measure alfa diversity (a measure of richness and species distribution in a sample) and beta diversity (a measure of diversity between samples) of microbial communities (McMurdie et al., 2013). Alpha diversity in each sample was estimated with Phyloseq using the Chao1 index at the taxa-level. The Bray–Curtis index was used to measure beta diversity. Differential abundance in atopic and non-atopic subjects was assessed using EdgeR version 3.2.1 (Robinson et al., 2010).

4.5 Ethics

The Ethics Committee of Satakunta Central Hospital and the Ethics Committee of the Hospital District of Southwest Finland approved the study protocols. Written informed consent was obtained from all the study patients or their guardians.

5 Results

5.1 Improved HBoV1 infection diagnostics (I)

5.1.1 Study population and patient characteristics

Initially, enrolling involved 200 patients (Boka-study), of whom 12 had insufficient sample material. In total, 188 patients had adeno-/tonsillectomy (n=143) or an adenoidectomy performed (n=45), yielding good-quality biopsy samples for microbial and immunological studies. The median age of this study group was 12 (range 1–65). The main indications for tonsillectomy were tonsillar hypertrophy in 48/143 (34%) and recurrent tonsillitis in 43/143 (30%) of the patients. Adenotonsillar hypertrophy was the most common indication for adenotonsillectomy in 40/143 (28%) of the cases. Other indications for adeno-/tonsillectomy were peritonsillar abscess, recurrent fever, and food remnants in tonsils and teeth braces in 12/143 (8%). The main indications for sole adenoidectomy were recurrent otitis in 28/45 (62%) and adenoid hypertrophy in 17/45 (38%) of the patients. On operation day, 127/188 (67%) had no respiratory tract symptoms, and 37/188 (20%) reported mild respiratory tract symptoms such as mild rhinitis, cough, symptoms of otitis, throat pain, and upper airway obstruction symptoms. No data was available for 24 (13%) study patients.

5.1.2 HBoV1 DNA load and mRNA

HBoV1 DNA in NPA, tonsillar tissue, or both samples was detected in 40 patients (21%) with a median age of 5 (range 1–22 years). Of these HBoV1-positive patients, 15/40 (38%) were sole adenoidectomy patients and 25/40 (63%) adeno-/tonsillectomy patients. Mild respiratory tract symptoms were reported by 12 of the 40 patients (30%). In 28 patients, the HBoV1 result was only positive in NPA; in seven patients, only in tonsillar; and in five, with both samples. In the sole adenoidectomy group, five patients had a high ($>10^6$ copies/mL) viral load in NPA using qPCR, but the mRNA result was negative. Concerning the adeno-/tonsillectomy group, nine patients had a relatively high ($>10^4$ copies/mL) viral load in NPA, but the mRNA results were negative (Table 1). Furthermore, all 29 NPA and the eight tonsil samples analysed were HBoV1 mRNA-negative, despite HBoV1 DNA-positive results. Beta actin-mRNA PCR was strongly positive in the eight NPA samples tested (with HBoV1 DNA load $>10^4$ copies/mL).

5.1.3 HBoV IgM and IgG

An HBoV1 DNA finding was accompanied by IgG positivity in all but three patients, signifying a prior infection. The corresponding sera available for the nine patients with a relatively high HBoV1 DNA load in NPA and/or tonsils were HBoV1 IgM-negative (Table 1). Only one patient with a positive HBoV1 PCR result in tonsils had an IgM-positive test result (barely, with an absorbance level of 0.147). However, the IgG absorbance in paired samples was stable, indicating a recent but nonacute HBoV1 infection.

The three HBoV1 DNA-positive but seronegative children were HBoV2-IgG-positive. This finding suggests their HBoV1 IgG-negative results can be explained by an immunological phenomenon called original antigenic sin, meaning that a past infection by a similar virus inhibits the immune response towards a subsequent, slightly different virus (Kantola et al., 2015). Also, in the seven paired serum samples of HBoV1 DNA-positive patients, HBoV1-IgG levels did not increase.

Table 1. Adenoidectomy (n=5) and adeno-/tonsillectomy patients (n=9) with high (>10^6 copies/mL) or relatively high (>10^4 copies/mL) HBoV1 DNA-load in NPA and/or tonsil tissue samples. Modified from study I.

Case no.	Age (y)	Indication for the operation	Symptoms[a] on the operation day	HBoV1 DNA load (cp/ml), NPA	HBoV1 PCR result, tonsils	HBoV1 DNA load (cp/g), tonsils	mRNA NPA/ tonsils	HBoV 1 IgG	HBoV 1 IgM (abs.)
B073	3	ROM	no	100396800	-	-	neg/-	-	-
B087	3	ROM	yes	28269400	-	-	neg/-	-	-
B100	6	AH	yes	19708800	-	-	neg/-	-	-
B182	2	ROM	no	20537600	-	-	neg/-	-	-
B184	1	ROM	no	2227000	-	-	neg/-	-	-
B051	7	ATH	no	133200	neg	0	-/-	pos[b]	neg
B113	12	ATH	-	119200	neg	0	neg/-	pos[b]	neg
B130	5	ROM, ATH	-	30400	neg	0	neg/-	pos	neg
B162	6	ATH	no	210600	neg	0	neg/-	pos	neg
B056	5	ATH	yes	307800	pos	58400	neg/neg	-	-
B082	4	ATH	no	32200	pos	2000	neg/neg	pos[c]	neg
B106	4	ATH	no	220800	pos	2400	neg/neg	pos[c]	neg
B150	5	RT, ATH	-	92400	pos	4400	neg/-	pos[b,d]	neg
B197	3	ATH	no	202600	pos	1400	neg/neg	neg[d]	neg

Abbreviations: y, years; ATH, adenotonsillar hypertrophy; TH, tonsillar hypertrophy, ROM, recurrent otitis media; RT, recurrent tonsillitis; NPA, nasopharyngeal aspirate; cp, copies; -, not available; abs., absorbance (cutoff ≥0.131)

[a] Mild rhinitis, cough, symptoms of otitis, throat pain or upper airway obstruction symptoms

[b] Paired serum samples; no increase in IgG

[c] No acute-phase serum sample available

[d] HBoV2 IgG-positive; may influence induction of HBoV1 IgG through original antigenic sin (Kantola et al., 2015)

5.2 HBoV1 and tonsillar cytokines expression (II)

5.2.1 Study population and patient characteristics

Of the 200 enrolled patients, 45 were sole adenoidectomy patients, and 12 had otherwise insufficient sample material. Therefore, 143 suitable tonsil and NPA samples from adenotonsillectomy or tonsillectomy patients were analysed. HBoV1 DNA in NPA, tonsil or both samples was detected by PCR in 25/143 (17%). The rest, 118/143 (83%), were HBoV1 DNA-negative. The median age of the HBoV1-positive patients was 6, and of the negative ones, 18 ($p < 0.001$).

The sex distribution between the study groups was equal. There was no difference in sensitisation, allergic diseases, self-reported allergy, active/passive smoking, or symptoms on the operation day between the groups. Tonsillar hypertrophy was a more common indication for operation in the HBoV1-positive group (64%) compared to the HBoV1-negative group (27%) ($p < 0.001$). Conversely, recurrent tonsillitis was a more common indication in the HBoV1-negative group compared to the positive group (34% vs 12%) ($p = 0.03$).

5.2.2 Virus findings and serology

Eighteen of the 143 patients (13%) were positive for HBoV1 in NPA samples, 12 (8%) in tonsil tissue, and five (3%) in both. RV was the most common co-infection with HBoV1 in both sample types. HBoV-specific serology was analysed from 123 of the 143 study patients. In 100 of the 123 (81%) patients, HBoV1-specific IgG was detected, indicating a prior infection. Of the 25 HBoV1 DNA-positive patients, 18 showed a positive IgG and a negative IgM result.

5.2.3 Cytokine and transcription factor findings

The cytokine and transcription factor results were diverse between HBoV1-positive and -negative study patients. The expressions of Th17 type transcription factor RORC2 and Treg type transcription factor FOXP3 were significantly lower in the HBoV1-positive group ($p = 0.021$, $p = 0.045$) using linear regression analysis (Table 2, Figure 5). After adjusting for age, recurrent tonsillitis, tonsillar hypertrophy, RV in NPA, AdV in NPA, tonsillar AdV, tonsillar EV, tonsillar RSV, AdV in tonsil and NPA, RV in tonsil and NPA, two or more viruses in NPA, tonsil or both, the results persisted. A negative correlation existed between tonsil HBoV1 DNA load and IL-28 ($p=0.004$), IL-29 ($p=0.019$), and IL-13 expression levels ($p=0.013$).

Table 2. Cytokine or transcription factor expression in tonsils of patients with HBoV1 DNA-positive or -negative NPA or tonsil tissue. Tbet, T-box transcription factor; TGF, transforming growth factor. Modified from study II.

Cytokine or transcription factor	HBoV1 positive n=25	HBoV1 negative n=118	*p* value univariate	*p* value multivariate	Adjustments
Th1 -type					
IFN-γ	65 (35, 104)	65* (34, 103)	0.96	0.58	RV NPA and tonsil
Tbet	54 (22, 79)	45 (20, 71)	0.65	0.14	age, RV NPA, and tonsil
Th2 -type					
IL-13	1.4 (0.3, 3.2)	0.5 (0.02, 4.1)	0.19		-
GATA3	24 (10, 31)	24 (11, 39)	0.27		-
Th17 -type					
IL-17	10 (7, 19)	10 (5, 18)	0.18	0.47	age, RSV tonsil
RORC2	11 (7, 19)	21 (11, 32)	***0.021***		-
Treg -type					
IL-10	57 (25, 73)	42 (23, 67)	0.20	0.55	age
IL-37	0.24 (0.14, 0.35)	0.17*** (0.11, 0.34)	0.10	0.91	age
FOXP3	35 (14, 60)	51 (28, 86)	***0.045***		-
TGF-β	140 (104, 234)	163 (105, 217)	0.89		-
Type I/III interferons					
IFN-α	20 (1, 181)	10** (0.4, 128)	0.23		-
IFN-β	37 (7, 154)	16* (3, 100)	0.23		-
IL-28	40 (8, 78)	17* (2, 76)	0.11		-
IL-29	16 (3, 36)	6 (2, 28)	0.59		-

Values are arbitrary units x 10^4 relative to EF1α.
Data are expressed as the median (interquartile range).
Adjustments are selected backward and stepwise from significant differences between groups
* n=117, ** n=116, *** n=115

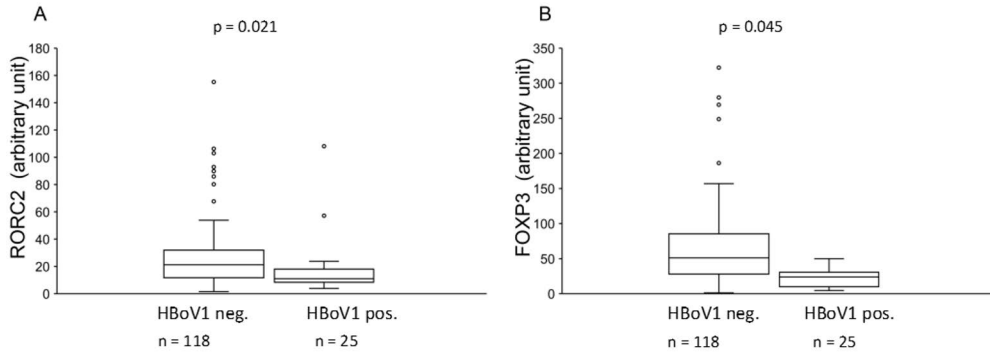

Figure 5. Tonsillar expression of transcription factors RORC2 and FOXP3 between HBoV1-negative and -positive (PCR pos. in NPA and/or tonsil) patients. Modified from study II.

5.3 Viruses in adenoid and tonsil tissue (III)

5.3.1 Study population and patient characteristics

Of the 200 patients originally enrolled, we included all the adenoidectomy (n=45) and adenotonsillectomy (n=44) ones. Altogether, 89 patients had sufficient sample material and were included. We analysed 45 autologous adenoid and NPA samples and 44 autologous NPA, adenoid, and tonsil samples. Adenoid hypertrophy, tonsillar hypertrophy or both in 43 of 89 (48%) patients were the most common reasons for the operation. A mixed indication of otitis media with effusion, tonsillar/adenoid hypertrophy, and recurrent tonsillitis was found in 33/89 (37%) of the cases. Mild upper respiratory tract symptoms were reported by 24/77 (31%) of the study patients.

5.3.2 Virus findings

At least one virus was detected in 97% (86/89) of the adenoid samples. Also, 95% (42/44) of the tonsil samples and 94% (84/89) of the NPA samples were positive for more than one virus. Regarding overall prevalence, at least one of the seven herpesviruses detected was found in 89% of the samples, and one of the 16 respiratory viruses was detected in 94%.

HHV6 was the most common intra-adenoid herpesvirus (33%), the second was HHV7 (26%), and then EBV (25%). CMV and HSV1 were detected in one sample each. HSV2 and VZV were not found in adenoid samples. CMV was found in 15% (13/89) of the NPA samples; similar detection rates of herpesviruses were found in NPA samples compared to adenoid samples (Table 3). The intra-tonsil herpesvirus detection rates were HHV6, 45%; HHV7, 52%; and EBV, 23% (Table 3). The intra-

adenoid genome levels of HHV6 and HHV7 were significantly higher compared to genome levels of HHV6 and HHV7 in tonsils, with p-values of 0.002 and <0.001, respectively. Intra-adenoid HHV6-positive patients were younger than negative ones (median age 3 vs 6 years, respectively, p=0.017).

RV (60%) and HBoV1 (58%) were the two most prevalent respiratory viruses in adenoid tissue and NPA samples (63% and 35%, respectively). HBoV1 (23%) was the most prevalent virus detected in tonsils, followed by AdV (18%) (Table 3).

Table 3. Detected viruses in NPA, intra-adenoid, and intratonsillar samples. Values are expressed as the number of positive findings (%). Modified from study III.

Virus	Intra-adenoid n=89		Intratonsillar n=44		Nasopharyngeal n=89	
	With or without other virus	Sole	With or without other virus	Sole	With or without other virus	Sole
HHV6	29 (33%)	1 (1%)	20 (45%)	5 (11%)	41 (46%)	0
HHV7	23 (26%)	1 (1%)	23 (52%)	6 (14%)	34 (38%)	1 (1%)
EBV	22 (25%)	2 (2%)	10 (23%)	0	22 (25%)	1 (1%)
CMV	1 (1%)	0	0	0	13 (15%)	0
HSV1	1 (1%)	0	0	0	2 (2%)	0
HSV2	0	0	0	0	0	0
VZV	0	0	0	0	0	0
RV	53 (60%)	4 (4%)	3 (7%)	0	56 (63%)	9 (10%)
HBoV	52 (58%)	3 (3%)	10 (23%)	1 (2%)	31 (35%)	1 (1%)
AdV	11 (12%)	1 (1%)	8 (18%)	0	23 (26%)	1 (1%)
EV	6 (7%)	0	7 (16%)	2 (5%)	9 (10%)	0
CoV**	1 (1%)	1 (1%)	0	0	8 (9%)	1 (1%)
PIV1- 4	12 (13%)	2 (2%)	3 (7%)	0	6 (7%)	0
FLU A/B	1 (1%)	0	0	0	4 (4%)	1 (1%)
RSV	8 (9%)	0	2 (5%)	0	2 (2%)	0
MPV	0	0	1 (2%)	0	0	0
no virus findings	3 (3%)		2 (5%)		5 (6%)	
≥1 viruses	86 (97%)		42 (95%)		84 (94%)	

*Coronavirus types 229E, NL63, OC43, or HKU1

5.3.3 Agreement results

Detecting HHV7 in adenoids was agreeable with detecting the virus in corresponding tonsils (kappa value 0.55, 95% confidence interval 0.34–0.77). EBV was frequently detected in the adenoid and tonsil tissue of the same patient (kappa value 0.67, 95% confidence interval 0.39–0.94). Moreover, EBV detection agreed between NPA and tonsils (kappa value 0.53) and between adenoid and NPA samples (kappa value 0.58). There was no significant agreement in detecting any respiratory virus among different sample types (Table 4).

Table 4. Agreement analysis of adenoid vs tonsil, adenoid vs NPA, and NPA vs tonsil virus detections. Modified from study III.

	Adenoid vs. Tonsil, n=44				**Adenoid vs NPA, n=89**				**NPA vs Tonsil, n=44**			
	Adenoid	Tonsil PCR		Kappa coefficient	Adenoid	NPA PCR		Kappa coefficient	NPA	Tonsil PCR		Kappa coefficient
Virus	PCR	neg	pos	(95% CI)	PCR	neg	pos	(95% CI)	PCR	neg	pos	(95% CI)
HHV6	neg	20 (83%)	12 (60%)		neg	36 (75%)	24 (58%)		neg	13 (54%)	12 (60%)	
	pos	4 (17%)	8 (40%)	0.24 (-0.029, 0.51)	pos	12 (25%)	17 (42%)	0.17 (-0.03, 0.37)	pos	11 (46%)	8 (40%)	-0.059 (-0.35, 0.23)
HHV7	neg	21 (100%)	10 (43%)		neg	48 (87%)	18 (53%)		neg	15 (71%)	8 (35%)	
	pos	0 (0%)	13 (57%)	***0.55 (0.34, 0.77)***	pos	7 (13%)	16 (47%)	0.37 (0.17, 0.56)	pos	6 (29%)	15 (65%)	0.37 (0.091, 0.64)
EBV	neg	32 (94%)	3 (30%)		neg	60 (90%)	7 (32%)		neg	31 (91%)	4 (40%)	
	pos	2 (6%)	7 (70%)	***0.67 (0.39, 0.94)***	pos	7 (10%)	15 (68%)	***0.58 (0.38, 0.77)***	pos	3 (9%)	6 (60%)	***0.53 (0.23, 0.83)***
RV	neg	18 (44%)	1 (33%)		neg	19 (58%)	17 (30%)		neg	14 (34%)	0 (0%)	
	pos	23 (56%)	2 (67%)	0.024 (-0.11, 0.15)	pos	14 (42%)	39 (70%)	0.27 (0.063, 0.47)	pos	27 (66%)	3 (100%)	0.066 (-0.012, 0.14)
HBoV1	neg	21 (63%)	2 (20%)		neg	33 (57%)	4 (13%)		neg	23 (67%)	5 (50%)	
	pos	13 (37%)	8 (80%)	0.301 (0.056, 0.55)	pos	25 (43%)	27 (87%)	0.38 (0.21, 0.55)	pos	11 (33%)	5 (50%)	0.146 (-0.14, 0.44)

CI Confidence interval

Values are expressed as n (%).

5.4 Microbiome of tonsils (IV)

5.4.1 Study population

Altogether, the Boka- and Tons2-studies enrolled 551 patients. Of the 200 patients enrolled in the Boka-study, 45 were excluded because of a sole adenoidectomy and 12 because of low-quality samples. Of the 143 patients, 62 had sufficient sample material available for RNA sequencing. The Tons2-study enrolled 351 patients, of which 183 had tonsil viruses analysed. We selected three cases with HBoV1-positive results in tonsil tissue to carry on with RNA sequencing. RNA sequencing was used to analyse the tonsil tissue transcriptome profile of 65 patients. Serum samples were obtained from 55 of the 65 RNAseq cases, and the sensitisation (atopy) status was defined by a positive IgE antibody to common food or aeroallergens. Outliers were removed from the data, leaving 53 samples for study IV (Figure 6).

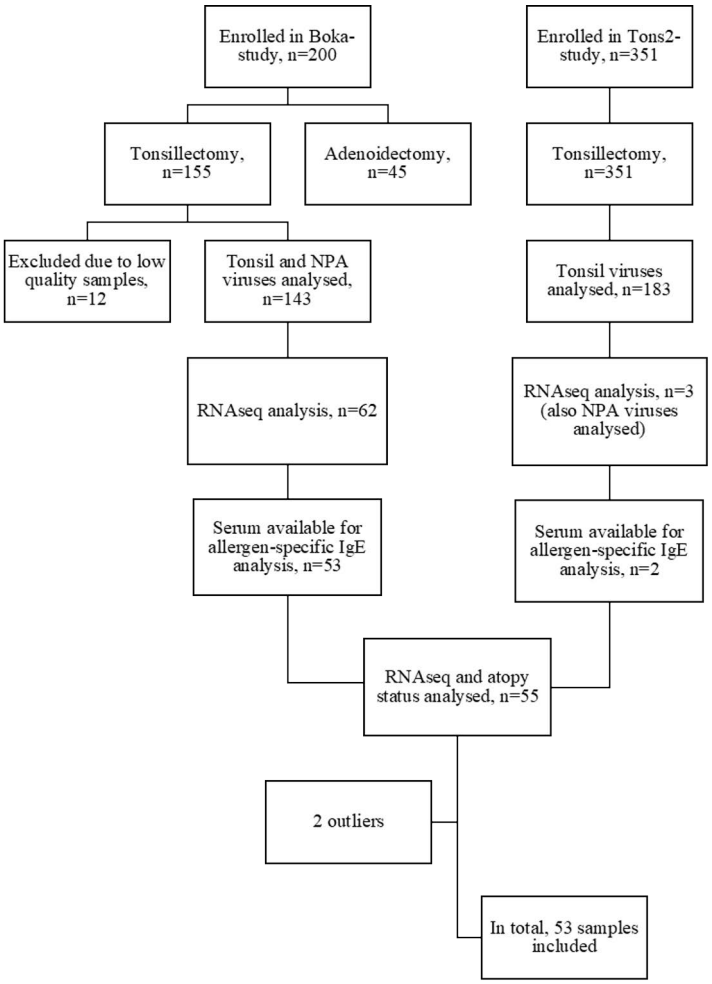

Figure 6. Flowchart of the study. Two cohorts (Boka- and Tons2-study) were combined to find suitable cases for RNA sequencing analysis. The cases included for RNA sequencing analysis were men and women, preferably under 21 years, with different combinations of tonsillar and NPA viruses. Modified from study IV.

5.4.2 Patient characteristics

The median age of the study patients (n=53) was 11 (range 2–38 years). The division into two groups was made by a sensitisation result (allergen-specific IgE), and 24/53 (45%) were considered atopics. Recurrent tonsillitis and tonsillar hypertrophy were the most common indications of the operation. The operations were not performed during acute tonsillitis or any other acute infection. See patient characteristics in Table 5.

Table 5. Patient characteristics. Modified from the study IV.

Factor	Atopic n=24	Non-atopic n=29	p
Median age (range), years	12.4 (2.0–38.1)	7.5 (3.0–21.1)	.14
Male	12 (50%)	16 (55%)	.70
Indication for adeno-/tonsillectomy			
Recurrent tonsillitis	12 (50%)	13 (45%)	.70
Tonsillar hypertrophy	11 (46%)	13 (45%)	.94
Other indication†	1 (4%)	3 (10%)	.62
Self-reported allergy	12 (50%)	8 (28%)	.07
Sensitisation			
Food	6 (25%)	0 (0%)	
Aero (i.e. pollen, animal, or house dust mite)	14 (58%)	0 (0%)	
Food and aero	4 (17%)	0 (0%)	
Physician-diagnosed asthma	6/22 (27%)	3/26 (12%)	.16
Self-reported allergic rhinitis	11/20 (55%)	3/27 (11%)	.003
Physician-diagnosed atopic dermatitis (AD)	2/20 (10%)	6/29 (21%)	.32
No clinical allergic disease (asthma, rhinitis, AD)	8/22 (36%)	17/29 (59%)	.12
Respiratory viruses in tonsillar tissue	12 (50%)	15 (52%)	.90
Respiratory viruses in nasopharynx	18 (75%)	24 (83%)	.49
Smoking or exposure to smoking	11/23 (48%)	15/28 (54%)	.68
Use of antibiotics (ab) within 1 year	16/20 (80%)	18/28 (64%)	.24
Season of sampling			
April–August	10 (42%)	11 (38%)	.78
September– March	14 (58%)	18 (62%)	

†Chronic white patches in tonsils (n=1), teeth braces (n=1), periodic fever (n=2)

5.4.3 Microbial diversity and abundance between the groups

Alpha diversity measures the species richness and diversity in a sample. The alpha diversity was lower in the tonsil samples of atopic patients compared to non-atopic (Figure 7A). Beta diversity measures the variation of microbial diversity between group levels. There was no significant difference in tonsil tissue microbial beta diversity between atopic and non-atopic groups. The most abundant phylum was Proteobacteria, but no significant difference in overall bacterial abundance between atopic and non-atopic study patients existed (Figure 7B). In general, the microbiome was not dependent on the sex or age of the study subjects. The interrelations of pathogenic bacteria were stronger in the tonsils of atopic study patients than in non-

atopic, as shown in the heatmap (Figure 8). The correlations of *Burkholderia cenocepacia* and *Morganella morganii* were positive in the atopic group but negative or indifferent in the non-atopic group (Figure 8).

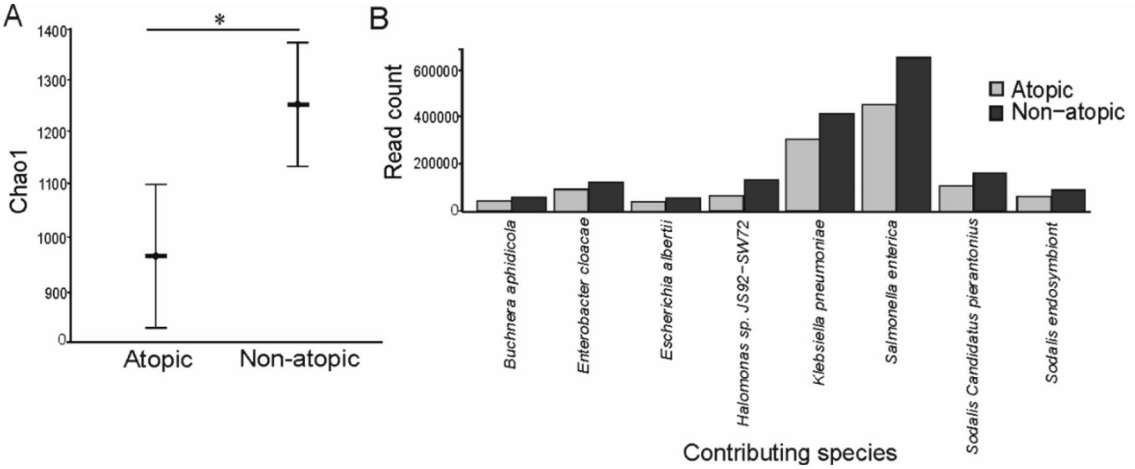

Figure 7. (**A**) Microbial diversity (alpha diversity) between atopic and non-atopic patients. Shown in the mean and the standard error of the mean (SEM). * p=0.02 (**B**) Atopic subjects had non-significantly lower abundance of Proteobacteria (largest phylum) than non-atopic subjects (p=0.3). Modified from study IV.

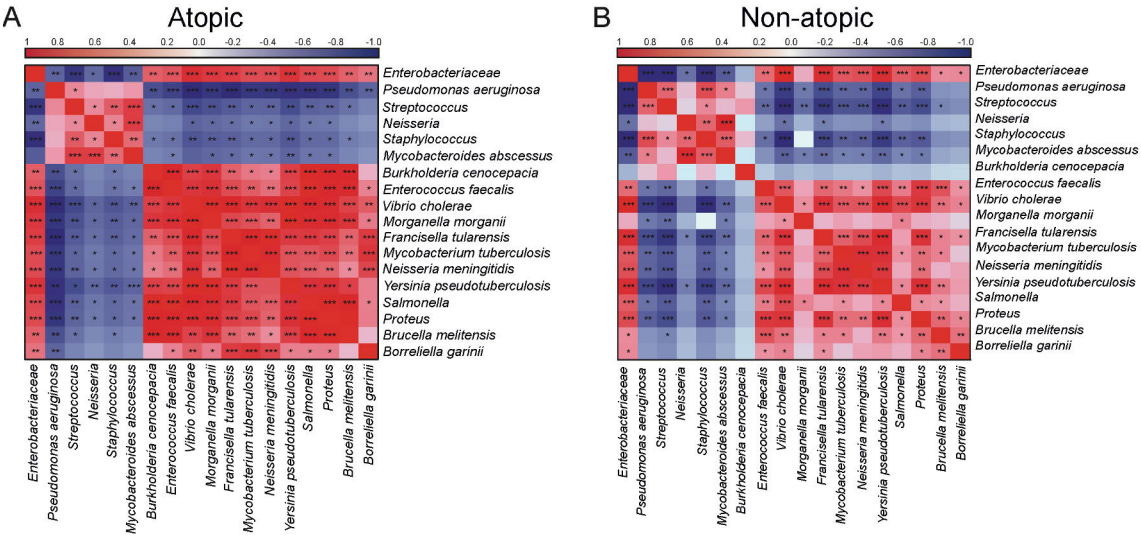

Figure 8. The heatmaps show stronger interrelations of pathogenic bacteria in tonsils of atopic compared to non-atopic study patients. The blue-red colour scale shows the strength and direction of the correlation (red=positive). p values < 0.001, 0.01, and 0.05 are indicated with ***, ** and *, respectively. Modified from study IV.

5.4.4 Diversity of tonsil microbiome in relation to sensitisation and clinical allergy

In the atopy group, 12 of 24 (50%), and the non-atopy group, 8 of 29 (28%) patients reported an allergy (yes/no). Conversely, 8/22 (36%) of the atopy group reported no asthma, rhinitis, or atopic dermatitis, and 17/29 (59%) of the non-atopic, respectively. Comparing allergic diseases between the study groups there was a significant difference only in self-reported allergic rhinitis between the atopic and non-atopic groups. Therefore, we performed another analysis of the microbial diversity between groups: Patients who reported allergic disease and were sensitised and those who reported no allergic disease and were not sensitised. Alpha diversity was computed using the Chao index (estimate of diversity in the microbiome). In the allergic sensitised group, alpha diversity was lower than in the non-allergic non-sensitised group. (Figure 9).

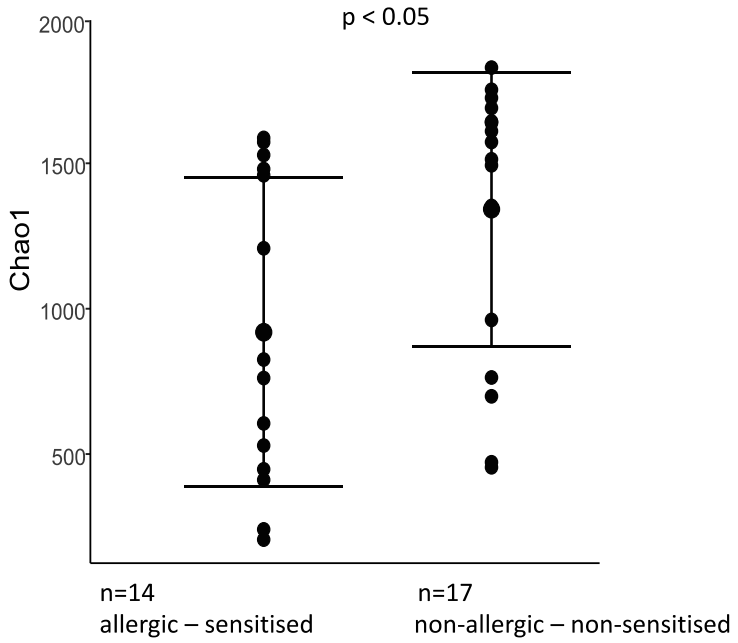

Figure 9. Alpha diversity compared in allergic and sensitised samples (n = 14) with non-allergic and non-sensitised samples (n = 17) computed with Chao 1 index. Shown in mean and quartiles.

6 Discussion

6.1 Baseline characteristics

All the Boka-study patients were operated on according to clinical indications and consecutively recruited year-round in a central hospital. The study population represents typical patients with chronic adenotonsillar diseases. Earlier studies have been made for similar patients (Berger et al., 2007; Jensen et al., 2013; Kourieh et al., 2019; Proenca-Modena et al., 2012, 2014). Furthermore, the most common indications for the operations were recurrent tonsillitis and hypertrophic tonsils/adenoid, which align worldwide (Mitchell et al., 2019). The median age of all 200 patients enrolled was 12. Approximately 7000 (adeno)tonsillectomies and 4000 adenoidectomies are performed in Finland annually, according to the statistics from the Finnish Institute for Health and Welfare.

6.2 Human bocavirus diagnostics

The first aim of this thesis was to evaluate if patients without acute infection have a high HBoV1 genome load in the nasopharynx and tonsils and whether it associates with mRNA detection and serology findings. The diagnostics of the HBoV1 is complicated since PCR often detects the virus from the nasopharynx of children suffering RTI. However, HBoV1 is also commonly found in asymptomatic children. Conversely, HBoV1 is a respiratory tract pathogen, which can cause even severe infections (Christensen et al., 2019; Longtin et al., 2008; Söderlund-Venermo et al., 2009; Xu et al., 2017). The risk factors for HBoV1 infection are age under two years, immunosuppression, and a chronic medical condition such as cardiac or pulmonary disease (Christensen et al., 2010; Ishiguro et al., 2020; Koskenvuo et al., 2008; Martin et al., 2010; Windisch et al., 2016). HBoV1 is often found as a co-infection with other respiratory tract viruses (Petrarca et al., 2020; Proenca-Modena et al., 2012). However, whether the HBoV1 found in a symptomatic patient is an actual pathogen remains challenging.

We studied mainly asymptomatic adeno-/tonsillectomy patients; the prevalence rate of HBoV1 in tonsil tissue or NPA was 21%. The median age of the HBoV1-positive

patients was five, aligning with the earlier literature and reflecting the fact that children under six are more prone to have HBoV1 infection (Christensen et al., 2019). Even a higher prevalence (43%) has been detected in younger children undergoing adeno-/tonsillectomy or myringotomy (Longtin et al., 2008). All of our study patients suffered from chronic adenotonsillar diseases, but no evidence that HBoV1 would be a causative agent for these conditions exists (Proenca-Modena et al., 2012, 2014). Of the HBoV1-positive patients, 30% reported mild respiratory tract symptoms. However, these study patients did not suffer from fever or other severe symptoms associated with acute infection and fulfilled the criteria for general anaesthesia.

HBoV1 is known to persist in the respiratory tract and the tonsils/adenoids for weeks or even months after a symptomatic infection (Blessing et al., 2009; Norja et al., 2012; Qiu et al., 2017; Szalmás et al., 2013; Xu et al., 2017). The prolonged shedding of the virus has been shown, but the profound mechanisms of the virus's persistence are not fully understood (Martin et al., 2010; Wagner et al., 2016). Interestingly, a recent study found persistent HBoV1 DNA in the germinal centres of the adenoid tissue, mainly in monocytes and B-cells (Xu et al., 2021).

Due to the prolonged persistence of the virus in the respiratory tract, the standard qualitative PCR test from the nasopharynx is an insufficient diagnostic tool for HBoV1. Quantitative PCR was thought to solve the problem. Earlier studies have suggested that the virus DNA reduces over time, and the high HBoV1 DNA loads ($>10^4$ to 10^6 copies/mL) would signal acute bocavirus infection (Christensen et al., 2010; Söderlund-Venermo et al., 2009; Zhao et al., 2013). Our study compared three diagnostic methods for HBoV1 on patients without acute infection. Even high ($>10^6$ copies/mL) HBoV DNA loads were detected from the nasopharynx of elective adenoidectomy patients. Relatively high virus loads ($>10^4$ copies/mL) were detected from tonsil, nasopharynx aspirate or both samples of adeno-/tonsillectomy patients. However, the mRNA results or the serology results did not show signs of acute infection in any of the study patients.

Messenger RNA reverse transcription PCR (mRNA RT-PCR) detects spliced viral mRNA produced during active virus replication, meaning mRNA is a marker for viral activity. HBoV1 mRNA has been used as a marker of viral activity for patients with acute infection but not symptomless controls. Also, an association between a high viral load and a positive mRNA result has been suggested (Christensen et al., 2013; Proença-Modena et al., 2011; Xu et al., 2017). Contrary to other studies, our asymptomatic patients had negative mRNA results, suggesting no active virus replications, despite a high viral load.

Concerning serology, most of the HBoV1 DNA PCR-positive patients with sera available were HBoV1 IgG-positive, indicative of a prior infection. The infection is usually acquired during childhood, and almost 100% of the adults are seropositive for HBoV1-specific IgG (Söderlund-Venermo et al., 2009). Three HBoV1 DNA-positive but seronegative patients had HBoV2 immunity, probably due to antigenic sin (Kantola et al., 2011; Li et al., 2015).

In conclusion, no correlation existed between a high HBoV1-DNA load and mRNA or serology results in chronic adenotonsillar disease patients. This study strengthens the earlier results that qualitative and even quantitative PCR tests may be inadequate for diagnosing acute HBoV1 infection. Due to the complexity of the HBoV1 diagnostics, a recent review recommended that at least two of the five components should be fulfilled when diagnosing an acute HBoV1 infection: high ($>10^6$) HBoV1 DNA load from NPA, positive HBoV1 mRNA from NPA, positive IgM, low IgG avidity, or at least a 4-fold increase in IgG titre in paired serum samples (Christensen et al., 2019).

6.3 HBoV1 infection and immune reactions

The second aim of this thesis was to evaluate if persistent HBoV1 infection causes alterations in tonsillar cytokine responses. Local immunological consequences of HBoV1 are not fully understood. In general, Th cells participate in antiviral immunity by producing cytokines and helping B-cells and other T-cells (Guidotti et al., 2003). HBoV1 infection increases Th1 and Th2 type cytokine levels in the nasopharyngeal aspirate of small children with bronchiolitis (Chung et al., 2008). Furthermore, HBoV1 has been associated with elevated Th2 type cytokine levels in bronchoalveolar lavage fluid of patients with lung fibrosis (Khalfaoui et al., 2016). *In vitro* studies have shown that HBoV1 stimulates IFN-γ, IL 10, and IL-13 responses in asymptomatic adults (Kumar et al., 2011; Lindner et al., 2008). Also, a nearly full-length HBoV clone could modulate the IFN-β pathway *in vitro* (Luo et al., 2013; Zhang et al., 2012). Interestingly, Lukkarinen et al. studied the co-infections of HBoV1 and RV confirmed serologically. HBoV1 infection in wheezing children resulted in lower serum proinflammatory and Th2 type cytokine response than in RV-positive wheezing children. Co-infection of HBoV1 and RV resulted in altered non-Th2-type cytokine response. This study suggests a significant interaction between HBoV1 and RV, with HBoV1 having a protective effect (Lukkarinen et al., 2014). Different RV species have been reported to associate with distinct tonsil tissue cytokine responses. An RV-C infection associated with decreased expression of RORC2 (Mikola et al., 2019).

Considering the previous literature, we wanted to study whether tonsillar cytokine response differs between HBoV1-positive and -negative adeno-/tonsillectomy patients without symptoms of acute infection. The prevalence of HBoV1 in tonsil and NPA samples using PCR was 17%, and the HBoV1 IgG seroprevalence was 81%; both results aligned with earlier studies (Proenca-Modena et al., 2012; Xu et al., 2017). The major finding of our study was that HBoV1 positivity was associated with the suppression of tonsillar cytokine and transcription factor expression. Significant differences of RORC2 and FOXP3 transcription factor expressions between HBoV1-positive and -negative study patients were observed using univariate and multivariant analysis.

RORC2 is one of the main transcription factors for Th17 cell differentiation. Th17 cells originate from naïve T-cells exposed to antigen-presenting cells, differentiation cytokines, and RORC2. IL-17 and IL-22 expression is mainly produced by Th17 cells. Furthermore, Th17 derived cytokines contribute to mediating host defence for infections and in the pathogenesis of autoimmune and inflammatory processes (Akdis et al., 2016; Hymowitz et al., 2001).

We observed that HBoV1-positive study patients had a lower expression of RORC2 in their tonsil tissue than HBoV1-negative patients, suggesting a link between HBoV1 and Th17 type cytokine reactions via RORC2. As described, RORC2 is a crucial player in cell-mediated immune responses, making our finding intriguing. However, the expression levels of IL-17 between HBoV1-positive and -negative groups did not differ significantly. NK cells, neutrophils, CD8+ T cells, and γð T cells also produce IL-17, which may explain the results (Akdis et al., 2016). A recent study collected *in vivo* differentiated Th17 cells from IBD (inflammatory bowel disease) and non-IBD subjects. RORC2 was inhibited by an inverse agonist, increasing the production of anti-inflammatory IL-10 (Boardman et al., 2020). These findings justify the future studies of RORC2 related to chronic inflammatory diseases.

FOXP3 is the transcription factor conducting the differentiation of naïve T-cells into functioning Treg cells. Furthermore, specific Treg cells produce suppressive cytokines such as IL-10 and TGF-ß. The anti-inflammatory function of IL-10 is associated with allergic diseases, whereas TGF-ß balances the expression of pro- and anti-inflammatory cytokines. At the disease level, TGF-ß is linked to autoimmune diseases, allergic rhinitis, fibrosis, Alzheimer's disease, cardiovascular pathologies, and cancer (Akdis et al., 2016). An earlier study identified functional allergen-specific FOXP3+ T-reg cells in palatine and lingual tonsils at higher levels than in peripheral blood (Palomares et al., 2011). We discovered that the expression of

FOXP3 was lower in the tonsil tissue of HBoV1-positive patients. These findings support the active role of tonsil tissue in the first line of immune tolerance.

Furthermore, we observed a negative correlation among IL-28, IL-29, IL-13, and intra-tonsillar HBoV1-DNA loads. The IFN-λ family (IL-28, IL-29) acts against respiratory viral infections, especially in the epithelium of the respiratory tract (Lazear et al., 2010). A clear negative correlation has been shown between RV load and IFN-λ expression in the bronchoepithelial cells (Contoli et al., 2006). IL-13 has been related to allergic diseases and virus-induced immune reactions (Akdis et al., 2016; Lucas et al., 2020). Does the high amount of HBoV1 consume all the cytokines mentioned above, resulting in a negative correlation? Or is it other way around: High cytokine levels keep the virus load low? This must be studied more.

In light of earlier studies, HBoV1 is evidently associated with immunomodulatory effects. Furthermore, our study showed that HBoV1 positivity is associated with the immune modulation of major transcription factors at the tonsil tissue level. Our study patients had chronic adenotonsillar diseases but were not acutely ill. This finding raises questions: Does HBoV1 participate in the pathogenesis of chronic adenotonsillar disease?

6.4 Viruses of tonsil and adenoid tissue

In recent years, respiratory viruses of tonsil and adenoid tissue have been frequently studied. Adenoidectomy, adenotonsillectomy, and tonsillectomy are common surgeries among children and young adults everywhere, offering plenty of sample material. It is known that the virus prevalence is high in the secondary lymphoid tissue of patients with chronic adenotonsillar diseases. However, the virus prevalence decreases with age (Jartti et al., 2014). The most common respiratory viruses detected in tonsil and adenoid tissues have been AdV, EV, HBoV1, and RV, with the prevalence rates depending on the study (Faden et al., 2016; Jartti et al., 2014; Proenca-Modena et al., 2012; Proença-Módena et al., 2014; Vinícius et al., 2014). Furthermore, human herpesviruses in tonsils and adenoids have been studied somewhat (Berger et al., 2007; Kourieh et al., 2019; Seishima et al., 2017; Silvoniemi et al., 2020). There is a lack of information concerning respiratory virus and herpesvirus co-infections in adenoids and tonsils. Therefore, the third aim of this thesis was to characterise respiratory and herpesvirus findings in up to three sample types of adenoidectomy/adenotonsillectomy patients: adenoids, tonsils, and NPA.

The overall virus rate was high in our study. There were only two adenoid, three tonsil, and five NPA samples without any virus finding, and 100% of the study

subjects had at least one virus in at least one of the sample types. An earlier study found respiratory viruses in 86% of the adenoids, 69% of tonsils, and 79% of NPA samples. Another study reported a 100% detection rate of at least one herpesvirus in tonsil tissue (Berger et al., 2007; Proenca-Modena et al., 2012). HHV6, HHV7, and EBV were commonly found in adenoid and tonsil tissues in our study. These results reveal the abundance of different viruses in secondary lymphoid tissue in patients with chronic adenotonsillar disease.

The most common herpesviruses in adenoid tissue were HHV6 (33%), followed by HHV7 (26%), and then EBV (25%). Intratonsillar prevalence rates were similar: 45%, 52%, and 23%, respectively, aligning with earlier studies (Berger et al., 2007; Kourieh et al., 2019; Sato et al., 2009; Silvoniemi et al., 2020). Interestingly, we found that HHV6-positive patients were younger than HHV6-negative ones (3 years vs 6), opposing the results of Comar et al., who reported that intra-adenoid HHV-positivity was higher in the >5-year-old group than the younger one (Comar et al., 2010). Our results are logical as they consider that HHV6 causes childhood *exanthema subitum* – usually experienced in early childhood between 6 and 12 months of age (Mullins et al., 2021). In immunocompromised adults and children, HHV6 can cause severe infections (Caserta et al., 1993).

Moreover, HHV7 may act as an etiologic agent for childhood *exanthema subitum*. The clinical presentation is unclear, but fever and seizures have been associated with viremic HHV7 infection in small children (Hall et al., 2006; Kono et al., 2020). We discovered that HHV7 was the most prevalent virus in the tonsils detected. Moreover, intra-tonsillar HHV6 and HHV7 DNA levels were higher than in adenoid tissue. Concerning HHV7, similar results have been reported (Berger et al., 2007). In the same sample type, HHV6- and HHV7-DNA load levels were similar, possibly due to the interaction between HHV6 and HHV7 in children (Hall et al., 2006).

EBV was detected in similar prevalence rates in all three sample types. There was a strong agreement between EBV detection in the adenoids and corresponding tonsils in the same patient. Furthermore, an agreement was observed between detecting EBV in adenoids and NPA samples and between the tonsils and NPA samples. This can be interpreted that if the subject has the virus, it can be detected in all three sample types. Considering the agreement results, EBV seems to infect and persist in lymphocytes in all sample types. The EBV positivity of the NPA samples might be due to EBV-infected lymphocytes in the nasopharyngeal exudate.

In the present study, RV was the most prevalent respiratory virus in adenoids (60%) and NPA samples (63%). The intratonsillar RV detection rate was only 7%. Earlier

studies using PCR have seen similar results, where RV is more common in the adenoid tissue than in the tonsil tissue (Faden et al., 2016; Proenca-Modena et al., 2012). Whether the differences in RV's prevalence are due to the location of the secondary lymphoid tissue (adenoid in nasopharynx and tonsils in oropharynx) or because of the differences in histological structures, is unknown.

Agreement between RV detection in tonsils and corresponding NPA samples was unobserved. For example, most of the RV-positive NPA samples were virus-negative in tonsils. Interestingly, RV was detected in 14 adenoid samples without concurrent virus detection in NPA samples, which may be explained by the faster resolution of RV from the nasopharynx than from the adenoids after infection. Conversely, 17 NPA samples with positive RV findings were virus-negative in corresponding adenoid samples. These results show that the true persistence of infectious RV in adenoids is unknown.

The most common respiratory virus in tonsil tissue was HBoV1. However, the virus frequency was even higher in adenoid and NPA samples. Despite the high prevalence rates of HBoV1 in all sample types, no strong agreement was observed, demonstrating the diverse distribution of HBoV1 in different sample materials. However, the tropism of HBoV1 for adenoid tissue was observed earlier (Faden et al., 2016; Proenca-Modena et al., 2012). In our study, 38% of the HBoV1-positive cases were found only in adenoid tissue samples, confirming earlier results: HBoV1 is likelier found in adenoid tissue than in tonsils or NPA.

In conclusion, to our knowledge, this was the first study to detect 7 of 8 human herpesviruses from adenoid, tonsil, and NPA samples. Also, common respiratory viruses were detected from the same sample types. This study offers vital information about virus prevalence and herpesvirus persistence in secondary lymphoid tissue. Tonsils and adenoids seem to serve as a virus reservoir in patients with chronic adenotonsillar disease. How these chronic virus infections influence the hosts' immunity and the pathogenesis of chronic adenotonsillar diseases remains unsolved.

6.5 Tonsillar microbiome in atopic and non-atopic patients

Allergic diseases are a growing problem worldwide, and recent studies have revealed the connection between allergies and the host's microbiome (Huang et al., 2017). For example, the skin microbiota diversity in atopic eczema is deprived compared to healthy skin. Moreover, an association of skin microbiota between age and one's

living environment has been suggested (Haahtela, 2019; Lehtimäki et al., 2017). Asthma has been linked to, for example, alterations in lower respiratory tract microbiota and the enrichment of Proteobacteria (Aho et al., 2015; Durack et al., 2017). The studies of gut microbiota indicate that dysbiosis is associated with allergic disorders and other non-communicable diseases (Haahtela et al., 2013).

The fourth aim of this thesis was to study the tonsillar microbiome and compare the findings between atopic and non-atopic adeno-/tonsillectomy patients. Since tonsil tissue is active secondary lymphoid tissue in close contact with inhaled and ingested antigens, we hypothesised that a difference in tonsillar microbiome between the study groups might exist. Our study was the first to describe the differences in tonsillar microbial diversity, abundance, and interrelations between atopic and non-atopic.

The main finding was that microbial alpha diversity was lower in the tonsils of atopic patients than in the tonsil samples of non-atopic, aligning with earlier results comprising other tissues (Huang et al., 2017). As tonsil tissue contains a rich microbiome in close contact with the immune system, it is logical that the sensitisation status is associated with microbial diversity. In this study, the most abundant genera were *Salmonella*, *Klebsiella,* and *Sodalis,* belonging to the most abundant phylum Proteobacteria. Earlier studies concerning microbiota of tonsil tissue have discovered that Proteobacteria are highly associated with tonsillar hyperplasia and recurrent tonsillitis in children (Jensen et al., 2013; Johnston et al., 2018a). The mean age of our study patients was 11, and tonsillar hyperplasia and recurrent tonsillitis were the main indications for the operation. *Streptococcus. pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis* are exclusively found in adenoids but not in the tonsils of children with secretory otitis media or adenotonsillar hyperplasia (Fagö-Olsen et al., 2019). A recent study compared the tonsillar core and surface microbiota between chronic tonsillitis and tonsillar hypertrophy patients. Four different tonsillar microbial types were found, in which *Haemophilus* and *Neisseria* were especially associated with tonsillar hyperplasia and *Dialister* and *Parvimonas* with chronic tonsillitis (S. Wu et al., 2021). Also, comparing the tonsil microbiota of PFAPA patients with hypertrophic tonsils, a difference was observed in the relative abundance of the phylum Cyanobacteria (Tejesvi et al., 2016).

No significant difference existed in the overall bacterial abundance between the study groups. However, the differential abundance of two specific taxas – *Moraxella Osloensis* and *Clostridium Botulinum* – was lower in the atopic group. At the genus level, *Moraxella* in the respiratory tract has been associated with asthma and

Clostridia in the gut microbiome, with the resolution of food allergies later in life (Bunyavanich et al., 2016; Huang et al., 2017).

The atopy was defined by allergen specific IgE in serum samples. Sensitisation does not automatically mean the subject has a clinically relevant allergic disease. Moreover, we did not use skin prick tests: Some atopy patients are IgE-negative but skin prick test positive (Ansotegui et al., 2019). The questionnaire, which the study patients filled in, was thorough regarding allergic diseases. However, clinical allergies were not cross-checked with medical history; the information was based on the questionnaire. Assessing clinical illness is difficult, so we believed the grouping based on the sensitisation status was more objective. We did another analysis, considering allergic symptoms. The diversity was lower in the allergic and sensitised compared to the non-allergic and non-sensitised study patients.

In conclusion, RNA sequencing makes studying the tonsil microbiome possible, even at the species level. Our study shows distinct differences in an intratonsillar microbiome between atopic and non-atopic adenotonsillar surgery patients. These results suggest an association between atopy status and a tonsillar microbiome, encouraging more extensive study of the tonsil tissue microbiome.

6.6 Limitations

All the study patients were operated on due to clinical indications. Thus, they all had chronic adenotonsillar diseases. Ethically, operating on the adenoid or tonsils of healthy control patients would be inappropriate. On the other hand, most of the earlier studies concerning the adenoid and tonsil tissues were done for similar patients, making our study comparable with earlier ones. Boka-study patients were recruited from Satakunta Central Hospital by otorhinolaryngologists working at the clinic, so some variation concerning the indications exists. The study patients were a heterogeneous group and we included patients of all ages. This can be considered as a limitation. Furthermore, study subjects filled in a standard questionnaire for the researchers to obtain information about medication, allergic symptoms/diseases, and respiratory symptoms 30 days before the operation. Unfortunately, not all the questions were answered appropriately. However, the information from the questionnaire was comprehensive, resulting in thoroughly characterised study subjects. The data set of the serum samples was incomplete; paired serum samples were obtained from 63 of the 200 study patients.

Regarding study IV, a methodological aspect must be considered. Most of the earlier studies concerning the tonsillar microbiome have used 16S rRNA sequencing for

detecting bacteria at the genus or even species level (Fagö-Olsen et al., 2019; Jensen et al., 2013; Johnston et al., 2019; Johnston et al., 2018a, 2018b; S. Wu et al., 2021). This technique uses PCR amplification of the 16S rRNA genes – present in all bacteria – to describe microbes. However, non-targeted RNA sequencing analyses transcriptomes extensively, so detecting bacterial species and diversity is improved compared to the 16S rRNA sequencing method. The RNA sequencing approach lacks the risk of biases concerning primers used in 16S rRNA sequencing. Previously, RNA sequencing has been used to detect microbiomes from whole blood and nasal epithelium (Hanif et al., 2019; Olde Loohuis et al., 2018).

We studied common respiratory viruses and human herpesviruses. However, HPV was not included in the studies. Considering the role of HPV in the oropharyngeal region, analysing HPV from adenoid and tonsil tissues would have been interesting.

The virus diagnostics for NPA and tonsils were done earlier than the adenoids. Therefore, a new test kit version replaced by the manufacturer was used for adenoid samples. The HBoV1 DNA load was not measured from the adenoid tissue, and serum samples of sole adenoidectomy patients were not analysed. The limitation concerning all the studies includes the relatively small number of study subjects (n=53–188).

7 Summary and Conclusions

7.1 Main Findings

First, even high loads of HBoV1 DNA were found in the nasopharynx and tonsils of adeno-/tonsillectomy patients without acute infection. Active virus replication was not observed using mRNA detection, and HBoV1-specific IgM was not detected from the serum samples. There is no association among the HBoV1 DNA load, mRNA, and serology findings in chronic adenotonsillar disease patients. Quantitative PCR detecting HBoV1 appears insufficient for diagnosing acute HBoV1 infection.

Second, persistent HBoV1 infection was associated with alterations in tonsillar cytokine response. The tonsillar expression of the main transcription factors – RORC2 and FOXP3 – was suppressed in the HBoV1-positive group. This finding indicates that HBoV1 may have immunomodulatory effects.

Third, a broad spectrum of different herpes viruses and common respiratory viruses were detected from the autologous adenoid and tonsil tissues of non-acutely ill adenotonsillar surgery patients. HHV6, HHV7, and EBV were commonly found in the corresponding adenoids and tonsils. Furthermore, differences in herpesvirus prevalence among adenoid, tonsil, and NPA samples were observed. Adenoid and tonsil tissues seemingly serve as virus reservoirs in non-acutely ill patients.

Fourth, a rich tonsillar microbiome was detected using RNA sequencing. The bacterial diversity was lower in the tonsils of atopic than non-atopic subjects. We observed stronger bacterial interrelations in the atopic group. Dysbiosis in atopic patients appears to extend to the local tissue level.

Overall, persistent and prolonged respiratory virus and herpesvirus infections are common in adenoid and tonsil tissues in adenotonsillar surgery patients. Virus infections in close contact with the immune system may have immunomodulatory effects. Furthermore, low tonsillar microbiome richness appears to associate with systemic atopic illness. Tonsil (and adenoid) tissue is being developed as a new *in*

vivo model to study local immune responses in health and disease. However, virus and bacterial infections must be considered essential modulators. Local lymphoid tissue is immunologically active.

7.2 Future considerations

Adenotomy, adenotonsillectomy, and tonsillectomy are common operations everywhere. However, the pathogenesis underlying some of the conditions leading to these operations is still unclear. The immunological function of secondary local lymphoid tissue (i.e. tonsils and adenoids) has been under investigation for decades (Perry et al., 1998; Surjan et al., 1978). The function of tonsils producing antibodies and activating T-cells is well-known, but whether the immune functions change after an adeno-/tonsillectomy remains debatable. A recent review stated that tonsillectomy has no negative influence on humoral or cellular immunity in children (Altwairqi et al., 2020).

Conversely, a large nationwide population-based study reported an association between tonsillectomy and an increased risk of IBD (M. C. Wu et al., 2020). Changes in immune function after tonsillectomy have been reported. However, the results are incompatible and must be studied more. Furthermore, a shift has occurred in the operation technique towards tonsillotomy instead of tonsillectomy (Hultcrantz et al., 2013). Since tonsils are immunologically active, studying whether the potential postoperative immunological changes differ after tonsillotomy when compared to tonsillectomy would be interesting (Geißler et al., 2020).

In allergic diseases, the peripheral T-cell tolerance is lost. It has been shown *in vitro* that the allergen-specific T-cell tolerance can be disrupted by activating Toll-like receptors and proinflammatory cytokines (Kücüksezer et al., 2013). Understanding the mechanisms and breaking mechanisms of peripheral T-cell tolerance is crucial to inventing new treatment modalities for allergic disorders and cancers. Intralymphatic immunotherapy is under investigation to offer shorter allergen immunotherapy treatments (Hellkvist et al., 2018). Earlier results and the immunological activity of the tonsils raise the question of intratonsillar immunotherapy. Using tonsils as an *in vitro* model to study immunotherapy could be the first step in this field. Concerning patient studies, injection into the tonsil is unpleasant and might compromise the airway. Therefore, administering the allergen by nasal or mouth spray directed at the tonsils and adenoids could be possible.

Immunotherapy is a hot topic in cancer studies. Novel treatment modalities require a fundamental understanding of the immunology behind the tumour's genesis and

host response. Concerning HPV-positive tonsil carcinoma, immune checkpoint inhibitors have been widely studied recently (Stern et al., 2021). Tonsils as an *in vivo* model could be used in cancer studies. An association between HBoV in tonsil tumour development has been speculated (Höpken et al., 2018) and would be an interesting topic to study more. Are the virus-induced immune reactions similar in cancer tissue compared to non-cancerous tonsils?

Next-generation sequencing methods have expanded the field of studying microbes. DNA- and RNA-based analyses provide enormous data sets, and microbiome and metagenome studies are run by bioinformatic tools. Tonsil tissue provides an excellent niche for metagenomic studies. Furthermore, studying the whole virome of tonsil tissue could give answers about the mechanisms behind chronic adenotonsillar disease. Virome study could reveal the potential of, for example, herpesviruses in the pathogenesis of noncommunicable diseases. Our study found that HBoV1 is associated with tonsillar immune reactions. Other viruses are also presumed to have immunomodulatory effects, considering the prolonged virus infections in the tonsils and adenoids. A recent paper proposed that children might have milder symptoms of COVID-19 due to more active secondary lymphoid tissue in tonsils and adenoids than adults (Onal et al., 2020). Studies of SARS-CoV-2 in tonsil and adenoid tissue will reveal relevant information about the virus's persistence. Furthermore, immune alterations caused by SARS-CoV-2 at the local tissue level must be studied.

Although tonsillectomy is an extremely common procedure, especially in the pediatric population, there are risks, the most significant and potentially fatal being postoperative haemorrhage in 0.22%–3.5% of cases (Walner et al., 2017). Postoperative pain, especially in the adult population, is a significant adverse event (Burton et al., 2014). New operation techniques are introduced to reduce these risks (Sakki et al., 2019). Profoundly understanding the mechanisms behind chronic adenotonsillar diseases and even developing non-surgical treatments against these common problems by utilising the knowledge of local immune responses would be interesting. Future studies using tonsils as an *in vivo* model are warranted to achieve these goals and ambitions.

Acknowledgements

This study was conducted at the Department of Paediatrics in collaboration with the Department of Otorhinolaryngology – Head and Neck Surgery in the University of Turku and Turku University Hospital during 2017–2022.

I thank Professor Erika Isolauri for providing me the opportunity for PhD studies. I am grateful to Professor Jaakko Pulkkinen for creating an inspiring atmosphere for conducting research at the Department of Otorhinolaryngology in the Turku University Hospital.

I would like to express my deepest gratitude to my supervisor Professor Tuomas Jartti. You guided me into the intriguing world of science and through this thesis. In the beginning I was a little lost in the field of paediatric allergology and immunology but you taught me and gently pushed me to do my best. I admire you as a scientist and I have sincerely enjoyed collaborating with you.

I would also like to express my deepest gratitude to my supervisor, Associate professor Tuomo Puhakka. You encouraged me to start this project and you have encouraged me to go forward. I really respect you as a colleague and otorhinolaryngologist. Thank you for your comments on the manuscripts from the clinical and ENT points of view.

I warmly thank the reviewers of this thesis, Professor Marjo Renko and Professor Anne Pitkäranta, for their time and expertise. I truly enjoyed our scientific discussions and your valuable comments certainly improved the quality of this thesis.

I am very grateful to my co-authors for their valuable contribution to the publications of this thesis. Associate professor Tytti Vuorinen and Associate professor Matti Waris, your help concerning virology on the manuscripts was priceless. I warmly thank you for your time and effort. Associate professor Andreas Christensen (University of Science and Technology, Trondheim), Professor Tobias Allander (Karolinska Institute, Stockholm), and Associate professor Maria Söderlund-

Venermo (Department of Virology, University of Helsinki), I thank you for your help regarding the mysterious human bocavirus. ENT specialist, colleague Antti Silvoniemi, PhD, I have learned much from our chats and Saturday night e-mails. You were the voice of reason when my ideas got sidetracked. Tanzeela Hanif, MSc, and Freed Ahmad, MSc (Department of Biology, University of Turku), I warmly thank you for your collaboration concerning Study IV. I really admire your expertise in bioanalytics. Regarding studies II and IV, I want to thank the following persons: Professor Cezmi A. Akdis; Professor Mubeccel Akdis; Ge Tan, PhD; Can Altunbulakli, PhD (all from the Swiss Institute of Allergy and Asthma Research, University of Zürich, Davos); Professor Oscar Palomares (Department of Biochemistry and Molecular Biology, Complutense University of Madrid); Riitta Turunen, PhD; and Associate professor Sanna Toppila-Salmi (Skin and Allergy Hospital, Helsinki University Hospital and University of Helsinki).

Colleague, friend, co-author Emilia Mikola, I am truly grateful for your friendship and your peer support which has been more than important to me.

A special thanks to study nurse Heidi Jokinen who has not only done fine laboratory work but who also did some excellent detective work tracking down some missing samples. I am also grateful to biostatistician Tero Vahlberg, MSc, who got me excited (at least a little) about statistics. A warm thanks to my friend and colleague Katariina Tamminen, for drawing a figure for this book.

I want to thank warmly all my dear colleagues and other staff at the Department of Otorhinolaryngology – Head and Neck Surgery at the Turku University Hospital. You all are the best work mates I can imagine. There is no day at work without laughter and you offer support when needed. Associate professor Ilpo Kinnunen, head of the department, Professor Heikki Irjala, and Lotta Haavisto, PhD, thank you for giving me the opportunity to combine clinical work and research. I am grateful to “Tulevaisuuden Tohtorit” group for their peer support and friendship.

I am tremendously lucky to have made many dear friends along the way. Thank you all for making my life better. Päivi, Emmi, and Niina, we all have the ”savolaisuus” spirit in our hearts and common history in Kuopio. Let’s cherish that! My good friends from medical school with whom I always meet up with pleasure. Anna Pärtty, thank you for your friendship during these years, I value our walks and talks. KS group, sisters, thank you! Eeva Haapio and Miia Ruuskanen, besides of being wonderful colleagues you are also true friends. I truly appreciate your support at work and fun company outside of work. Let’s keep on dancing in Dynamo! Aino Kalske, thank you for your friendship and accompanying me to outdoor activities.

I am deeply grateful to my loving parents Markku and Leena Saaranen: you have always believed in me and encouraged me to go after my dreams. I want to thank the Ivaska family for participating in our everyday life. I am especially grateful to the best grandparents (all four) that our children have. Your help has been crucial and has really supported the completion of this thesis! A warm thanks to my brothers Otto and Joonas and their lovely families for sharing many enjoyable moments together.

Finally, I express my warmest thanks to the love of my life, Lauri. You have always supported me, and you cope with all my wild ideas with calmness and great sense of humour. Unto and Valto, even though you sometimes make me "ragee," I still love you the most!

This work was financially supported by the Turku University Foundation, the TYKS Foundation, the University of Turku Graduate School, the Research Funds from Specified Government Transfers (EVO), the Finnish Cultural Foundation, the Helsinki Allergy Foundation, the Finnish ORL-HNS Foundation, the Finnish Medical Foundation, and the Paulo Foundation.

Turku, June 2022
Lotta Ivaska

References

Aho, V. T. E., Pereira, P. A. B., Haahtela, T., Pawankar, R., Auvinen, P., & Koskinen, K. The microbiome of the human lower airways: a next generation sequencing perspective. *The World Allergy Organization Journal*. 2015; 8(23): 1–13

Akdis, M., Aab, A., Altunbulakli, C., Azkur, K., Costa, R. A., Crameri, R., et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. *Journal of Allergy and Clinical Immunology.* 2016; 138(4): 984–1010.

Akdis, M., Palomares, O., Van De Veen, W., Van Splunter, M., & Akdis, C. A. TH17 and TH22 cells: A confusion of antimicrobial response with tissue inflammation versus protection. *Journal of Allergy and Clinical Immunology*. 2012; 129(6): 1438–1449.

Allander, T. Jartti, T., Gupta, S., Niesters, H. G. M., Lehtinen, P., Usterback, et al. Human Bocavirus and Acute Wheezing in Children. *Clinical Infectious Diseases*. 2007; 44(7): 904–910.

Allander, T. Tammi, M. T., Eriksson, M., Bjerkner, A., Tiveljung-Lindell, A., & Andersson, B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(36): 12891–12896.

Altwairqi, R. G., Aljuaid, S M, & Alqahtani, A S. Effect of tonsillectomy on humeral and cellular immunity: a systematic review of published studies from 2009 to 2019. *European Archives of Oto-Rhino-Laryngology*. 2020; 277(3): 1–7.

Alvaro-Lozano, M., Akdis, C. A., Akdis, M., Alviani, C., Angier, E., Arasi, S., et al. EAACI Allergen Immunotherapy User's Guide. *Pediatric Allergy and Immunology*. 2020; *31*(Suppl 25): 1-101

Andrews, S. *Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data : http://www.bioinformatics.babraham.ac.uk/projects/fastqc*. 2019

Ansotegui, I. J., Melioli, G., Walter Canonica, G., Caraballo, L., Villa, E., Ebisawa, et al. IgE allergy diagnostics and other relevant tests in allergy*, a World Allergy Organization position paper*. 2019; 13: 1–50

Arambula, A., Brown, J. R., & Neff, L. Anatomy and physiology of the palatine tonsils, adenoids, and lingual tonsils. *World Journal of Otorhinolaryngology-Head and Neck Surgery*. 2021; *7*: 155–160.

Bai, X., Rogers, B. B., Harkins, P. C., Sommerauer, J., Squires, R., Rotondo, K., et al. Predictive Value of Quantitative PCR-Based Viral Burden Analysis for Eight Human Herpesviruses in Pediatric Solid Organ Transplant Patients. *The Journal of Molecular Diagnostics*. 2000; *2*(4): 191–201.

Bamgbose, B. O., Ruprecht, A., Hellstein, J., Timmons, S., & Qian, F. Clinical Study The Prevalence of Tonsilloliths and Other Soft Tissue Calcifications in Patients Attending Oral and Maxillofacial Radiology Clinic of the University of Iowa. *ISRN Dentistry*. 2014; ID 839635: 1–9

Barratt, J., Tang, S. C. W. Treatment of IgA Nephropathy: Evolution Over Half a Century. *Seminars in Nephrology*. 2018; 38(5): 531–540.

Bathala, S., Eccles, R. A review on the mechanism of sore throat in tonsillitis. *The Journal OfLaryngology & Otology*. 2013; 127: 227–232.

Berger, C., Hug, M., Gysin, C., Molinari, L., Frei, M., Bossart, W., & Nadal, D. Distribution patterns of β- and γ-herpesviruses within Waldeyer's ring organs. *Journal of Medical Virology*. 2007; 79(8): 1147–1152.

Bjornevik, K., Cortese, M., Healy, B. C., Kuhle, J., Mina, M. J., Leng, Y., et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science*. 2022; 375(6578): 296–301.

Blessing, K., Neske, F., Herre, U., Kreth, H.-W., Weissbrich, B. Prolonged detection of human bocavirus DNA in nasopharyngeal aspirates of children with respiratory tract disease. *The Pediatric Infectious Disease Journal*. 2009; *28*(11): 1018–1019.

Boardman, D. A., Garcia, R. V., Ivison, S. M., Bressler, B., Dhar, T. M., Zhao, Q., & Levings, M. K. Pharmacological inhibition of RORC2 enhances human Th17-Treg stability and function. *European Journal of Immunology*. 2020; 50(9): 1400–1411.

Bohnacker, S., Esser-Von Bieren, J., Troisi, F., Jimenez, M. What Can Parasites Tell Us About the Pathogenesis and Treatment of Asthma and Allergic Diseases. *Frontiers in Immunology.* 2020; 11(2106): 1–8.

Bolger, A. M., Lohse, M., Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014; 30(15); 2114–2120.

Bowers, I., Shermetaro, C. Adenoiditis. *Tratado de Otorrinolaringología y Patología Cervicofacial*. StatPearls 2021; 467–476. https://www-ncbi-nlm-nih-gov.ezproxy.utu.fi/books/NBK536931/

Brambilla, I., Pusateri, A., Pagella, F., Caimmi, D., Caimmi, S., Licari, A., et al. Adenoids in children: Advances in immunology, diagnosis, and surgery. *Clinical Anatomy*. 2014; 27(3): 346–352.

Brandtzaeg, P. Immune Functions of Nasopharyngeal Lymphoid Tissue. *Advances in oto-rhino-laryngology.* 2011; 72: 20–24.

Brandtzaeg, P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. *International Journal of Pediatric Otorhinolaryngology*. 2003; 67(1): 69–76.

Bruning, A. H. L., Susi, P., Toivola, H., Christensen, A., Söderlund-Venermo, M., Hedman, et al. Detection and monitoring of human bocavirus 1 infection by a new rapid antigen test. *New Microbes and New Infections*. 2016; 11: 17–19.

Bunyavanich, S., Shen, N., Grishin, A., Wood, R., Burks, W., Dawson, P., et al. Early-life gut microbiome composition and milk allergy resolution. *The Journal of Allergy and Clinical Immunology*. 2016; *138*(4): 1122–1130.

Burton, M. J., Glasziou, P. P., Chong, L. Y., Venekamp, R. P. Tonsillectomy or adenotonsillectomy versus non-surgical treatment for chronic/recurrent acute tonsillitis. *Cochrane Database of Systematic Reviews.* 2014; 11. Art. No.: CD001802.

Byington, C. L., Ampofo, K., Stockmann, C., Adler, F. R., Herbener, A., Miller, T., et al. Community Surveillance of Respiratory Viruses Among Families in the Utah Better Identification of Germs-Longitudinal Viral Epidemiology (BIG-LoVE) Study. *Clinical Infectious Diseases*. 2015; 61(8), 1217–1224.

Calvo, C., García-García, M. L., Pozo, F., Carballo, D., Martínez-Monteserín, E., Casas, I. Infections and coinfections by respiratory human bocavirus during eight seasons in hospitalized children. *Journal of Medical Virology*. 2016; 88(12): 2052–2058.

Caserta, M. T., Hall, C. B. Human herpesvirus-6. *Annu. Rev. Med*. 1993; 44: 377–383.

Chan, T. V. The Patient with Sore Throat. *Med Clin N Am*. 2010; *94*: 923–943.

Chang, L., Chi, H., Chiu, N. C., Huang, F. Y., Lee, K. S. Deep Neck Infections in Different Age Groups of Children. *Journal of Microbiology, Immunology and Infection*. 2010; 43(1): 47–52.

Chen, S., Wang, W.-W., Wang, Y., Li, Y.-Q., Zhu, L.-X. Cellular senescence in recurrent tonsillitis and tonsillar hypertrophy in children. *International Journal of Pediatric Otorhinolaryngology.* 2020; *133*(10004): 1–8

Cho, K.-S., Kim, S. H., Hong, S.-L., Lee, J., Mun, S. J., Roh, Y. E., et al. Local Atopy in Childhood Adenotonsillar Hypertrophy. *American Journal of Rhinology & Allergy*. 2018; *32*(3): 160–166.

Christensen, A., Døllner, H., Skanke, L. H., Krokstad, S., Moe, N., Nordbø, S. A. Detection of Spliced mRNA from Human Bocavirus 1 in Clinical Samples from Children with Respiratory Tract Infections. *Emerging Infectious Diseases*. 2013; 19(4): 574–580.

Christensen, A., Kesti, O., Elenius, V., Eskola, A. L., Døllner, H., Altunbulakli, C., et al. Human bocaviruses and paediatric infections. *Lancet Child Adolesc Health*. 2019; 3: 481–426.

Christensen, A., Nordbø, S. A., Krokstad, S., Gro, A., Rognlien, W., Døllner, H. Human bocavirus in children: Mono-detection, high viral load and viraemia are associated with respiratory tract infection. *Journal of Clinical Virology*. 2010; 49: 158–162.

Chung, J.-Y., Han, T. H., Kim, J.-S., Kim, S. W., Park, C.-G., Hwang, E.-S. Th1 and Th2 cytokine levels in nasopharyngeal aspirates from children with human bocavirus bronchiolitis. *Journal of Clinical Virology*. 2008; 43: 223–225.

Comar, M., Grasso, D., Dal Molin, G., Zocconi, E., Campello, C. HHV-6 infection of tonsils and adenoids in children with hypertrophy and upper airway recurrent infections. *International Journal of Pediatric Otorhinolaryngology*. 2010; 74: 47–49.

Contoli, M., Message, S. D., Laza-Stanca, V., Edwards, M. R., Wark, P. A. B., Bartlett, N. W., et al. Role of deficient type III interferon-λ production in asthma exacerbations. *Nature Medicine.* 2006; 12(9): 1023–1026.

Cruz, A. A., Cooper, P. J., Figueiredo, C. A., Alcantara-Neves, N. M., Rodrigues, L. C., Barreto, M. L. Global issues in allergy and immunology: Parasitic infections and allergy. *The Journal of Allergy and Clinical Immunology*. 2017; 140(5): 1217–1228.

De Corso, E., Galli, J., Di Cesare, T., Lucidi, D., Ottaviano, G., Seccia, V., et al. A systematic review of the clinical evidence and biomarkers linking allergy to adeno-tonsillar disease. *International Journal of Pediatric Otorhinolaryngology*. 2021; 147(110799)

Dogru, M., Evcimik, M. F., Calim, O. F. Does adenoid hypertrophy affect disease severity in children with allergic rhinitis? *European Archives of Oto-Rhino-Laryngology*. 2017; 274(1): 209–213.

Durack, J., Lynch, S. V., Nariya, S., Bhakta, N. R., Beigelman, A., Castro, M., et al. Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment. *Journal of Allergy and Clinical Immunology*. 2017; 140(1): 63–75.

Ebell, M. H., Call, M., Shinholser, J., Gardner, J. Does This Patient Have Infectious Mononucleosis?: The Rational Clinical Examination Systematic Review. *JAMA*. 2016; 315(14): 1502–1509.

Ekici, N. Y., Görgülü, O., Yucel, G., Külahcı, Ö., Arıkan, O. K., & Durmaz, C. Can the number of eosinophils in adenoid and tonsil tissue determine the allergy in children? *International Journal of Pediatric Otorhinolaryngology*. 2018; 108: 35–39.

Elenius, V., Palomares, O., Waris, M., Turunen, R., Puhakka, T., Rückert, B.,et al. The relationship of serum vitamins A, D, E and LL-37 levels with allergic status, tonsillar virus detection and immune response. *PloS One*. 2017; 12(2): 119–124.

Faden, H., Callanan, V., Pizzuto, M., Nagy, M., Wilby, M., Lamson, D., et al. The ubiquity of asymptomatic respiratory viral infections in the tonsils and adenoids of children and their impact on airway obstruction. *International Journal of Pediatric Otorhinolaryngology*. 2016; 90: 128–132.

Fagö-Olsen, H., Dines, L. M., Sørensen, C. H., Jensen, A. The Adenoids but Not the Palatine Tonsils Serve as a Reservoir for Bacteria Associated with Secretory Otitis Media in Small Children. *MSystems*. 2019; 4:e00169-1.

Fang, P., Zhou, P., Zhou, L., Kolls, Y. K., Zheng, J. K. Immune Modulatory Effects of IL-22 on Allergen-Induced Pulmonary Inflammation. *PLoS ONE*. 2014; 9(9): e107454.

Ferguson, M., Aydin, M., Mickel, J. State of the Art Review Halitosis and the Tonsils: A Review of Management. *Otolaryngology-Head and Neck Surgery*. 2014; 151(4): 567–574.

Fossum, C. C., Chintakuntlawar, A. V., Price, D. L., Garcia, J. J. Characterization of the oropharynx: anatomy, histology, immunology, squamous cell carcinoma and surgical resection. *Histopathology*. 2017; 70(7),:1021–1029.

Friedrichs, I., Rowe-Jones, J. Acute and chronic infections of the ear, nose and throat. *Surgery. Oxf.* 2004; 22(8): 178.

Fry, A. M., Lu, X., Chittaganpitch, M., Peret, T., Fischer, J., Dowell, S. F., et al. Human Bocavirus: A Novel Parvovirus Epidemiologically Associated with Pneumonia Requiring Hospitalization in Thailand. *The Journal of Infectious Diseases*. 2007; 195(7): 1038–1045.

Galioto, N. J. Peritonsillar Abscess. 2017; *95*(8). Retrieved from www.aafp.org/afp

Galli, S. J., Tsai, M., Piliponsky, A. M., Stephen J., G., Tsai, M., Piliponsky, A. M. The development of allergic inflammation. *Nature*. 2013; 454(7203): 445–454.

Geiger, Z., Gupta, N. Adenoid Hypertrophy. StatPearls 2021 Retrieved from https://www-ncbi-nlm-nih-gov.ezproxy.utu.fi/books/NBK536984/

Geißler, K., Weigel, C., Schubert, K., Rubio, I., Guntinas-Lichius, O. Cytokine production in patients with recurrent acute tonsillitis: analysis of tonsil samples and blood. *Sci Rep.* 2020; 10: e13006

Gesualdo, L., Leo, V. Di, Coppo, R. The mucosal immune system and IgA nephropathy. *Seminars in Immunopathology*. 2021; *43*: 657–668.

Gorfien, J. L., Noble, M. B. Comparison of the microanatomical distributions of macrophages adn dendritic cells in normal and diseased tonsils. *In Ann Otol Rhinol Laryngol.* 2001; 110: 173–182

Griffith, M., Walker, J. R., Spies, N. C., Ainscough, B. J., Griffith, O. L. Informatics for RNA Sequencing: A Web Resource for Analysis on the Cloud. *PLoS Computational Biology*. 2015; 11(8): e1004393

Guay, M.E., Lavertu, P. Tonsillar carcinoma. *European Archives of Oto-Rhino-Laryngology.* 1995; *252*(5): 259–264.

Guidotti, L. G., Chisari, F. V. Noncytolytic Control of Viral Infections by the Innate and Adaptive ImmuneResponse. *Annu. Rev. Immunol.* 2003; 19: 65–91.

Günel, C., Kırdar, S., Ömürlü, İ. K., Ağdaş, F. Detection of the Epstein–Barr virus, Human Bocavirus and novel KI and KU polyomaviruses in adenotonsillar tissues. *International Journal of Pediatric Otorhinolaryngology*. 2015; 79(3): 423–427.

Haahtela, T. A biodiversity hypothesis. *Allergy*. 2019; 74(8): 1445–1456.

Haahtela, T., Holgate, S., Pawankar, R., Akdis, C. A., Benjaponpitak, S., Caraballo, L. et al. The biodiversity hypothesis and allergic disease: world allergy organization position statement. *The World Allergy Organization Journal*. 2013; 6(3): 1–18.

Hall, C. B., Caserta, M. T., Schnabel, K. C., McDermott, M. P., Lofthus, G. K., Carnahan, J. A.et al. Characteristics and Acquisition of Human Herpesvirus (HHV)–7 Infections in Relation to Infection with HHV-6. *The Journal of Infectious Diseases*. 2006; 193(8): 1063–1069.

Hanif, T., Dhaygude, K., Kankainen, M., Renkonen, J., Mattila, P., Ojala, T., et al. Birch pollen allergen immunotherapy reprograms nasal epithelial transcriptome and recovers microbial diversity. *Journal of Allergy and Clinical Immunology*. 2019; 143(6): 2293-2296.

Hellkvist, L., Hjalmarsson, E., Kumelien Georen, S., Karlsson, A. Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial. *Journal of Allergy and Clinical Immunology*. 2018; 142, 1338–1341.

Höpken, M., Förster, I., Maune, S., Brockmann, M., Schildgen, O., Schildgen, V. Association of the Human Bocavirus With Tonsil Squamous Cell Carcinomas. *Frontiers in Microbiology*. 2018; *9*: 2450.

Huang, C.-C., Wu, P.-W., Lee, T.-J., Chen, C.-L., Wang, C.-H., Tsai, C.-N., Chiu C-H. Differential IL-17A response to S. pneumoniae in adenoid tissue of children with sleep disordered breathing and otitis media with effusion. *Scientific Reports (Nature research)*. 2019; 9: 19839.

Huang, Y.J., Marsland, B. J., Bunyavanich, S., O'mahony, L., Leung, D. Y. M., Muraro, A., Fleisher, T. A. The microbiome in allergic disease: Current understanding and future opportunities—2017 PRACTALL document of the American Academy of Allergy, Asthma & Immunology and the European Academy of Allergy and Clinical Immunology. *Journal of Allergy and Clinical Immunology*. 2017; 139(4), 1099–1110.

Hultcrantz, E., Ericsson, E. Factors Influencing the Indication for Tonsillectomy: A Historical Overview and Current Concepts Hultcrantz et al.: Factors Influencing the Indication for

Tonsillectomy: A Historical Overview and Current Concepts. *ORL J Otorhinolaryngol Relat Spec* . 2013; *75*(3): 184–191.

Hymowitz, S. G., Filvaroff, E. H., Yin, J. P., Lee, J., Cai, L., Risser, P., et al. IL-17s adopt a cystine knot fold: Structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO Journal*. 2001; 20(19): 5332–5341.

Ishiguro, T., Hirota, S., Kobayashi, Y., Takano, K., Kobayashi, Y., Shimizu, Y., Takayanagi, N. Fatal Primary Human Bocavirus Pneumonia in an Immunocompetent Adult. *Internal Medicine.* 2020; 59(3): 421–424.

Jartti, T., Palomares, O., Waris, M., Tastan, O., Nieminen, R., Puhakka, T., et al. Distinct regulation of tonsillar immune response in virus infection. *Allergy.* 2014; 69(5): 658–667.

Jartti, T., Lehtinen, P., Vuorinen, T., Österback, R., van den Hoogen, B., Osterhaus, A. D. M. E., Ruuskanen, O. Respiratory Picornaviruses and Respiratory Syncytial Virus as Causative Agents of Acute Expiratory Wheezing in Children. *Emerging Infectious Diseases*. 2004: 10(6); 1095–1101.

Jensen, A., Fagö-Olsen, H., Sørensen, C. H., Kilian, M. Molecular Mapping to Species Level of the Tonsillar Crypt Microbiota Associated with Health and Recurrent Tonsillitis. *PLoS ONE*. 2013: 8(2); e56418.

Johnston, J., Hoggard, M., Biswas, K., Astudillo-García, C., Radcliff, F. J., Mahadevan, M., Douglas, R. G. Paired analysis of the microbiota between surface tissue swabs and biopsies from pediatric patients undergoing adenotonsillectomy. *International Journal of Pediatric Otorhinolaryngology*. 2018a; 113: 51–57.

Johnston, J., Hoggard, M., Biswas, K., Astudillo-García, C., Radcliff, F. J., Mahadevan, M., Douglas, R. G. Pathogen reservoir hypothesis investigated by analyses of the adenotonsillar and middle ear microbiota. *International Journal of Pediatric Otorhinolaryngology*. 2019; 118: 103–109.

Johnston, J., Hoggard, M., Biswas, K., Astudillo-García, C., Waldvogel-Thurlow, S., Radcliff, F. J., et al. The bacterial community and local lymphocyte response are markedly different in patients with recurrent tonsillitis compared to obstructive sleep apnoea. *International Journal of Pediatric Otorhinolaryngology*. 2018b; 113: 281–288.

Johnston, J., Douglas, R. Adenotonsillar microbiome: An update. *Postgraduate Medical Journal*. 2018; 94: 398–403.

Kadriyan, H., Dirja, B. T., Suryani, D., Yudhanto, D. COVID-19 infection in the palatine tonsil tissue and detritus: the detection of the virus compartment with RT-PCR. *BMJ Case Rep*. 2021; 14: e239108

Kantola, K., Hedman, L., Arthur, J., Alibeto, A., Delwart, E., Jartti, T., et al. Seroepidemiology of human bocaviruses 1-4. *The Journal of Infectious Diseases*. 2011; 204(9): 1403–1412.

Kantola, K., Hedman, L., Tanner, L., Simell, V., Mäkinen, M., Partanen, J., et al. B-Cell Responses to Human Bocaviruses 1–4: New Insights from a Childhood Follow-Up Study. *PLOS ONE*. 2015; 10(9): e0139096.

Khalfaoui, S., Eichhorn, V., Karagiannidis, C., Bayh, I., Brockmann, M., Pieper, M., et al. Lung Infection by Human Bocavirus Induces the Release of Profibrotic Mediator Cytokines In Vivo and In Vitro. *PLoS ONE*. 2016; 11(1): e0147010

Kim, D., Langmead, B., Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. *Nature Methods*. 2015; 12(4): 357–360.

Koch, C. M., Chiu, S. F., Akbarpour, M., Bharat, A., Ridge, K. M., Bartom, E. T., Winter, D. R. A beginner's guide to analysis of RNA sequencing data. *American Journal of Respiratory Cell and Molecular Biology*. 2018; 59(2): 145–157.

Kono, T., Hattori, F., Kawamura, Y., Miura, H., Sudo, Y., Suzuki, M., Yoshikawa, T. Three adolescents with primary human herpesvirus 7 infection during a measles outbreak. *Pediatric Infectious Disease Journal*. 2020; 39(8): E209–E211.

Koskenvuo, M., Möttönen, M., Waris, M., Allander, T., Salmi, T. T., Ruuskanen, O. Human bocavirus in children with acute lymphoblastic leukemia. *European Journal of Pediatrics*. 2008; 167(9), 1011–1015

Kourieh, A., Gheit, T., Tommasino, M., Dalstein, V., Clifford, G. M., Lacau St Guily, J., et al. Prevalence of human herpesviruses infections in nonmalignant tonsils: The SPLIT study. *Journal of Medical Virology*. 2019; 91(4): 687–697.

Kücüksezer, U. C., Palomares, O., Ruckert, B., Jartti, T., Puhakka, T., Nandy, A., et al. Triggering of specific Toll-like receptors and proinflammatory cytokines breaks allergen-specific T-cell tolerance in human tonsils and peripheral blood. *Journal of Allergy and Clinical Immunology*. 2013; 131(3): 875–885

Kumar, A., Filippone, C., Lahtinen, A., Hedman, L., Söderlund-Venermo, M., Hedman, K., Franssila, R. Comparison of Th-cell Immunity Against Human Bocavirus and Parvovirus B19: Proliferation and Cytokine Responses are Similar in Magnitude but More Closely Interrelated with Human Bocavirus. *Scandinavian Journal of Immunology*. 2011; 73: 135–140.

Landis, J. R., Koch, G. G. The measurement of observer agreement for categorical data. *Biometrics*. 1977; *33*(1): 159–174

Lantto, U., Koivunen, P., Tapiainen, T., Glumoff, V., Hirvikoski, P., Uhari, M., Renko, M. Microbes of the tonsils in PFAPA (Periodic Fever, Aphtous stomatitis, Pharyngitis and Adenitis) syndrome - a possible trigger of febrile episodes. *APMIS*. 2015: 123(6); 523–529.

Lazear, H. M., Nice, T. J., Diamond, M. S. Interferon-l: Immune Functions at Barrier Surfaces and Beyond. *Immunity.* 2010; 43: 15–28

Lehtimäki, J., Karkman, A., Laatikainen, T., Paalanen, L., von Hertzen, L., Haahtela, T., et al. Patterns in the skin microbiota differ in children and teenagers between rural and urban environments *Nature Publishing Group, Scientific Reports*. 2017; 7: srep45651

Leung, D., Akdis, C., Bacharier, L., Cunningham-Rundles, C., Sicherer, S., Sampson, H. Pediatric Allergy: *Principles and Practice - 4th Edition 2020 Elsevier Health*.

Leung, S., Liu, X., Fang, L., Chen, X., Guo, T., Zhang, J. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells andregulatory T cells in autoimmune disease. *Cellular and Molecular Immunology*. 2010; 7(3): 182–189.

Li, X., Kantola, K., Hedman, L., Arku, B., Hedman, K., Söderlund-Venermo, M. Original antigenic sin with human bocaviruses 1-4. *The Journal of General Virology*. 2015; 96(10): 3099–3108.

Lindner, J., Zehentmeier, S., Franssila, R., Barabas, S., Schroeder, J., Deml, L., Modrow, S. CD4+ T Helper Cell Responses against Human Bocavirus Viral Protein 2 Viruslike Particles in Healthy Adults. *The Journal of Infectious Diseases*. 2008; 198(11): 1677–1684.

Longtin, J., Bastien, M., Gilca, R., Leblanc, E., de Serres, G., Bergeron, M. G., Boivin, G. Human Bocavirus Infections in Hospitalized Children and Adults. *Emerging Infectious Diseases*. 2008; 14(2): 217–221.

Lucas, C., Wong, P., Klein, J., Castro, T. B. R., Silva, J., Sundaram, M., et al. Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature*. 2020; 584(7821): 463–469.

Lukkarinen, H., Söderlund-Venermo, M., Vuorinen, T., Allander, T., Hedman, K., Simell, O. et al. Human bocavirus 1 may suppress rhinovirus-associated immune response in wheezing children. *Journal of Allergy and Clinical Immunology*. 2014; 133(1): 256–258.

Luo, H., Zhang, Z., Zheng, Z., Ke, X., Zhang, X., Li, Q., et al. Human bocavirus VP2 upregulates IFN-β pathway by inhibiting ring finger protein 125-mediated ubiquitination of retinoic acid-inducible gene-I. *Journal of Immunology.* 2013; 191(2): 660–669.

Mäkela, M. J., Puhakka, T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimäki, M. et al. Viruses and Bacteria in the Etiology of the Common Cold. *Journal of clinical microbiology.* 1998; 36(2): 538–542

Mannonen, L., Vainionpää, R., Kauppinen, J., Lienhard, R., Tritten, M.-L., Cannon, G., et al. Evaluation of multiplex polymerase chain reaction and microarray-based assay for rapid herpesvirus diagnostics. *Diagnostic Microbiology and Infectious Disease*. 2012; 73: 74–79

Marcus, C. L., Moore, R. H., Rosen, C. L., Giordani, B., Garetz, S. L., Taylor, H. G.,et al. A Randomized Trial of Adenotonsillectomy for Childhood Sleep Apnea. *New England Journal of Medicine*. 2013; 368(25): 2366–2376.

Marseglia, G.L., Poddighe, D., Caimmi, D., Marseglia, A., Caimmi, S., Ciprandi, G. et al. Role of adenoids and adenoiditis in children with allergy and otitis media. *Current Allergy and Asthma Reports*. 2009; 9(6), 460–464.

Marshall, G. S., Edwards, K. M., Butler, J., Lawton, A. R. Syndrome of periodic fever, pharyngitis, and aphthous stomatitis. *The Journal of Pediatrics*. 1987; 110: 43–46.

Martin, E. T., Fairchok, M. P., Kuypers, J., Magaret, A., Zerr, D. M., Wald, A., Englund, J. A. Frequent and Prolonged Shedding of Bocavirus in Young Children Attending Daycare. *The Journal of Infectious Diseases*. 2010; 201(11): 1625–1632

Masieri, S., Trabattoni, D., Incorvaia, C., De Luca, M. C., Dell'Albani, I., Leo, G., Frati, F. A role for Waldeyer's ring in immunological response to allergens. *Current Medical Research and Opinion*. 2014; 30(2): 203–205.

Masters, K. G., Lasrado, S. Anatomy, Head and Neck, Tonsils. In StatPearls. 2019 StatPearls Publishing. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/30969614

McLeish, N. J., Witteveldt, J., Clasper, L., McIntyre, C., McWilliam Leitch, E. C., Hardie, A., et al. Development and Assay of RNA Transcripts of Enterovirus Species A to D, Rhinovirus Species A to C, and Human Parechovirus: Assessment of Assay Sensitivity and Specificity of Real-Time Screening and Typing Methods. *Journal of Clinical Microbiology*. 2012; 50(9): 2910–2917.

McMurdie, P. J., Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS One*. 2013; 8(4), e61217.

Mikola, E., Elenius, V., Saarinen, M., Palomares, O., Waris, M., Turunen, R., et al. Tonsillar cytokine expression between patients with tonsillar hypertrophy and recurrent tonsillitis. *Clinical and Translational Allergy*. 2018; 8(1).

Mikola, E., Palomares, O., Turunen, R., Waris, M., Ivaska, L. E., Silvoniemi, A. et al. Rhinovirus species and tonsillar immune responses. *Clin Transl Allergy*. 2019; 9(63)

Mitchell, R. B., Archer, S. M., Ishman, S. L., Rosenfeld, R. M., Coles, S., Finestone, et al. Clinical Practice Guideline: Tonsillectomy in Children (Update)- Executive Summary. *Otolaryngology--Head and Neck Surgery.* 2019; 160(2): 187–205.

Mnatsakanian, A., Heil, J. R., Sharma, S. Anatomy, Head and Neck, Adenoids. *StatPearls*. 2021. Retrieved from https://www-ncbi-nlm-nih-gov.ezproxy.utu.fi/books/NBK538137/

Mullins, T. B., Krishnamurthy, K. Roseola Infantum. *StatPearls*. 2021. Retrieved from https://www-ncbi-nlm-nih-gov.ezproxy.utu.fi/books/NBK448190/

Näsman, A., Du, J., Dalianis, T. A global epidemic increase of an HPV-induced tonsil and tongue base cancer - potential benefit from a pan-gender use of HPV vaccine. *Journal of Internal Medicine*. 2020; 287(2): 134–152.

Nave, H., Gebert, A., Pabst, R. Morphology and immunology of the human palatine tonsil. *Anatomy and Embryology*. 2001; 204(5): 367–373.

Nistico, L., Kreft, R., Gieseke, A., Coticchia, J. M., Burrows, A., Khampang, P., et al. Adenoid Reservoir for Pathogenic Biofilm Bacteria. *Journal of Clinical Microbiology*. 2011; 49(4): 1411–1420.

Niu, X., Wu, Z. H., Xiao, X. Y., Chen, X. The relationship between adenoid hypertrophy and gastroesophageal reflux disease: A meta-analysis. *Medicine*. 2018; 97(41).

Nora-Krukle, Z., Vilmane, A., Xu, M., Rasa, S., Ziemele, I., Silina, E., et al. Human Bocavirus Infection Markers in Peripheral Blood and Stool Samples of Children with Acute Gastroenteritis. *Viruses*. 2018; 10(11).

Norja, P., Hedman, L., Kantola, K., Kemppainen, K., Suvilehto, J., Pitkäranta, A., et al. Occurrence of human bocaviruses and parvovirus 4 in solid tissues. *Journal of Medical Virology*. 2012; 84(8): 1267–1273.

Nyström, K., Biller, M., Grahn, A., Lindh, M., Larson, G., Olofsson, S. Real time PCR for monitoring regulation of host gene expression in herpes simplex virus type 1-infected human diploid cells. *Journal of Virological Methods*. 2004; 118(2): 83–94.

Ogasawara, N., Kojima, T., Go, M., Takano, K.-I., Kamekura, R., Ohkuni, T., et al. Epithelial barrier and antigen uptake in lymphoepithelium of human adenoids. *Acta Oto-Laryngologica.* 2010; 131: 116–123.

Olde Loohuis, L. M., Mangul, S., Ori, A. P. S., Jospin, G., Koslicki, D., Yang, H. T., et al. Transcriptome analysis in whole blood reveals increased microbial diversity in schizophrenia. *Translational Psychiatry*. 2018; 8(96)

Onal, M., Onal, O., Turan, A. Can secondary lymphoid organs exert a favorable effect on the mild course of COVID-19 in children? *Acta Oto-Laryngologica*. 2020; 141(1): 83–84

Önal, M., Yılmaz, T., Bilgiç, E., Müftüoglu, S.F., Kusç, U.,Günaydın, R. Apoptosis in chronic tonsillitis and tonsillar hypertrophy. *International Journal of Pediatric Otorhinolaryngology*. 2015; 79: 191–195.

Osterback, R., Tevaluoto, T., Ylinen, T., Peltola, V., Susi, P., Hyypia, T., Waris, M. Simultaneous Detection and Differentiation of Human Rhino- and Enteroviruses in Clinical Specimens by Real-Time PCR with Locked Nucleic Acid Probes. *Journal of Clinical Microbiology*. 2013; 51(12): 3960–3967.

Palomares, O., Jartti, T., Can, U. K., Puhakka, T., Gomez, E., Fahrner, H. B., et al. Induction and maintenance of allergen-specific FOXP3 1 Treg cells in human tonsils as potential first-line organs of oral tolerance. *Journal of Allergy and Clinical Immunology*. 2011; 129(2): 510–520.

Paloniemi, M., Lappalainen, S., Salminen, M., Kätkä, M., Kantola, K., Hedman, L., et al. Human bocaviruses are commonly found in stools of hospitalized children without causal association to acute gastroenteritis. *Eur J Pediatr*. 2014; 173: 1051–1057.

Patel, A., Brook, C. D., Levi, J. R. Comparison of adenoid assessment by flexible endoscopy and mirror exam. *International Journal of Pediatric Otorhinolaryngology*. 2020; 134: 110073.

Pawankar, R., Holgate, S.T., Canonica, G.W. Lockey, R.F. WAO White Book of Allergy. *WAO White Book of Allergy*. 2011. Retrieved from http://www.worldallergy.org/UserFiles/file/WAO-White-Book-on-Allergy_web.pdf

Pelaez-Prestel, H. F., Sanchez-Trincado, J. L., Lafuente, E. M., Reche, P. A., Shimodaira, S. Immune Tolerance in the Oral Mucosa. *International Journal of Molecular Sciences*. 2021; 22: 12149

Peltola, V., Waris, M., Österback, R., Susi, P., Ruuskanen, O., Hyypiä, T. Rhinovirus Transmission within Families with Children: Incidence of Symptomatic and Asymptomatic Infections. *The Journal of Infectious Diseases*. 2008; 197(3): 382–389.

Pelucchi, C., Grigoryan, L., Galeone, C., Esposito, S., Huovinen, P., Little, P., Verheij, T. Guideline for the management of acute sore throat. *Clin Microbiol Infect*. 2012; 18: 1–27.

Pereira, L., Monyror, J., Almeida, F. T., Almeida, F. R., Guerra, E., Flores-Mir, C., Pacheco-Pereira, C. Prevalence of adenoid hypertrophy: A systematic review and meta-analysis. *Sleep Medicine Reviews.* 2018; 38: 101–112

Perry, M., Whyte, A. Immunology of the tonsils. *Immunology Today*. 1998; 19(9): 414–421.

Petra, D., Petra, K., Michaela, K., Daniela, K., Petr, H., Ladislav, K., et al. Polyclonal, newly derived T cells with low expression of inhibitory molecule PD-1 in tonsils define the phenotype of lymphocytes in children with Periodic Fever, Aphtous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome. *Molecular Immunology*. 2015; 65(1): 139–147.

Petrarca, L., Nenna, R., Frassanito, A., Pierangeli, A., Di Mattia, G., Scagnolari, C., Midulla, F. Human bocavirus in children hospitalized for acute respiratory tract infection in Rome. *World Journal of Pediatrics*. 2020; 16: 293–298.

Proença-Módena, J.L., Buzatto, G. P., Paula, F. E., Saturno, T. H., Delcaro, L. S., Prates, M. C., et al. Respiratory viruses are continuously detected in children with chronic tonsillitis throughout the year. *International Journal of Pediatric Otorhinolaryngology*. 2014; 78(10): 1655–1661.

Proenca-Modena, J.L., de Souza Cardoso, R., Criado, M. F., Milanez, G. P., de Souza, W. M., Parise, P. L., et al. Human adenovirus replication and persistence in hypertrophic adenoids and palatine tonsils in children. *Journal of Medical Virology*. 2019; 91(7): 1250–1262.

Proença-Modena, J.L., Gagliardi, T. B., Escremim de Paula, F., Iwamoto, M. A., Criado, M. F., Camara, A. A., et al. Detection of Human Bocavirus mRNA in Respiratory Secretions Correlates with High Viral Load and Concurrent Diarrhea. *PLoS ONE*. 2011; 6(6): e21083.

Proenca-Modena, J.L., Paula, F. E., Buzatto, G. P., Carenzi, L. R., Saturno, T. H., Prates, M. C.,et al. Hypertrophic Adenoid Is a Major Infection Site of Human Bocavirus 1. *Journal of Clinical Microbiology*. 2014; 52(8): 3030–3037.

Proenca-Modena, J.L., Pereira Valera, F. C., Jacob, M. G., Buzatto, G. P., Saturno, T. H., Lopes, L., High rates of detection of respiratory viruses in tonsillar tissues from children with chronic adenotonsillar disease. *PLoS ONE*. 2012; 7(8): e42136

Qiu, J., Söderlund-Venermo, M., Young, N. S. Human Parvoviruses. *Clinical Microbiology Reviews*. 2017; 30(1): 43–113.

Renko, M., Lantto, U., Tapiainen, T. Towards better diagnostic criteria for periodic fever, aphthous stomatitis, pharyngitis and adenitis syndrome. *Acta Paediatrica*. 2019; 108(8): 1385–1392.

Richardson, M. A. Sore Throat, Tonsillitis, and Adenoiditis. *Medical Clinics of North America* 1999; *83*: 75–83.

Rihkanen, H., Carpén, O., Roivainen, M., Vaheri, A., Pitkäranta, A. Rhinovirus in adenoid tissue. *International Journal of Pediatric Otorhinolaryngology.* 2004; 68: 903–908.

Robinson, M. D., McCarthy, D. J., Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010; 26(1), 139–140.

Rosenfeld, R. M., Tunkel, D. E., Schwartz, S. R., Anne, S., Bishop, C. E., Chelius, D. C. Clinical Practice Guideline: Tympanostomy Tubes in Children (Update). *Otolaryngology-Head and Neck Surgery*. 2022; 166(1S): 1–55.

Rout, M. R., Mohanty, D., Vijaylaxmi, Y., Bobba, K., Metta, C. Adenoid Hypertrophy in Adults: A case Series. *Indian Journal of Otolaryngology and Head & Neck Surgery*. 2013; *65*(3): 269–274

Sakki, A., Mäkinen, L. K., Roine, R. P., Nokso-Koivisto, J. Changing trends in pediatric tonsil surgery. *International Journal of Pediatric Otorhinolaryngology*. 2019; 118: 84–89.

Sánchez-Ponce, Y., Varela-Fascinetto, G., Romo-Vázquez, J. C., López-Martínez, B., Sánchez-Huerta, J. L., Parra-Ortega, et al. Simultaneous Detection of Beta and Gamma Human Herpesviruses by Multiplex qPCR Reveals Simple Infection and Coinfection Episodes Increasing Risk for Graft Rejection in Solid Organ Transplantation. *Viruses*. 2018; 10(12): 730

Sato, M., Li, H., Ikizler, M. R., Werkhaven, J. A., Williams, J. V., Chappell, J. D., et al. Detection of Viruses in Human Adenoid Tissues by Use of Multiplex PCR. *Journal of Clinical Microbiology*, 2009; 47(3): 771–773

Schildgen, V., Malecki, M., Tillmann, R.-L., Brockmann, M., Schildgen, O. The Human Bocavirus Is Associated with Some Lung and Colorectal Cancers and Persists in Solid Tumors. *PLoS ONE.* 2013; 8(6); e68020

Schildgen, V., Pieper, M., Khalfaoui, S., Arnold, W. H., Schildgen, O. Human Bocavirus Infection of Permanent Cells Differentiated to Air-Liquid Interface Cultures Activates Transcription of Pathways Involved in Tumorigenesis. *Cancers*. 2018; 10(410)

Schlaberg, R., Ampofo, K., Tardif, K. D., Stockmann, C., Simmon, K. E., Hymas, et al. Human Bocavirus Capsid Messenger RNA Detection in Children With Pneumonia. *The Journal of Infectious Diseases*. 2017; 216(6): 688–696.

Seishima, N., Kondo, S., Wakisaka, N., Kobayashi, E., Imoto, T., Moriyama-Kita, M., et al. EBV infection is prevalent in the adenoid and palatine tonsils in adults. *Journal of Medical Virology*. 2017; 89(6): 1088–1095.

Shah, S., Garritano, F. G. Pediatric oral anatomy. *Operative Techniques in Otorhinolaryngology*. 2015; *26*: 2–7.

Shannon-Lowe, C., Rickinson, A. The Global Landscape of EBV-Associated Tumors. *Frontiers in Oncology*. 2019; 9: 713.

Shulman, S. T., Bisno, A. L., Clegg, H. W., Gerber, M. A., Kaplan, E. L., Lee, G., et al. Clinical Practice Guideline for the Diagnosis and Management of Group A Streptococcal Pharyngitis: 2012 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases.* 2012; *55*(10): e86.

Silvoniemi, A., Mikola, E., Ivaska, L., Jeskanen, M., Löyttyniemi, E., Tuomo Puhakka, et al. Intratonsillar detection of 27 distinct viruses: A cross-sectional study. *J Med Virol*. 2020; 92: 3830–3838.

Smithard, A., Cullen, C., Thirlwall, A. S., Aldren, C. Tonsillectomy may cause altered tongue sensation in adult patients. *The Journal of Laryngology & Otologo.* 2009; 123(5): 545–549.

Söderlund-Venermo, M., Lahtinen, A., Jartti, T., Hedman, L., Kemppainen, K., Lehtinen, P., et al. Clinical assessment and improved diagnosis of bocavirus-induced wheezing in children, Finland. *Emerging Infectious Diseases*. 2009; 15(9): 1423–1430.

Stern, P. L., Dalianis, T. Oropharyngeal Squamous Cell Carcinoma Treatment in the Era of Immune Checkpoint Inhibitors. *Viruses.* 2021; 13: 1234.

Stojanov, S., Lapidus, S., Chitkara, P., Feder, H., Salazar, J. C., Fleisher, T. A., et al. Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) is a disorder of innate immunity and Th1 activation responsive to IL-1 blockade. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108(17): 7148–7153.

Subtil, J., Rodrigues, J. C., Reis, L., Freitas, L., Filipe, J., Santos, A., et al. Adenoid bacterial colonization in a paediatric population. *European Archives of Oto-Rhino-Laryngology.* 2017; 274(4): 1933–1938.

Surjan, L. Jr,, Brandtzaeg, P., Berdal, P. Immunoglobulin systems of human tonsils. II. Patients with chronic tonsillitis or tonsillar hyperplasia: quantification of Ig-producing cells, tonsillar morphometry and serum Ig concentrations. *Clinical and Experimental Immunology*. 1978; 31(3): 382–390

Suvilehto, J., Roivainen, M., Seppänen, M., Meri, S., Hovi, T., Carpén, O., Pitkäranta, A. Rhinovirus/enterovirus RNA in tonsillar tissue of children with tonsillar disease. *Journal of Clinical Virology*. 2006; 35: 292–297.

Swidsinski, A., Göktas, O., Bessler, C., Loening-Baucke, V., Hale, L. P., Andree, H., et al. Spatial organisation of microbiota in quiescent adenoiditis and tonsillitis. *Journal of Clinical Pathology*. 2007; *60*(3): 253–260.

Szalmás, A., Papp, Z., Csomor, P., Kónya, J., Sziklai, I., Szekanecz, Z., Karosi, T. Microbiological profile of adenoid hypertrophy correlates to clinical diagnosis in children. *BioMed Research International*. 2013; 2013:ID 629607.

Tejesvi, M. V, Uhari, M., Tapiainen, T., Pirttilä, A. M., Suokas, M., Lantto, U., et al. Tonsillar microbiota in children with PFAPA (periodic fever, aphthous stomatitis, pharyngitis, and adenitis) syndrome. *European Journal of Clinical Microbiology & Infectious Diseases*. 2016; *35*: 963–970.

Thorley-Lawson, D. A., Gross, A. Persistence of the Epstein–Barr Virus and the Origins of Associated Lymphomas. *N Engl J Med.* 2004; 350: 1328–1337.

Tiveljung-Lindell, A., Rotzén-Ostlund, M., Gupta, S., Ullstrand, R., Grillner, L., Zweygberg-Wirgart, B., Allander, T. Development and implementation of a molecular diagnostic platform for daily rapid detection of 15 respiratory viruses. *Journal of Medical Virology*. 2009; 81(1): 167–175.

Toivonen, L., Karppinen, S., Schuez-Havupalo, L., Teros-Jaakkola, T., Vuononvirta, J., Mertsola, J., et al. Burden of recurrent respiratory tract infections in children: A prospective cohort study. *Pediatric Infectious Disease Journal*. 2016; *35*(12): e362–e369.

Torretta, S., Capaccio, P., Gaffuri, M., Gaini, L. M., Borin, M., Maruca, A., et al. ENT management of children with adenotonsillar disease during COVID-19 pandemic. Ready to start again? *International Journal of Pediatric Otorhinolaryngology*. 2020; *138*: 110145

van Kempen, M. J. P., Rijkers, G. T., van Cauwenberge, P. B. The Immune Response in Adenoids and Tonsils. *International Archives of Allergy and Immunology*. 2000; *122*(1): 8–19.

Vinícius, O., Primo, B., Lourenço, E. A., Passos, S. D. Detection of Respiratory Viruses in Nasopharyngeal Swab and Adenoid Tissue from Children Submitted to Adenoidectomy: Pre-and Postoperative Analysis. *Int Arch Otorhinolaryngol*. 2014; 18: 150–154.

Wada, K., Mizoguchi, S., Ito, Y., Kawada, J. I., Yamauchi, Y., Morishima, T. et al. Multiplex real-time PCR for the simultaneous detection of herpes simplex virus, human herpesvirus 6, and human herpesvirus 7. *Microbiology and Immunology*. 2009; 53(1): 22–29.

Wagner, J. C., Pyles, R. B., Miller, A. L., Nokso-Koivisto, J., Loeffelholz, M. J., Chonmaitree, T. (Determining Persistence of Bocavirus DNA in the Respiratory Tract of Children by Pyrosequencing. *The Pediatric Infectious Disease Journal*. 2016; 35(5): 471–476.

Waldeyer, H. W. *Waldeyer W* . 1884; 10(20): 315

Walner, D. L., Mularczyk, C., Sweis, A. Utilization and trends in surgical instrument use in pediatric adenotonsillectomy. *International Journal of Pediatric Otorhinolaryngology*. 2017; *100*: 8–13.

Wang, B., Tang, X., Xu, J., Yao, H. Differential expression of Immunoglobulin A in the adenoids of children with and without exudative otitis media. *International Journal of Pediatric Otorhinolaryngology*. 2012; 76, 728–730.

Wang, H. Chronic adenoiditis. *The Journal of International Medical Research*. 2020; *48*(11): 1–8

Windfuhr, J. P., Toepfner, N., Steffen, G., Waldfahrer, F., Berner, R. Clinical practice guideline: tonsillitis I. Diagnostics and nonsurgical management. *European Archives of Oto-Rhino-Laryngology*. 2016; 273: 973–987.

Windisch, W., Pieper, M., Ziemele, I., Rockstroh, J., Brockmann, M., Schildgen, O., Schildgen, V. Latent infection of human bocavirus accompanied by flare of chronic cough, fatigue and episodes of viral replication in an immunocompetent adult patient, Cologne, Germany. *JMM Case Reports*. 2016; 3(4): e005052.

Wood, D. E., Salzberg, S. L. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*. 2014; *15*(3): R46.

Wu, M. C., Ma, K. S. K., Wang, Y. H., Wei, J. C. C. Impact of tonsillectomy on irritable bowel syndrome: A nationwide population-based cohort study. *PLoS ONE*. 2020; 15(9): e0238242

Wu, S., Hammarstedt-Nordenvall, L., Jangard, M., Cheng, L., Radu, S. A., Angelidou, P., et al. Tonsillar Microbiota: a Cross-Sectional Study of Patients with Chronic Tonsillitis or Tonsillar Hypertrophy. *MSystems*. 2021; 6(2): e01302-20

Xu, M., Arku, B., Jartti, T., Koskinen, J., Peltola, V., Hedman, K., Söderlund-Venermo, M. Comparative Diagnosis of Human Bocavirus 1 Respiratory Infection With Messenger RNA Reverse-Transcription Polymerase Chain Reaction (PCR), DNA Quantitative PCR, and Serology. *The Journal of Infectious Diseases* 2017; 215: 1551–1557.

Xu, M., Perdomo, M. F., Mattola, S., Pyöriä, L., Toppinen, M., Qiu, J., et al. Persistence of Human Bocavirus 1 in Tonsillar Germinal Centers and Antibody-Dependent Enhancement of Infection. *MBio*. 2021; *12*(1): 1–15.

Zhang, P. C., Pang, Y. T., Loh, K. S., Wang, D. Y. Comparison of histology between recurrent tonsillitis and tonsillar hypertrophy. *Clinical Otolaryngology & Allied Sciences*. 2003; 28(3): 235–239.

Zhang, Z., Zheng, Z., Luo, H., Meng, J., Li, H., Li, Q., et al. Human Bocavirus NP1 Inhibits IFN-β Production by Blocking Association of IFN Regulatory Factor 3 with IFNB Promoter. *The Journal of Immunology*. 2012; *1*89(3), 1144–1153.

Zhao, B., Yu, X., Wang, C., Teng, Z., Wang, C., Shen, J., et al. High Human Bocavirus Viral Load Is Associated with Disease Severity in Children under Five Years of Age. *PLoS ONE*. 2013; *8*(4): e0062318

TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2022

ISBN 978-951-29-8932-4 (PRINT)
ISBN 978-951-29-8933-1 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)