

Elina Seppälä

**PICORNAVIRUS INFECTIONS AND
ACUTE OTITIS MEDIA AMONG YOUNG
FINNISH AND ESTONIAN CHILDREN**

Faculty of social sciences
Master's thesis
January 2019

ABSTRACT

Elina Seppälä: Picornavirus infections and acute otitis media among young Finnish and Estonian children
Master's thesis
Tampere University
Master's Degree Programme in Health Sciences (International Health)
January 2019

Background. Human rhinoviruses (HRV), human enteroviruses (HEV) and human parechoviruses (HPeV) have been linked to acute otitis media (AOM). This association was evaluated in a prospective birth cohort setting.

Methods. 542 healthy infants were followed up from birth to the age of 3 years in the DIABIMMUNE study in Finland and Estonia. Stool samples were collected monthly and analyzed for HRV, HEV and HPeV. Nasal swab samples were collected at the age of 3, 6, 12, 18, 24 and 36 months, and screened for HRV and HEV. Parents recorded all AOM diagnoses in a diary. The association between virus detection in samples and AOM episodes was assessed by univariate and multivariate logistic regression.

Results. Altogether 1066 AOM episodes were recorded during the study period. 21%, 5% and 4% of the stool samples tested positive for HRV, HEV and HPeV, respectively. Of the nasal swab samples, 23% were positive for HRV and 2% for HEV. HRV and HPeV positivity of stool samples was associated with experiencing an AOM episode within two weeks before or after obtaining the stool sample (adjusted OR 1.42 [95% CI 1.00-2.01] for HRV and adjusted OR 2.00 [95% CI 1.12-3.55] for HPeV).

Conclusion. HRV, HEV and HPeV infections occur frequently in early childhood. These viruses may play an independent role in the development of AOM in children.

Keywords: Acute otitis media, picornaviruses, human rhinovirus, human enterovirus, human parechovirus, child

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REFERENCE TO THE ORIGINAL PAPER

This thesis is based on the original article: Seppälä E., Oikarinen S., Lehtonen J., Neupane S., Sillanpää S., Laranne J., Knip M., Hyöty H., DIABIMMUNE Study Group. Picornavirus infections are associated with acute otitis media in a prospective Finnish-Estonian birth cohort study.

ABBREVIATIONS

AOM	Acute otitis media
CI	Confidence interval
COME	Chronic otitis media with effusion
CSOM	Chronic suppurative otitis media
ET	Eustachian tube
HEV	Human enterovirus
HPeV	Human parechovirus
HRV	Human rhinovirus
IgA	Immunoglobulin A
MEE	Middle ear effusion
MEF	Middle ear fluid
OM	Otitis media
OME	Otitis media with effusion
OR	Odds ratio
PCV	Pneumococcal conjugate vaccine
qPCR	Quantitative polymerase chain reaction
rAOM	Recurrent acute otitis media
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
URI	Upper respiratory infection

1. INTRODUCTION

Acute otitis media (AOM) occurs worldwide and primarily affects pediatric populations. Among children, AOM is the most common reason for the prescription of antibiotics, and along with upper respiratory infections (URIs), the leading cause for doctors' visits.[1, 2] URIs and AOM are closely linked: URIs, most of which have a viral etiology, are often complicated by AOM, especially among children.[3, 4] In studies carried out among pediatric populations, approximately one third of all URIs have been complicated by AOM, which usually occurs within the first seven days of URI onset.[3-6] The significant burden of viral URIs leads to the high incidence of AOM, which is one of the most common diseases in childhood. Furthermore, children encountering AOM early in life have been shown to be at increased risk of recurrent AOM later on.[7] Otitis media results in the widespread use of antibiotics as well as otologic surgery, causing significant financial losses to the families of the children and society.[8]

Generally, AOM is acknowledged to be a bacterial complication of viral URI. However, viruses alone or together with bacterial co-pathogens have also been shown to cause AOM.[1, 9, 10] Of the large variety of viruses causing URIs, picornaviruses have been found to be among the most common causes, with human rhinoviruses (HRV) accounting for up to 73% of all respiratory infections recorded during the first year of life.[11, 12] HRVs as well as human enteroviruses (HEV) and human parechoviruses (HPeV) have been linked to AOM in a number of studies. In Finnish and Australian studies, HRVs and HEVs were the predominant viruses detected in children with AOM and recurrent AOM.[9, 13, 14] HPeVs have been found to play a smaller role in the development of AOM.[15-17]

The aforementioned studies have mostly relied on methods based on reverse transcriptase polymerase chain reaction (RT-PCR) assays, the high sensitivity of which has led to the questioning of the clinical relevance of the results. These viruses detectable with PCR-based methods have also been found in children without concurrent respiratory symptoms.[18] Hence, the presence of HRVs, HEVs and HPeVs in respiratory or stool samples alone does not establish causality of the concurrent illness. In order to further investigate the possible causal relationship between virus findings and clinical symptoms,

it is important to conduct studies in which the overall frequency of HRV, HEV and HPeV infections is compared between children who develop AOM and those who do not.

The aim of this study was to assess the association between HRV, HEV and HPeV infections and AOM in a cohort of healthy children who were followed from birth to the age of 3 years. Nasal swab and stool samples were collected regularly from the children and analyzed for HRVs, HEVs and HPeVs using sensitive RT-PCR methods. The infections diagnosed during the first year of life were of special interest, as AOM experienced early in life can predispose to recurrent AOM later. Furthermore, the inclusion of nasal swab samples in this study was expected to bring additional information on the epidemiology of HRV, HEV and HPeV infections as compared to the study recently published by our study group.[19] AOM episodes were recorded during the follow-up, allowing the evaluation of the association between virus-positive findings to AOM episodes in an unbiased manner.

2. REVIEW OF LITERATURE

This chapter presents a summary of selected aspects of AOM most relevant to the topic of the study. Above all, the review of literature focuses on the role of viruses, and especially picornaviruses, in the development of AOM.

2.1 Definitions of otitis media

In general, the term otitis media (OM) refers to the inflammation of the middle ear. AOM is defined as the presence of middle ear effusion (MEE) together with the acute onset of signs and symptoms of inflammation of the middle ear and tympanic membrane, such as otalgia, otorrhea, fever, irritability and other symptoms typical of URIs.[1, 20] Recurrent AOM (rAOM) is referred to when a person encounters three or more AOM episodes in a period of six months, or four or more in 12 months.[21] Otitis media with effusion (OME), another stage of the OM continuum, is a condition with MEE, but without acute signs or symptoms of infections as seen in AOM. The term chronic OME (COME) is used when the condition persists for at least three months. In the case of chronic suppurative otitis media (CSOM), continuous or intermittent otorrhea is present due to a perforation in the tympanic membrane.[1, 22] This study and review of literature focus on AOM.

2.2 Epidemiology of acute otitis media

2.2.1 Incidence

AOM occurs worldwide, affecting especially pediatric populations. Among children, AOM is the most common reason for the prescription of antibiotics, and along with URIs, the leading cause for doctors' visits.[1, 2] Based on a systematic review and hypothetical model, Monasta et al. estimated 709 million AOM episodes to occur each year, 51% of these among children under 5 years of age. There is large variation in the incidence and prevalence of OM between regions: the study group calculated the global incidence rate of AOM (new episodes per 100 person-years) to range from 4 in Central and Eastern Europe to 43 in Western and Central Sub-Saharan Africa. In Western Europe, including Finland, the incidence rate was estimated to be 6, i.e. slightly higher than in other parts

of Europe. Globally, the incidence rates were highest in the youngest age groups: 61 among 1-4-year-olds and 45 in the under 1-year age group.[23] Indeed, a Finnish review summarizing the results of Scandinavian and Northern American studies reported the incidence of AOM to be highest at the age of 6-24 months, with the peak incidence at 10-12 months.[20] Birth cohort studies have suggested that more than 60% and 80% of the children experience at least one AOM episode by the age of two and three years, respectively.[24, 25]

Experiencing AOM at an early age increases the risk of recurrent AOM later on.[7] For example, a Danish birth cohort study found that children who experienced their first AOM episode under the age of six months were more likely to encounter four or more AOM episodes by the age of seven years than those whose first AOM episode occurred at a later age.[26]

2.2.2 Risk factors

In addition to young age, several other risk factors for AOM have been identified. Regarding environmental risk factors, a meta-analysis published more than 20 years ago suggested day care attendance, having one or more siblings, parental smoking, pacifier use and positive family history of OM to be risk factors for the occurrence and recurrence of AOM.[27] The meta-analysis also suggested breastfeeding to protect from OM. However, this finding has later been questioned. While some studies have linked male sex and low socioeconomic status with OM, others have not been able to confirm these findings. In addition, adenoid hypertrophy, allergy and atopy, weight, gastroesophageal reflux disease and vitamin D appear to be associated with OM.[26, 28-30] Heritability also plays a role, especially in rAOM and COME.[31]

2.3 Pathogenesis of acute otitis media

URI is undoubtedly one of the most important risk factors of AOM, as most cases of AOM occur simultaneously with or after URI.[5, 32] More than 9 out of 10 children with AOM experience concurrent URI symptoms. Furthermore, during AOM episodes, respiratory viruses are found in the majority of nasopharyngeal samples and up to 70% of middle ear specimens also test positive for these viruses.[9]

Indeed, respiratory viruses play an important role in the development of AOM. These viruses cause inflammation of the nasopharynx and Eustachian tube (ET), which connects the middle ear to the nasopharynx. This, in turn, leads to the generation of host inflammatory and immune responses. In addition, viral infections increase bacterial colonization and the adherence of bacteria to epithelial cells in the nasopharynx. The inflammatory and immune responses lead to swelling of the nasopharyngeal mucosa, diminished mucociliary clearance, obstruction of the ET and negative middle ear pressure. This facilitates the entrance of colonizing bacteria as well as viruses into the middle ear where inflammation develops, leading ultimately to the production of middle ear fluid (MEF), and signs and symptoms of AOM.[33]

2.4 Etiology of acute otitis media

As explained previously, AOM is considered a polymicrobial disease, in which bacteria and viruses interact in the pathogenesis.[9, 34] The role of bacteria in the pathogenesis of AOM is well established. The three most frequent bacterial pathogens of AOM, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*, belong to the normal flora and colonize the nasopharynx from an early age.[35] These pathogenic bacteria cause disease only when the milieu of the nasopharynx changes, most often as a consequence of viral URI. There is not much geographical variation in bacterial pathogens causing OM. A recently performed systematic review studying the frequency of detection of bacteria in MEF specimens obtained from children experiencing OM suggested that, globally, *S. pneumoniae* is most commonly found during AOM (26.4-30.2% of MEF samples), and it is closely followed by *H. influenzae* (22.2-27.8% of MEF samples). The detection rates of *M. catarrhalis*, on the other hand, range from 2.7% in South America to 11.6% and 13.8% in Europe and North America, respectively. Other bacteria less commonly found in patients with AOM include *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[36]

The role of viruses in the development of AOM is equally important, however, as virtually any virus causing URI can lead to the development of AOM. Some studies have suggested certain viruses to be more likely to cause AOM, and some viruses have been shown to cause AOM also without bacterial coinfection. The detection rates of different viruses

during AOM have varied between studies, depending e.g. on the study setting, detection technique used, viruses included in the assay, and timing of the study period. Viruses frequently linked to AOM include respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, adenovirus, coronavirus, polyomavirus, human bocavirus (HBoV), metapneumovirus, and picornaviruses.[3, 9, 10, 13, 14, 37-39]

The association between RSV, influenza and parainfluenza viruses, adenovirus, enterovirus and AOM has already been seen in earlier studies relying on viral culture and/or antigen detection methods.[38, 40] Newer studies based on more sensitive molecular detection methods have suggested also other viruses to be of importance in the development of AOM. An Australian hospital-based case-control study investigating the microbiology of rAOM in children undergoing surgery for the installation of tympanostomy tubes found HRV, HBoV, polyomaviruses, parainfluenza viruses, adenovirus, and RSV significantly more often in the nasopharynx of children with a history of rAOM compared to healthy controls.[14] A prospective, longitudinal study of infants in the first year of life assessing the prevalence and risks for URTI and AOM found that AOM development was associated with RSV, HRV, HEV, adenovirus and HBoV infection, and the rate of AOM complicating URTI in this age group was 27%. Of these viruses, RSV has been found to be associated with increased risk of AOM (OR 6.50) without concurrent presence of bacterial copathogens, emphasizing the independent role of RSV in the pathogenesis of AOM.[10]

More recent studies utilizing PCR-based detection methods have also provided more information on the role of picornaviruses in AOM. HRVs, HEVs and HPeVs will be discussed separately in the following chapters. It should be noted, however, that some controversy exists regarding the significance of the positive findings of these more sensitive molecular methods. This is discussed in chapter 2.5.2.

2.4.1 Human rhinovirus

As HRVs are among the most common causes of URI, accounting for up to 73% of all respiratory infections recorded during the first year of life [11, 12], it can be expected that HRVs play a significant role also in the development of AOM. Indeed, HRVs have been linked to AOM in a number of studies. In the previously mentioned study carried out

among 0-12-month-old infants living in the United States, HRV was the most common virus detected during URI and accounted for 40% of all detected viruses. HRV was significantly associated with the development of AOM (OR 5.08), which complicated 27% of all URI episodes.[3] In the Australian hospital-based study, HRV was predominant in the middle ear and was detected in MEF samples of 46% of children with rAOM.[14] Similarly, in a recent birth cohort study conducted in South-Western Finland, HRV was associated with 50% of AOM episodes diagnosed among children aged 0-2 years.[41]

2.4.2 Human enterovirus

Compared to HRVs, HEVs appear to play a somewhat smaller role in the development of AOM. A Finnish study including children with otorrhea from tympanostomy tubes, detected enteroviruses with PCR in 10% of the subjects.[9] The aforementioned study conducted in the United States found HEV to account for 11% of all detected viruses among symptomatic and asymptomatic 0-12-month-old infants. HEV was marginally associated with the development of AOM, however, HEV was also found in asymptomatic individuals.[3] The Finnish Otitis Media Vaccine Trial, a randomized, double-blinded cohort study, utilized PCR for the detection of picornaviruses in MEF and nasopharyngeal aspirate samples. HEVs were the second most common viruses found (in 25% of AOM events) after HRVs (32%). It is noteworthy, however, that other viruses included in the study were sought by less sensitive antigen detection methods, hence the role of picornaviruses in this study may have been overestimated.[13]

2.4.3 Human parechovirus

More recently, HPeVs have also been suggested to play a role in the development of AOM. A small Finnish birth cohort study found 11% and 15% of MEF and nasopharyngeal aspirate samples to be PCR positive for HPeV, and HPeV seropositivity was associated with OM.[17] Similarly, HPeV infection was diagnosed in the MEF, nasal cavity and/or stool in 14% of AOM cases in a prospective, out-patient-based study conducted in Finland.[16] A retrospective study conducted in the Netherlands found that 19% of children under the age of five years who tested positive for HPeV type 4-6 in fecal samples, had otitis media.[15]

2.5 Diagnosis of acute otitis media

2.5.1 Clinical diagnosis

In clinical practice, the diagnosis of AOM is based on three criteria: acute symptoms of infection, signs of middle ear inflammation and presence of MEE.[20, 42] According to the Finnish Current Care Guidelines, the verification of signs of inflammation on the tympanic membrane and presence of MEE is done with pneumatic otoscopy performed by a doctor.[20] Pneumatic otoscopy includes the visual assessment of the mobility and appearance of the tympanic membrane. It can be supplemented with tympanometry, a quantitative method of evaluating the mobility of the tympanic membrane and middle ear function.[1] If a tympanostomy tube has been inserted or in the case of perforation of the tympanic membrane, the diagnosis of AOM is based on the detection of otorrhea through the tube or perforation.[20]

2.5.1 Microbiological diagnosis

In every day practice, the diagnosis of AOM is based solely on clinical evaluation, and the decision to treat is usually made without microbiological information on the cause of the infection. In Finland, microbiological samples are recommended to be considered in the case of AOM in children with otorrhea through a previously inserted tympanostomy tube. Similarly, MEF samples are recommended to be obtained through tympanocentesis (puncture of the tympanic membrane) from children with immunocompromising conditions, with suspected complications of AOM, or requiring hospitalization.[20]

While tympanocentesis is not suitable for every day practice, other sample types such as nasopharyngeal swabs and aspirates, and even stool samples could be considered for assessing the pathogens potentially causing AOM episodes. As viral URI and nasopharyngeal carriage of certain pathogenic bacteria are strongly associated with the development of AOM, nasopharyngeal samples would be the logical, more feasible diagnostic tool of choice. Indeed, numerous studies assessing the microbiology of AOM have utilized nasal or nasopharyngeal specimens, either alone or in combination with MEF samples.[3-5, 13, 16, 37, 38] In the Finnish Otitis Media Cohort Study and the Finnish Otitis Media Vaccine Trial, the same virus was detected in both MEF and

nasopharyngeal aspirate specimens in 41% and 49% of the virus-positive AOM events, respectively. In the former study, HRV, influenza virus A and RSV were found concurrently in both sample types in 36%, 40% and 59% of the virus-positive events, respectively. In the latter study, the concurrence for HRV, HEV and RSV was 42%, 42% and 61%, respectively. In both studies, MEF samples were more often positive for these viruses than nasopharyngeal samples.[13] Hence, the choice of specimen type may have an impact on the number of virus-positive findings, as well as the variety of pathogens that can be detected. This was true for a smaller Finnish study assessing the presence of AOM pathogens in three specimen types: MEF, nasal swabs and stool samples. HPeV was detected in at least one of the sample types in 12 subjects (14%), and the specimen most commonly positive for HPeV was stool (50%). In only one of the subjects HPeV could be found in two sample types, namely MEF and stool.[16] This finding is understandable, as stool specimens have been considered as the classical sample type for HEVs and HPeVs replicating primarily in the intestinal and oropharyngeal mucosa.[43, 44] Also HRVs have been detected frequently in stool samples.[45]

After the sample or samples of choice have been obtained, several analytical methods can be utilized for the detection of AOM pathogens. Studies using conventional methods, including bacterial and viral cultures and antigen detection methods, have suggested bacteria alone to be the leading cause of AOM, and viruses to play only a minor role.[46] These methods, however, have been criticized for being vulnerable and insensitive. Furthermore, certain viruses commonly causing URI, such as coronavirus, human metapneumovirus and HBoV cannot be detected with conventional methods.[46] The use of molecular diagnostics such as PCR assays has increased the sensitivity for viral detection and enabled the detection of viruses not identifiable by culture. Consequently, studies utilizing molecular methods have suggested the majority of AOM episodes to be caused by bacteria and viruses together, and viruses alone have also been suggested to lead to the development of AOM.[9]

However, as Chonmaitree et al.[46] point out, the use of more sensitive methods has raised questions regarding the true pathogenicity of the detected viruses. Firstly, the detection of multiple viruses simultaneously has led to difficulties in determining which one of the detected viruses is the actual pathogen behind the symptoms. Secondly, nucleic

acids of certain viruses, including HRVs and HEVs, have been detected repeatedly in respiratory samples collected from children, which indicates prolonged presence of these viruses in the respiratory tract. Lastly, respiratory viruses have been commonly found in asymptomatic individuals as well, which could either suggest acute asymptomatic infection, recent symptomatic infection with prolonged shedding of the virus or viral persistence from previous infection.[3] Indeed, in two prospective longitudinal studies, viruses were detected in 27-40% of respiratory samples obtained during asymptomatic periods.[3, 47] Even though one of these studies found that asymptomatic viral infection did not result in the development of AOM [3], the significance of viral findings in respiratory samples during AOM remains somewhat controversial.

2.6 Treatment of acute otitis media

Traditionally, antibiotics have been the cornerstone of the treatment of AOM, and AOM continues to be the most common reason for children to consume antibiotics.[48] However, according to a recently updated systematic review and meta-analysis, most cases of AOM in high income countries heal spontaneously without complications, and antibiotics may only slightly speed up the resolution of symptoms.[49] This conclusion is supported by the previously presented information on the importance of viruses in AOM. Indeed, some cases of AOM may not require antibiotic therapy as AOM may be caused by viruses alone. It is also possible that the MEE containing bacteria and viruses may drain spontaneously in a few days' time through the Eustachian tubes once the function of the tubes has returned to normal.[33]

Recent studies and guidelines emphasize accurate diagnosis of AOM, effective treatment of ear pain and consideration of antibiotic therapy on an individual basis. Factors supporting antibiotic therapy include age less than two years, bilateral infection, otorrhea, bulging tympanic membrane and severe symptoms (ear pain ≥ 48 h, fever $\geq 39^{\circ}\text{C}$). Watchful waiting can be considered for children with non-severe symptoms and unilateral disease, provided that the clinical status of the child can be reassessed after 2-3 days if symptoms persist. Amoxicillin is considered the first-line choice of antibiotic, followed by amoxicillin-clavulanate. Options for pain management include paracetamol,

ibuprofen, naproxen or local anesthetics.[20, 42] As viral infections generally resolve spontaneously, the use of antiviral drugs is not recommended.

2.7 Prevention of acute otitis media

Considering the pathogenesis and etiology, the prevention of AOM episodes is ultimately based on the prevention of viral URI and reduction or prevention of nasopharyngeal carriage of bacterial otopathogens. To achieve this, current guidelines emphasize the reduction of environmental risks, and encourage the use of bacterial and viral vaccines.[20, 42]

2.7.1 Environmental risk factors

Regarding environmental risk factors, the American Academy of Pediatrics (AAP) recommends clinicians to encourage exclusive breastfeeding for at least six months and avoidance of tobacco smoke exposure.[42] The Finnish Current Care Guidelines also identify daycare attendance and pacifier use as possible targets for intervention.[20]

2.7.2 Vaccines

The polymicrobial nature of AOM poses significant challenges to the development and use of vaccines for preventive purposes. Of the three most important bacterial otopathogens, only vaccines against *S. pneumoniae* are currently widely available and used. The authors of a recently published systematic review[50] concluded that pneumococcal conjugate vaccines (PCVs), which have been used since year 2000, are somewhat effective for the prevention of pneumococcal OM and OM visits, as well as rOM and tympanostomy tube insertions. However, widespread use of PCVs has also been linked with changes observed in the distribution of other bacterial AOM pathogens and pneumococcal serotypes, with those not included in PCVs becoming more prevalent and thus reducing the effectiveness of PCVs against AOM. Despite this, the AAP strongly recommends the administration of PCV to all children.[42] While the 10-valent PCV containing *H. influenzae* protein D seems effective against pneumococcal AOM, its' effectiveness against AOM caused by *H. influenzae* is questionable. Other types of

vaccines against *H. influenzae* and *M. catarrhalis* also are being developed, but progress has been limited.[50]

As viruses play a significant role in the development of AOM, preventing viral URIs with vaccines would likely prevent AOM too. Currently, the only AOM-associated respiratory virus vaccine that is licensed and widely used is the influenza vaccine. A recently published systematic review suggested that the influenza vaccine may reduce the incidence of AOM in infants and small children[51], and the use of influenza vaccines is encouraged in Finnish and American AOM guidelines.[20, 42] Some other respiratory viruses, such as RSV and picornaviruses, have also been targeted in terms of vaccine development. While vaccines against RSV[52] and certain enteroviruses[53] have already been developed, attempts to produce an HRV vaccine have failed[54], and the effectiveness of the more successful vaccine candidates against AOM is not known. However, the findings of our previous study were encouraging: we found that the oral polio vaccine (OPV), a live attenuated enterovirus vaccine, may reduce the incidence of AOM by providing cross-protection from non-polio enteroviruses and other picornaviruses that have been associated with AOM.[55]

In conclusion, preventive methods are essential in reducing the burden of AOM on young children and their families. Verifying the significance of specific viruses in AOM would assist in the development of optimal antiviral strategies.

3. WORK DESCRIPTION

I began actively working on this study in the summer of 2016. Originally, this study was meant to be included in my doctoral dissertation only. Thus, the supervisor of my doctoral studies in the Doctoral Programme of Medicine and Life Sciences, professor Heikki Hyöty, had an important role in planning the study. Later, when the decision was made to include this study also in my master's thesis, Sami Oikarinen became my other supervisor. He also had a significant role in planning the study. Subas Neupane kindly accepted my request to be the other supervisor of this thesis.

The data collection period of the DIABIMMUNE study had already ended in October 2013, and all data collected during the visits to the study clinics and recorded by parents in the diaries had been saved in the DIAIMMUNE study database. After we had made the decision to include all Finnish and Estonian DIABIMMUNE study participants in this piece of work, I retrieved all data available on these subjects from the database, reorganized the data and created a Microsoft Excel -based database of my own. Iiris Tyni and Jussi Lehtonen assisted me with compiling the data on otitis media diagnoses, and Jussi Lehtonen was also responsible for merging the laboratory data with the dataset I had created.

Due to the relatively complicated study setting, Jussi Lehtonen performed all statistical analyses while I observed closely. Luckily, I had the opportunity to influence the conduction of all statistical analyses and could request additional analyses any time additional questions arose, or possible errors emerged. Heikki Hyöty, Sami Oikarinen and Subas Neupane kindly provided their input regarding the presentation of the results, namely the design of the tables and graphs included in the article manuscript.

I wrote the article manuscript on my own, utilizing literature I considered relevant to introduce and discuss the topic of our study. Sami Oikarinen informed me of the process used for the laboratory analyses of the nasal swab and stool samples obtained during the DIAIMMUNE study. Jussi Lehtonen checked the contents of the chapter describing the statistical methods. Sami Oikarinen and Subas Neupane also provided general feedback on the preliminary version of the article manuscript. After the submission of this thesis

and my version of the article manuscript, the manuscript will be circulated among the other authors to obtain more feedback.

4. ACKNOWLEDGMENTS

I am very grateful for the patience, support and guidance received from my supervisors Sami Oikarinen, Subas Neupane and professor Heikki Hyöty. Perhaps even more importantly, I would like to humbly thank Jussi Lehtonen for his priceless help with data management and analyses. Without Jussi's input this study could not have been conducted with such sophisticated methodology and preciseness.

I would also like to thank my bosses Jussi Sane and Taneli Puumalainen at the National Institute for Health and Welfare for being understanding and supportive of my efforts to graduate from this Master's Degree Programme in Health Sciences.

Last but not least, I am utterly grateful to Lauri, my family and friends for encouraging me to finish this degree. Without their support, this journey would have felt even harder.

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