Tampere University

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Association Between Enterovirus Infections During Early Life and Atopy

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty Council of the Faculty of Medicine and Health Technology
of the Tampere University,
for public discussion in the Lecture room F114
of the Arvo building, Arvo Ylpön katu 34, Tampere,
on 7 June 2019, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

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Cover design: Roihu Inc.

ISBN 978-952-03-1097-4 (print)
ISBN 978-952-03-1098-1 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-1098-1

PunaMusta Oy – Yliopistopaino
Tampere 2019

ACKNOWLEDGEMENTS

This has been a long journey. And now it has come to an end, yippee! The support and help from people around me have made this possible, I thank you all so much. You're great!

My deepest thanks go to my supervisors Professor Heikki Hyöty and Docent Maria Lönnrot. Your enthusiasm and insight have been the basis of everything. Thank you for always being so kind and patient. Heikki, your solid scientific knowledge and experience have supported me throughout this project. Your innovative approach to science and versatile academic reflections have been great to follow. I have learnt a lot. Maria, thank you for your tireless support through these years. Whether things were a bit entangled or at the moments of success, you were there for me. Special thanks for your quick and clear responses, you always found the time to help me. It feels great to share this achievement with you.

I sincerely thank the pre-examiners Docent Heli Harvala and Docent Anita Remitz for your careful review and valuable comments in the finalization of this thesis.

I would also like to thank the members of my dissertation advisory committee Professor Antti Lauerma and acting Professor Marko Pesu.

I thank all co-authors of the original papers, especially Mikael Knip, Jorma Ilonen, Riitta Veijola and Suvi Virtanen, for your valuable contribution. My special thanks belong to Tapio Seiskari for your collaboration with the serological analysis and to Heini Huhtala for your expertise with the statistical analysis.

I feel lucky to have had such skillful and helpful colleagues around me along the way. Jussi Lehtonen, warm thank you for your enormous help with the statistical analysis. Your relaxed way of approaching statistical issues has calmed me down a number of times. My special thanks go also to Sami Oikarinen for your admirable expertise with the PCR analysis and Anita Kondrashova for your valuable contribution with the

neutralization assays. Thank you Noora Nurminen for patiently helping me during these years. Leena Puustinen, thank you for skillful help with serological assays as well as your friendship, it means a lot to me.

One of the best parts of this thesis study was that I had the possibility to be a member of Heikki's superb virology team. This thesis book wouldn't exist without your excellent scientific and technical skills. Your cheerful company has refreshed me on numerous coffee breaks. So thank you Maarit Oikarinen, Jutta Laiho, Anni Honkimaa, Amirbabak Sioofy-Khojine, Eeva Tolvanen, Eveliina Paloniemi, Anne Karjalainen, Maria Ovaskainen, Mervi Kekäläinen, Tanja Kuusela, Minta Lumme, Jenna Ilomäki, Sari Valorinta and others.

This book would not have been possible without the time for it. I am grateful to Annikki Vaalasti, the head of the Department of Dermatology in TAYS, for your flexibility and patience in enabling me to have those (almost) endless research months. I also thank Professor Erna Snellman for the research friendly environment in the clinic.

I owe my warm thanks to my colleagues in TAYS. Your encouraging and supporting comments have helped me to push this project to an end. Thank you Ave Kokk, Eriika Mansikka, Sonja Suvinen, Rafael Pasternack, Kaisa Hervonen, Teea Salmi, Teija Kimpimäki, Tiina Ahti, Meri Lauha, Toni Karppinen, Maria Lagerstedt, Päivi Hantula and everyone else in the Department of Dermatology. Anna Alakoski, thank you for your friendship, support and sharp insight in any situation. Thank you Taina Hasan for teaching me so much, the spirit you created got me interested in allergology in the first place. I am also grateful for my colleagues in the Allergy Centre, it has always been nice to return to you from my scientific trips. Special thanks to Jussi Karjalainen, Terhi Rantalainen, Susanna Salmivesi, Johanna Vehmaa-Suoja and Minna Nosa for your support.

My deepest thanks also to my friends outside work, your understanding and support have helped me to carry on. Hanna, thank you for always being there, I am so happy to have (and continue to) grown up with you. Johanna, I am grateful for your friendship and relaxed moments with your family. Thank you Hanna, Mervi, Paula, Synnöve, Ulla, Sari, Merja and others, you are great company and I hold you dear. Special thanks to Hanna, for guiding me back to Heikki's group. Thank you Kirsti for your warm support and understanding.

Finally and most importantly, I wish to thank my family. Thank you my parents-in-law Kirsti and Antti for always making me feel at home and Aku, Nella and Asta for your friendship. Thank you my parents Vuokko and Timo-Erkki and my sister Anna. Your love and solid believe in me is the base everything is built on, thank you. And then the words end: thank you my husband Riku and my beloved sons Jaakko and Eero. Life with you is the best!

This study was financially supported by the Graduate Program of University of Tampere Medical School and Faculty of Medicine and Life Sciences, the Medical Research Fund of Tampere University Hospital, the Finnish Cultural Foundation, the Finnish Dermatological Society, the Finnish Allergy Research Foundation, Orion Research Foundation, Päivikki and Sakari Sohlberg Foundation, Tampere Tuberculosis Foundation, Sigrid Juselius Foundation, the Finnish Medical Foundation, the National Technology Agency in Finland (TEKES), the Academy of Finland, the European Comission PEVNET project, Estonia Research Council and the City of Tampere.

Tampere 2019

Laura Korhonen

ABSTRACT

The rapid increase in the prevalence of atopic diseases, such as asthma, allergic rhinitis and atopic eczema, over the recent decades suggests that environmental factors might play an important role in the pathogenesis of these diseases. According to the 'hygiene hypothesis', changes in the environmental microbial load and reduced microbial exposure during childhood, lead to an imbalance in the developing immune system and subsequently increase the risk of atopic diseases. The atopic reaction pattern often develops during early childhood, possibly already during the fetal period, suggesting that very early microbial contacts might be particularly important in the development of these diseases. Enteroviruses are common pathogens in childhood and previous studies have suggested an association between enterovirus infections and atopic diseases.

The aim of this study was to investigate the relations between early enterovirus infections and atopy. Enteroviruses were analyzed from serum and stool samples collected prospectively from children developing atopy and from non-atopic controls. In addition, the relationship between maternal enterovirus infections during pregnancy and the risk of atopic disease in the offspring was addressed.

The number of infections caused by different enterovirus types during early childhood was determined by analyzing neutralizing antibodies. Neutralizing antibodies against 12 different enterovirus types were measured and grouped as echoviruses and coxsackieviruses. The study showed that atopic case children had significantly fewer echovirus infections during the first two years of life than the non-atopic control children did. This finding supports previous data from cross-sectional studies suggesting an inverse relationship between echovirus infections and atopy.

Maternal enterovirus infection during pregnancy was diagnosed by an increase in the enterovirus antibody levels between serum samples drawn at the end of the first trimester of pregnancy and at birth. The results showed that mothers whose children later developed atopic diseases had significantly fewer enterovirus infections during pregnancy. No difference was observed between other microbial infections (influenza virus A, *Mycoplasma pneumoniae*, cytomegalovirus, *Helicobacter pylori*) and

atopy in the offspring. The relation between gestational enterovirus infections and atopy has not been studied previously.

In addition, the association between enteroviruses present in stool samples collected during the first year of life and atopic sensitization was studied. This study included rhinoviruses, belonging to the *Enterovirus* genus, enteroviruses, noroviruses and parechoviruses. Atopic sensitization was inversely associated with the number of rhinovirus positive samples, but this was detected only in boys. The reasons for this sex-dependent difference are currently not known but previous data have suggested that sex might affect the susceptibility to both viral infections and atopic diseases. Other included viruses showed no association with atopy.

In conclusion, the results from the present study demonstrate that early enterovirus infections are inversely associated with atopy in childhood and the results provide further support to the hygiene hypothesis. The pathogenesis of atopic diseases is a multifactorial process involving genetic predisposition and various environmental factors, and the results from this study suggest that enteroviruses represent one aspect of this pathogenetic entity. This study shows that enteroviruses are interesting viruses with respect to the development of atopy but further studies are still needed to clarify their possible causal relationship with atopy.

TIIVISTELMÄ

Atooppisten sairauksien eli astman, allergisen nuhan ja atooppisen ihottuman esiintyvyyden nopea kasvu viime vuosikymmeninä on herättänyt kiinnostusta ympäristötekijöiden osuudesta näiden sairauksien synnyssä. Ns. hygieniahypoteesin mukaan elinympäristön mikrobialtistuksen muuttuminen ja lapsuusiän mikrobikontaktien väheneminen johtavat kehittyvän immuunijärjestelmän säätelyn häiriintymiseen ja lisääntyneeseen allergiariskiin. Atooppinen reaktiotapa kehittyy usein varhaislapsuudessa, mahdollisesti jo sikiöaikana. Täten varsinkin varhaiset mikrobi-infektiot saattavat olla erityisasemassa atooppisten sairauksien synnyssä. Enterovirukset ovat yleisiä lapsuusiän infektioiden aiheuttajia ja aiempi tutkimusnäyttö on antanut viitteitä enterovirusten ja atooppisten sairauksien välisestä yhteydestä.

Tämän väitöskirjan tavoitteena oli tutkia varhaisten enterovirusinfektioiden yhteyttä atopiaan. Tutkimuksessa verrattiin enterovirusten esiintymistä prospektiivisesti kerätyissä veri- ja ulostenäytteissä atooppisilla tapauslapsilla ja ei-atooppisilla verrokkilapsilla. Lisäksi tutkimuksessa selvitettiin äidin raskaudenaikaisen enterovirusinfektion yhteyttä lapsen myöhempään atooppisen sairauden riskiin.

Varhaislapsuuden aikana sairastettujen eri enterovirustyyppien aiheuttamien infektioiden määrää selvitettiin tutkimalla neutraloivien vasta-aineiden esiintymistä 12 eri enterovirustyyppiä kohtaan. Tutkimuksessa enterovirukset ryhmiteltiin echoviruksiin ja coxsackieviruksiin. Tutkimus osoitti, että atooppiset tapauslapset olivat sairastaneet merkittävästi vähemmän echovirusinfektioita ensimmäisen kahden elinvuoden aikana kuin ei-atooppiset verrokkilapset. Tutkimustulos tukee aiempia poikkileikkausasetelmasta saatuja tuloksia, joissa echovirusinfektioiden ja atopian välillä on todettu käänteinen yhteys.

Äidin raskausaikana sairastaman enterovirusinfektion esiintyvyyttä selvitettiin mittaamalla enterovirusvasta-ainetasot alkuraskauden seeruminäytteestä ja synnytyksen yhteydessä otetusta napaverinäytteestä. Tutkimus osoitti, että raskausaikaiseen enterovirusinfektioon viittaavia vasta-ainetasojen nousuja oli vähemmän niillä äideillä, joiden lapsille kehittyi myöhemmin atooppinen sairaus. Muilla tutkituilla mikrobeilla (A-tyypin influenssavirus, *Mycoplasma pneumoniae*,

sytomegalovirus, *Helicobacter pylori*) ei todettu olevan yhteyttä lapsen atopiariskiin. Raskaudenaikaisen enterovirusinfektion yhteyttä atopiaan ei ole aiemmin tutkittu.

Lisäksi tutkittiin enterovirusten esiintymistä ensimmäisen elinvuoden aikana kerätyissä ulostenäytteissä ja niiden yhteyttä lapsen atooppiseen herkistymiseen. Ulostenäytteistä tutkittiin rinoviruksia, jotka kuuluvat *Enterovirus*-sukuun, enteroviruksia, noroviruksia ja parechoviruksia. Tulokset osoittivat, että atooppisesti herkistyneillä lapsilla oli vähemmän rinoviruspositiivisia näytteitä mutta tämä tulos oli nähtävissä vain pojilla. Todetun sukupuolieron merkitys on toistaiseksi epäselvä, mutta aiemmat tutkimustulokset ovat viitanneet sukupuolten välisiin eroihin sekä virusinfektioiden että atooppisten sairauksien osalta. Muut tutkitut virukset eivät yhdistyneet lapsen atooppiseen herkistymiseen.

Yhteenvetona voidaan todeta, että tutkimuksessa havaittiin varhaisten enterovirusinfektioiden olevan käänteisesti yhteydessä lapsen atopiaan ja tutkimustulokset tukevat siten osaltaan hygieniahypoteesia. Atooppisten sairauksien kehittyminen on kuitenkin monimutkainen tapahtuma, johon vaikuttavat sekä geneettinen alttius että erilaiset ympäristötekijät. Tutkimustulokset viittaavat siihen, että enterovirukset ovat osa tätä atopian syntyyn johtavaa kokonaisuutta. Tämä tutkimus osoitti enterovirusten olevan mielenkiintoinen virusryhmä atopian synnyssä mutta syy-seuraussuhteen varmistamiseksi tarvitaan vielä jatkotutkimuksia.

CONTENTS

ABBREVIATIONS

ATCC	American Type Culture Collection
CDHR3	cadherin-related family member 3
CI	confidence interval
CLIA	chemiluminescent immunoassay
CMV	cytomegalovirus
CV-A	coxsackievirus A
CV-B	coxsackievirus B
DC	dendritic cell
DNA	deoxyribonucleic acid
DIPP	Type 1 Diabetes Prediction and Prevention Study
E	echovirus
EIA	enzyme immunoassay
EV	enterovirus
FcεRI	high-affinity IgE receptor
FOXP3	forkhead box P3
HAV	hepatitis A virus
HFMD	hand-foot-mouth disease
H. pylori	Helicobacter pylori
HLA	human leukocyte antigen
IAV	influenza virus A
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISAAC	International Study on Asthma and Allergies in Childhood
kU/L	kilounit per liter
M. pneumoniae	Mycoplasma pneumoniae
MAP	mitogen-activated protein
NAB	neutralizing antibody
NoV	norovirus

OR	odds ratio
PV	poliovirus
QCMD	Quality Control for Molecular Diagnostics
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RV	rhinovirus
T1D	type 1 diabetes
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
Treg	regulatory T cell
T. gondii	Toxoplasma gondii
UTR	untranslated region
VP	viral protein

ORIGINAL PUBLICATIONS

This study is based on the following original publications, which are referred to in the text by Roman numerals I-III:

I Korhonen L, Kondrashova A, Tauriainen S, Haapala AM, Huhtala H, Ilonen J, Simell O, Knip M, Lönnrot M, Hyöty H. Enterovirus infections in early childhood and the risk of atopic disease – a nested case-control study. *Clin Exp Allergy* 2013 Jun;43(6):625-32.

II Korhonen L, Seiskari T, Lehtonen J, Puustinen L, Surcel HM, Haapala AM, Niemelä O, Virtanen S, Honkanen H, Karjalainen M, Ilonen J, Veijola R, Knip M, Lönnrot M, Hyöty H. Enterovirus infection during pregnancy is inversely associated with atopic disease in the offspring. *Clin Exp Allergy* 2018 Dec;48(12):1698-1704.

III Korhonen L, Oikarinen S, Lehtonen J, Mustonen N, Tyni I, Niemelä O, Honkanen H, Huhtala H, Ilonen J, Hämäläinen AM, Peet A, Tillmann V, Siljander H, Knip M, Lönnrot M, Hyöty H and the DIABIMMUNE Study Group. Rhinoviruses in infancy and risk of immunoglobulin E sensitization. *J Med Virol* 2019 (Epub ahead of print).

1 INTRODUCTION

There has been a dramatic increase in the prevalence of atopic diseases over the recent decades (1,2) and this 'allergy epidemic' has also been observed in Finland (3,4). For instance, in Finland, the prevalence of asthma increased from 0.3% to 3.5% between years 1966-2003 and the prevalence of allergic rhinoconjuctivitis increased from <0.1% to 9% by the year 2000 (4). This rapid increase suggests that environmental factors are important in this phenomenon. This is further supported by differences in the prevalence of atopic diseases in populations with genetic similarities but markedly different living environments (5-7).

The 'hygiene hypothesis' was introduced in the year 1989. The original study showed that a child's risk of allergic rhinoconjuctivitis was decreased in families with many older siblings (8). This and similar observations from other studies, led to the hypothesis stating that increased microbial contacts might protect from the development of atopy. The current concept of hygiene hypothesis suggests that recent changes in lifestyle in industrialized countries have led to reduced environmental microbial diversity and diminished infectious burden predisposing to the development of atopy (9).

Enteroviruses (EVs) belong to the *Picornaviridae* virus family and they are common viruses worldwide. Several EV types affect humans, *i.e.* EV types from species A to D and rhinoviruses (RVs), and they can cause a wide spectrum of diseases. Cross-sectional study settings have suggested that EVs might be interesting viruses with regard to protection from atopy (7,10,11) but prospective studies are lacking. For RVs, RV-associated wheezing episodes have been shown to predispose to the development of asthma in childhood (12) but there are limited data about the role of RVs in other atopic diseases and IgE sensitization.

The purpose of the present study was to evaluate the possible associations between EV infections and atopy in prospective study settings. The relation between cumulative exposure to different EV types during early childhood and atopy was assessed. In addition, the association between maternal EV infection during pregnancy and atopic disease in the offspring was analyzed. Further, the possible

role of repeated EV exposure in infancy was studied by detecting EVs (including RVs) from stools and analyzing the associations with atopic sensitization.

2 REVIEW OF THE LITERATURE

2.1 Atopic diseases

2.1.1 Definition

The nomenclature applied in allergology has been defined and published by international allergy organizations in 2004 (13). In that report, allergy is defined as "a hypersensitivity reaction initiated by specific immunologic mechanisms" (13). The same report defines hypersensitivity as "objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons" (13). Allergy can be further classified into antibody-mediated or cell-mediated allergy, according to the immunologic mechanisms behind the symptoms. In antibody-mediated allergy, the antibodies belong typically to the immunoglobulin (Ig) E isotype, and these patients have IgE-mediated allergy, *i.e.* atopic allergy.

The word "atopy" was introduced in 1923 and is derived from the Greek word atopia (unusualness, being out of the way) from *a-* (not, without) and *topos* (place) (14). Atopy is "a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjuctivitis or eczema" (13). In other words, atopy describes a tendency to produce IgE antibodies against common environmental allergens, such as pollens, animal dandruff and foods, which often manifests as atopic diseases. Atopic diseases include atopic eczema (also called atopic dermatitis), allergic asthma and allergic rhinoconjunctivitis. As also included in the definition of atopy, atopic diseases begin typically in childhood.

2.1.2 IgE and atopic diseases

In physiology, IgE-mediated immune reactions are thought to be central in host defense against parasites, such as helminths. One of the major pathologic roles of IgE involves immediate type 1 hypersensitivity reactions (15). In the sensitization phase of this IgE-mediated allergy, an antigen, *i.e.* an allergen, is presented by antigen-presenting cells to naïve T cells in the lymph nodes. In the presence of interleukin (IL)-4 and other T helper (Th)2 type cytokines, T cells are primed into effector Th2 cells, which stimulate the production of allergen-specific IgE antibodies in B cells. These IgE antibodies bind to high-affinity IgE receptor (FcεRI) expressed on the surface of mast cells and basophils. During an allergic reaction, contact with the specific allergen cross-links the receptor-bound IgE resulting in the degranulation of mast cells and basophils and secretion of active mediators such as histamine and tryptase. The release of these mediators leads *e.g.* to vasodilatation and contraction of bronchial smooth muscle, resulting in the clinical manifestations of an acute allergic reaction.

The role of IgE in the pathogenesis of chronic atopic diseases is less clear and is likely to depend on the type of the disease. Atopic diseases are usually considered to include asthma, atopic eczema and allergic rhinoconjuctivitis (13). However, it has become increasingly evident that these diseases are not uniform but rather a heterogeneous group of illnesses presenting with various phenotypes. These phenotypes share similarities in clinical manifestations but have different etiologies and pathogenetic mechanisms. Accordingly, also the role of IgE in atopic diseases varies. For instance, allergic asthma is a common asthma phenotype especially in childhood and it is associated with IgE sensitization and Th2-type immune response (16). On the other hand, especially in adults, the significance of other types of asthma is more important and the role of IgE in their pathogenesis is less clear. For atopic eczema, most patients have elevated levels of serum total IgE and many have IgE-mediated allergies such as hay fever and oral allergy syndrome (17). However, approximately 20% of patients with atopic eczema have normal IgE levels, a clinical phenotype sometimes referred as intrinsic atopic eczema (18). High total IgE has been shown to predict poor long-term outcome in atopic eczema (17) but the role of IgE in the pathogenesis of atopic eczema is unsolved. For instance, omalizumab, an anti-IgE antibody, is efficient in the treatment of severe allergic asthma but its efficacy in the treatment of atopic eczema is modest at best (19).

2.1.3 Epidemiology

Atopic diseases are the most common non-communicable diseases in childhood in affluent countries. Numerous epidemiological studies confirm a substantial increase in the incidence of atopic diseases during the past decades and the term "allergy epidemic" has been applied (2-4,20-22).

First reports of a sharply increasing incidence of childhood asthma were published in the 1960s (20) and the increase has continued, as demonstrated for instance by Haahtela *et al* for young men in Finland (3). In that study, the prevalence of asthma recorded at call-up examinations for military service increased from 0.08% in 1961 to 1.79% in 1989, and in the follow-up study in 2003, the prevalence was still increasing (4). A similar trend has also been observed for other atopic diseases. For example, in a British study the prevalence of eczema and hay fever in adolescents increased between 1974 and 1986 from 3% to 6% and from 12% to 23%, respectively (2). In Finland, the prevalence of atopic eczema in young men increased from the 1960s until the 1980s but has thereafter remained relatively constant (4). In the same study, the prevalence of allergic rhinoconjuctivitis remained <0.1% until the 1970s but increased thereafter being 9% in the year 2000 (4).

There are geographical variations in the prevalence of atopic diseases. In the International Study of Asthma and Allergies in Childhood (ISAAC) published in 1998, children aged 13-14 years worldwide answered a standardized questionnaire about symptoms of atopic diseases (23). Globally, there were 20-fold differences in the prevalence of asthmatic symptoms with the highest prevalence in the United Kingdom, New Zealand and Australia, and the lowest prevalence in Eastern Europe, Indonesia and China. For allergic rhinitis and atopic eczema there were no clear geographical trends as the centers with the highest prevalence were scattered around the world (23). The authors interpreted that this scattering might result *e.g.* from differences in diagnostic criteria or in the frequencies of risk factors, or from issues related to the validity of the questionnaires (23).

A large-scale study on the time trends of atopic diseases worldwide was published in 2006 (22). Based on questionnaires performed 4-9 years apart, it reported that most centers showed an increase in the prevalence of childhood atopic diseases (Figures 2-4 in reference 22). Finland was included in the study, and results from children aged 13-14 years showed a modest increase in the prevalence of asthma and atopic eczema, with an increase from 13% to 19% and from 13% to 16%, respectively. The prevalence of allergic rhinoconjuctivitis was 15% and did not change during the follow-up (22). The prevalence of asthma and allergic

rhinoconjuctivitis in Finland was in line with most other northern and western European countries whereas the prevalence of atopic eczema was higher than in most European countries (22). Subsequently, systematic reviews aiming to assess global trends in the prevalence of atopic eczema and asthma have been published (24,25). The prevalence of asthma was still increasing in most countries, including many European countries (24). The prevalence of atopic eczema continued to increase for instance in the United Kingdom, Estonia and Lithuania but decreased or stayed stable in Sweden and Norway (25).

A particular feature of atopic diseases is the marked variance in prevalence in populations with similar genetic backgrounds but living in adjacent countries with different environments. For example, the prevalence of hay fever among schoolchildren was 16 % in Finnish North Karelia and 1% in adjacent Russian Karelia (6). In the same way, atopic sensitization was found to be more common in Finland than in Russia (6,7). The same observation of more frequent aeroallergen sensitization and hay fever in westernized societies was reported previously in a study comparing children living in West Germany and East Germany (5). However, 5 years after the German unification, these disparities were diminishing, *i.e.* the prevalence of hay fever and atopic sensitization was increasing in children living in former East Germany (26). In contrast to Germany, the differences in the prevalence of atopic diseases and IgE sensitization between schoolchildren living in Finland and Russian Karelia have remained relatively constant, possibly reflecting the rather slow rate of urbanization in Russian Karelia (27).

2.2 Enteroviruses

Picornaviridae is a virus family comprised of small single-stranded ribonucleic acid (RNA) viruses. Currently the family consists of 35 genera containing 80 species (28). Picornaviruses can cause subclinical or symptomatic infections in both humans and animals. *Enterovirus* genus is one of the *Picornaviridae* genera and it comprises of 15 species; 12 EV species and 3 RV species.

2.2.1 Enterovirus species A-D

2.2.1.1 Classification and structure

EVs belong to the *Enterovirus* genus of the *Picornaviridae* family. Traditionally EVs have been classified into four groups, namely group A coxsackieviruses (CV-As), group B coxsackieviruses (CV-Bs), echoviruses (Es) and polioviruses (PVs), according to their antigenic and biological properties (29). The molecular characterization of the viral genome has led to the present classification where EVs are classified according to their genetic similarities (30). Currently, the *Enterovirus* genus consists of 15 species, namely *Enterovirus A-L* and *Rhinovirus A-C* (28). Viruses affecting humans belong to species *Enterovirus A-D* and *Rhinovirus A-C* (Table 1).

EVs have a simple structure with a protein capsid surrounding the single-stranded RNA genome. The capsid is a symmetrical icosahedral structure composed of 60 identical capsomeres, each formed by four structural proteins; viral protein (VP)1, VP2, VP3 and VP4, with VP1-3 on the surface of the capsid and VP4 located inside the capsid (28,30). There are also non-structural proteins that take part in viral genome replication and virus-host cell interactions (31).

2.2.1.2 Epidemiology and clinical disease

EVs are among the most common human viruses worldwide, and they are transmitted via the orofecal or respiratory route. It has been estimated that every year 10-15 million symptomatic EV infections occur in the United States (32). The incidence of symptomatic EV infections is highest among children with 44% of infections occurring in infants under the age of one year (32). EV infections follow a seasonal pattern, which is more pronounced in temper climates where infections peak in summer and fall (33). In addition to this annual seasonality, individual EV types show different long-term circulation patterns and can occur in sporadic epidemics (34).

Table 1. Enterovirus species and types affecting humans

Species	Types	Number of types
Enterovirus A	CV-A2 to CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, EV-A71, EV-A76, EV-A89, EV-A90, EV-A91, EV-A114, EV-A119 to EV-A121	20
Enterovirus B	CV-B1 to CV-B6, CV-A9, E1 (incl. E8) to E7, E9 (incl. CV-A23), E11 to E21, E24 to E27, E29 to E33, EV-B69, EV-B73 to EV-B75, EV-B77 to EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B111	59
Enterovirus C	PV1 to PV3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19 to CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116 to EV-C118	23
Enterovirus D	EV-D68, EV-D70, EV-D94, EV-D111	4
Rhinovirus A	RV-A1, RV-A2, RV-A7 to RV-A13, RV-A15, RV-A16, RV-A18 to RV-A25, RV-A28 to RV-A34, RV-A36, RV-A38 to RV-A41, RV-A43, RV-A45, RV-A46, RV-A47, RV-A49, RV-A50, RV-A51, RV-A53 to RV-A68, RV-A71, RV-A73 to RV-A78, RV-A80, RV-A81, RV-A82, RV-A85, RV-A88, RV-A89, RV-A90, RV-A94, RV-A96, RV-A100 to RV-A109	80
Rhinovirus B	RV-B3 to RV-B6, RV-B14, RV-B17, RV-B26, RV-B27, RV-B35, RV-B37, RV-B42, RV-B48, RV-B52, RV-B69, RV-B70, RV-B72, RV-B79, RV-B83, RV-B84, RV-B86, RV-B91 to RV-B93, RV-B97, RV-B100 to RV-B106	32
Rhinovirus C	RV-C1 to RV-C56	56

CV, coxsackievirus; EV, enterovirus; E, echovirus, PV, poliovirus; RV, rhinovirus

More than 100 EV types can infect humans causing a wide spectrum of clinical manifestations. Although most EV infections are asymptomatic or mild, EVs also cause upper respiratory infections, herpangina and hand-foot-mouth disease (HFMD), as well as more severe diseases, such as meningitis, encephalitis, flaccid paralysis and myocarditis (35). The severity of the infection depends on both the virus type and host-specific factors, for instance young age and male gender predispose to more severe disease (35). Certain species A EV types, especially EV-A71, CV-A10 and CV-A16, are typical pathogens causing HFMD (36), and CV-Bs are significant pathogens in acute myocarditis and chronic dilated cardiomyopathy (37). EVs, especially CV-Bs, have also been shown to cause subclinical chronic pancreatitis and they have been linked to the development of type 1 diabetes (T1D) (38).

2.2.1.3 Laboratory diagnostics

Laboratory methods applied to diagnose EV infections include virus isolation and culture, serology, such as enzyme immunoassay (EIA) and neutralization assay, and direct virus detection, such as reverse transcription polymerase chain reaction (RT-PCR). Virus isolation and culture are used primarily in research work. Analysis of neutralizing antibodies (NABs) requires cell culture facilities and a virus isolate, and is thereby rarely used in clinical diagnostics. NAB levels can remain elevated for years after an infection, and thereby reflect the infection history of the individual. Measuring NABs also allows serotype-specific identification of EVs.

Another serological method is the detection of IgM or IgG antibodies by EIA. Measuring of IgM antibodies can be applied to detect acute or recent infections. However, it may lead to false negative findings since EV infections do not always elicit clear IgM responses and/or these responses may not be detectable by the EV type used as an antigen in the assay (39). Detection of an increase in the IgG levels between paired sera can also be applied to diagnose acute EV infections. Nevertheless, the value of serological assays in clinical diagnostics is limited due to the high prevalence of EV antibodies in healthy background population and the cross-reactivity of antibodies between different EV types (39). Serological methods are also time consuming and often require paired sera making them impractical for the diagnosis of acute infections.

Direct detection of virus RNA with molecular methods, especially RT-PCR, has largely replaced virus culture and serology in EV diagnostics in clinical virus laboratories. RT-PCR is a sensitive and specific method for the detection of EVs (40) and it can be used for several types of samples, typically cerebrospinal fluid, respiratory samples and stool. Most RT-PCR applied in EV diagnostics target the highly conserved 5' untranslated region (UTR) and are suitable for the diagnosis of all EV types (39). Virus typing can be obtained by sequencing the viral genome or part of it, typically the regions coding for VP1, VP2 or VP4.

2.2.2 Rhinovirus species A-C

2.2.2.1 Classification and structure

RVs are single-stranded RNA viruses that belong to the *Enterovirus* genus of the *Picornaviridae* family. There are currently over 160 RV types identified capable of infecting humans and they are classified into three species: *Rhinovirus A-C* (Table 1).

RVs have a symmetrical icosahedral protein capsid encasing the RNA genome. The capsid is composed of four structural proteins (VP1-VP4), while nonstructural viral proteins are involved in genome replication and assembly of new viruses (41).

2.2.2.2 Epidemiology and clinical disease

RVs are among the most common pathogens causing upper respiratory tract infections. For example, RVs were detected in 71% of nasopharyngeal aspirate samples collected from Finnish children with common cold-like illnesses (42). A large number of different RV strains circulate each year with the highest incidence of infections from September to November and from April to May (43). RV species A and C were shown to be the most common RVs in young children and they were also associated with more severe respiratory infections in this age group (44). RVs are transmitted primarily through the respiratory route. They cause typically common cold-like illnesses but can also cause a wide range of other diseases, such as acute otitis media, bronchiolitis and pneumonia, as well as exacerbations of chronic pulmonary diseases including asthma (45).

2.2.2.3 Laboratory diagnostics

Culturing RVs is more complicated than that of EVs, and RV-Cs do not grow on regular cell cultures. Therefore, virus culture is mainly used for research purposes. RVs lack an antigen common to all strains, which also makes serology impractical for clinical use. Hence, RT-PCR is nowadays the method of choice in the detection of RVs in clinical settings (41,45). Many of the commercial RT-PCR assays target the 5'UTR region, a highly conserved genomic region among all RVs and EVs (41), but

RT-PCR tests able to differentiate between RVs and EVs have also been published (46). When specific information about the RV type or strain is needed, genotyping of the VP1 and/or VP4 region can be applied (41).

2.2.3 Pathogenesis

The primary replication site of EVs is the lymphoid tissue of small intestine and pharynx, whereas RV replication occurs typically in the nasal epithelium and nasopharynx. Viruses utilize a variety of cell surface receptors to enter the cell and the expression of these receptors in human cells contributes to the tissue-specific virulence of the virus. For example, E1, a prevalent EV among children, binds to α2β1 integrin, which is abundantly expressed on the cell surface of many cell types (47). On the other hand, EV-D68 shows respiratory tropism and it was found to bind to sialic acids containing cell surface receptors, which are extensively expressed on human airway tract epithelia (48). Upper respiratory epithelium also expresses intracellular adhesion molecule 1(ICAM-1), a cell surface receptor necessary for the cell entry of most RV-A and RV-B types (41). On the contrary, RV-Cs utilize a different receptor molecule, namely cadherin-related family member 3 (CDHR3) (49).

After entering the target cell, viral genomic RNA is released and acts as a template for protein synthesis and the formation of new virions. Multiple EV-encoded proteins and host-dependent factors are involved in protein translation and virus replication (31). In EV infections, after replication in the primary replication site, *e.g.* small intestine, a viremia ensues seeding multiple organ systems, such as the central nervous system. The subsequent replication at these sites results in signs and symptoms of an EV infection (29). In RV infections, viremia is less common and might be associated with a more severe disease (50).

The tissue tropism of EVs also depends on physical factors, such as environmental pH and temperature. For example, RVs were originally thought to replicate optimally at 33ºC with marked reduction of replication in temperatures of 37-39ºC. Accordingly, RVs have been considered clinically relevant pathogens only in the upper respiratory tract where temperatures are below the inner body temperature. However, newer data have shown that some RVs are capable of replicating almost similarly at 33ºC and 37ºC, and the virus titres at 37ºC have been still adequate to initiate an infection (51).

Upon virus infection, the host immune system activates and EV-derived proteins and nucleic acids are recognized by host pattern recognition receptors, such as Toll-like receptor (TLR) 7 and TLR8 (43). Activation of these receptors promotes gene expression of various mediators of inflammation, such as interferons (IFN) and chemokines that attract and activate immune cells at the site of infection. The initial activation of the host innate immune system augments pathogen-specific adaptive immune system, *i.e.* humoral and cell-mediated immunity. Viruses, including EVs, have been shown to induce a strong Th1-type immune reaction accompanied by IFN production (52).

2.3 Hygiene hypothesis

A rapid increase in the prevalence of atopic diseases over the past few decades implicates that environmental factors play an important role in the phenomenon. This is further supported by observations showing marked variations in the prevalence of atopic diseases in genetically similar groups living in different environments (5-7). The core of the 'hygiene hypothesis' lies in the idea that recent changes in lifestyle in affluent countries have led to the decreased burden of infectious diseases resulting in the increased prevalence of atopic diseases. The hygiene hypothesis was introduced by Strachan, who reported the risk of hay fever in childhood to be inversely associated with the number of older siblings (8). His hypothesis was that contact with siblings in early childhood predisposes the child to microbial infections, which in turn protects from atopic diseases. Since the initial introduction of the hygiene hypothesis, relation of atopy and different aspects of environmental exposures and microbial contacts have been studied abundantly (53,54). For instance, having older siblings has been shown to decrease neonatal gut colonization by Clostridium bacteria and to associate with a decreased risk of atopic eczema (55), and the amount of household dust and its' endotoxin load have been observed to inversely associate with atopic sensitization and asthma (56). The concept of hygiene hypothesis has also been extended to autoimmune diseases, such as T1D (57) and multiple sclerosis (58).

Recently, it has been suggested that the hygiene hypothesis should be expanded to include environmental microbiota in a broader sense. The proposed 'biodiversity hypothesis' connects the loss of global biodiversity with reduced human commensal microbiota and susceptibility to disease, including atopic diseases (59,60). In other

words, reduced contact with diverse environmental microbiota might hinder the development of robust host microbiota leading to immune dysregulation and increased risk of disease. For instance, IgE sensitized individuals have been shown to have lower environmental biodiversity in their immediate surroundings as well as lower bacterial diversity on their skin as compared to non-atopic individuals (61).

2.3.1 Environmental factors

2.3.1.1 Farming

Numerous studies have shown that living in a farm is inversely associated with atopic sensitization, hay fever and asthma. Examples of this 'farm effect' include the findings that regular contact with farm animals and regular farm milk consumption are associated with protection from atopic diseases (62-64). In addition, the combination of living in a farm and having a large family markedly decreased the risk of atopy with the lowest risk being in children living in farms and having three or more siblings (65).

The protective effect of the farm environment is probably mediated through various mechanisms, one of which might be increased microbial exposure. For instance, bacterial endotoxin levels in mattress dust were higher at homes of children living in farms, and endotoxin levels were inversely associated with atopic sensitization of the child (66). Another study showed that children living in farms were exposed to a wider range of microbial exposure than children in a non-farming environment were, and this microbial diversity was inversely related to asthma (67). It is possible that not only farming but also farming practices are important, as recently demonstrated in a study comparing the prevalence of asthma between Amish and Hutterite children (68). In that study, the Amish children had a significantly lower prevalence of asthma and atopic sensitization (68). As these two populations having similar genetic ancestries and lifestyles, with the exception of different farming traditions, the results were suggested to reflect the increased exposure to a diverse microbial environment associated with traditional farming practices in the Amish community as compared to the more industrialized farming practices among the Hutterites (68). The effect of urbanization on indoor microbiota has also been detected in a Finnish study, where the quantity of doormat debris and

the diversity of its bacterial communities decreased in accordance with the amount of built area around the home (69).

2.3.1.2 Pet keeping

Studies about the association between pet keeping and atopic diseases report conflicting results. The abundant data have yielded meta-analyses that have also ended up with different conclusions. A meta-analysis including cohort and case-control studies reported a slightly increased risk of asthma in children exposed to dogs and a decreased risk of asthma in children exposed to cats (70). Another meta-analysis including children participating in European birth cohort studies, found no association between contact with furry pets in early life and asthma or allergic rhinitis (71). For atopic eczema, an inverse association between exposure to dogs and the development of atopic eczema has been reported (72).

Similarly to farm environment, the possible effect of pets on atopy might be mediated by their influence on the microbial environment within the home. In fact, the microbial composition of house dust was found to be richer and more diverse in homes with dogs than in households with no furry pets (73). Further, living with furry pets was shown to associate with changes in the gut microbiota in infancy (74).

2.3.1.3 Family size

One of the first researchers to report an inverse association between family size and atopy was Strachan in 1989 (8). He performed a large questionnaire-based cross-sectional study and observed that the number of siblings, especially older siblings, was inversely related to hay fever at the age of 11 (8). Since that, an inverse association between family size and atopic disease or sensitization has been detected in many studies (65,75,76). The mechanisms behind this observation remain unclear but increased exposure to infections transmitted by siblings and maternal microbial pressure affecting the *in utero* environment have been suggested (76).

2.3.1.4 Day care attendance

Studies about the effect of day care attendance on atopic diseases have shown conflicting results. Attendance in day care during early childhood has been found both to inversely associate with the development of asthma (77) and not to associate with childhood asthma (78). Similarly, day care attendance was observed to be inversely associated with atopic eczema in one study (79) but in another study, a positive association was reported (80). Controversies are present also in relation to IgE sensitization, as day care attendance has been reported both to inversely associate with IgE sensitization (81) and to increase the risk of food sensitization (78). The reasons for these discrepancies may reflect differences in parental atopy history, type and intensity of day care and child's age at the beginning of day care.

In fact, there are results suggesting that the child's age at day care entry is especially important, as day care during the first six months of life was associated with protection from asthma whereas day care later in life was not (77). Similarly, atopic sensitization was more frequent among children who entered day care at the age of ≥ 12 months than in those entering at the age of 6-11 months (82).

Early day care attendance might reflect the effects of early microbial stimulus but the data are inconclusive. For example, attendance in day care has been associated with an increased frequency of respiratory infections but adjusting for infections did not change the associations between day care attendance and atopic disease (78,81).

2.3.2 Immunological aspects of the hygiene hypothesis

2.3.2.1 Innate immunity

Innate immunity system provides the first line of immune defense against pathogens. Innate immune responses are based on cellular expression of pattern recognition receptors, such as TLRs, RIG-I-like receptors and NOD-like receptors that recognize microbial components and activate immune reactions (83). In humans, 12 TLRs have been identified to date, and TLR2, 3, 4, 7, 8 and 9 are known to be activated by viral components, including activation of TLR7 and TLR8 by single-stranded RNA and TLR3 by double-stranded RNA (84). TLRs have also been linked to atopic diseases. Most data are derived from genetic variants of TLR2 and TLR4 (85) but also mutations in the TLR7 and TLR8 loci have been shown to associate

with asthma and other atopic diseases (86). Upon activation, different TLRs activate different signaling cascades and the type of TLR activated might contribute to the development of atopic diseases, as reviewed for asthma (84).

There are some data suggesting that the environment might modify the innate immune profile already *in utero*. TLR2 was upregulated in the cord blood of children born in Russian Karelia as compared to children born in Finland, which was suggested to reflect differences in the overall microbial load during pregnancy (87). In addition, maternal farming has been shown to associate with increased expression of TLR7 and TLR8 genes in cord blood cells (88) and maternal contact with farm animals during pregnancy was associated with increased expression of TLR2 and TLR4 in school-aged children (89). Furthermore, differences in cord blood TLR levels have been linked to atopy, as elevated levels of TLR5 and TLR9 in cord blood leucocytes were reported to associate with a reduced risk of atopic eczema (90).

2.3.2.2 Th1 - Th2 paradigm

Th cells are an integral part of the adaptive immune system. Classically, two types of activated Th cells have been characterized according to their cytokine production; Th1-type cells are characterized by the production of IFN-γ and Th2-type cells are characterized by the production of IL-4, IL-5 and IL-13. Typically, a Th1-type response is associated with autoimmune diseases like T1D, while allergic diseases are associated with a Th2-type response.

This Th1/Th2 dichotomy, also characterized by mutual inhibition of Th1 and Th2 cells, has long been considered a cornerstone of adaptive immunity. Accordingly, atopic allergies have been seen to result from a Th1/Th2 imbalance favoring the Th2-type immune response. In other words, stimulation of the Th1-type response, *e.g.* by a viral infection, might lead to decreased Th2 response and diminished risk of atopic diseases. However, as Th cell subtypes and functions have been shown to be far more diverse and plastic than originally postulated, the rigid Th1/Th2 dichotomy is proving to be an oversimplification (91). This might also explain why many studies have failed to show an inverse association between autoimmune disorders (*i.e.* alleged Th1-type diseases), such as T1D, and atopy (92).

2.3.2.3 Regulatory T cells

The immune system needs to regulate itself in order to respond appropriately to harmful pathogens while tolerating harmless antigens. Regulatory T cells (Tregs) play a central role in this balance and maintain tolerance by numerous mechanisms, such as promoting tolerogenic dendritic cell (DC) phenotypes, suppressing Th cell activation as well as reducing the production of IgE and increasing the production of IgG4 in antigen-specific B cells (93). The classification of Tregs is not fully established but they can be classified into at least 5 subtypes based on the expression of transcription factor forkhead box P3 (FOXP3) (94).

Tregs act in various ways, including secretion of soluble factors, such as IL-10 and transforming growth factor (TGF)-β, and direct cell-to-cell contact. Abnormal Treg function has been described in atopic diseases even though the overall picture remains yet to be elucidated (93). For example, it has been shown *in vitro* that the presence of IL-10 reduced the effector function of allergen-specific DCs upon activation by the allergen, *i.e.* IL-10 inhibited Th2 proliferation and cytokine production (95). Tregs are also important players in the remodeling of immune tolerance during allergen immunotherapy, and allergen-induced FOXP3 expression has been shown to increase in peripheral blood mononuclear cells derived from children on immunotherapy (96).

2.3.2.4 Epigenetics

Epigenetic mechanisms control gene expression without altering DNA sequence. The increase in the prevalence of atopic diseases has been too rapid to be explained by changes in DNA sequence but epigenetic mechanisms, such as DNA methylation and histone modification, provide means by which environmental factors might modulate gene expression.

Epigenetic regulation has also been studied in atopic diseases (97). For instance, pet keeping and exposure to tobacco smoke during childhood affected the degree of CD14 gene methylation, and CD14 is an important activator of innate immune responses (98). It is possible that epigenetic regulation begins already *in utero*. For example, altered DNA methylation at mitogen-activated protein (MAP) kinase signaling-associated genes, an important pathway for Th cell function, was observed in cord blood of children developing IgE-mediated food allergy as compared to non-allergic children (99).

2.3.2.5 Microbiota

The human microbiota is composed of microbial communities residing on or within the human body, for example skin, gut and airways. Alterations in the microbiota have been suggested to influence the susceptibility to atopic disease, particularly in early life when the immune system is maturating (54). Low diversity of gut microbiota in infancy was associated with subsequent atopic eczema (100). Similarly, bacterial diversity of intestinal microbiota at the age of 12 months was inversely associated with the risk of atopic sensitization and allergic rhinitis by the age of 6 years (101). On the skin, commensal staphylococci were found to be less abundant during the first 6 months of life in children developing atopic eczema than in those with no eczema (102). In addition, a recent study showed that extracts from neonatal gut microbiota were able to modulate T cell function *in vitro* (103). In that study, atopy-related gut microbiota promoted Th2-type immune response, *i.e.* increased the production of IL-4 and reduced the relative abundance of Tregs (103).

Although traditionally thought to form during birth and breastfeeding, new observations suggest that the microbiota might start to form already *in utero*. Aagaard *et al* found a distinct, low-abundance but metabolically rich microbiota in placentas collected under sterile conditions (104). Their results also suggest that maternal infections might affect the placental microbiota, since maternal infections during the first half of pregnancy were associated with distinct shapes of placental microbiota (104). Further, it has been reported that placenta and amniotic fluid harbor similar microbiota that shares features with the newborn infant's first intestinal discharge (meconium) (105). In addition, meconium microbiota has been found to differ from that at all other neonatal anatomic sites at birth (106). The shared features of microbiota in the placenta, amniotic fluid and neonatal gut led the authors to speculate that the early neonatal gastrointestinal microbiota might result from microbial transfer at the feto-placental interface (105,106).

2.4 Enteroviruses and atopy

Numerous microbes, including many viruses, have been studied in relation to atopic diseases and data are immense. Among the *Enterovirus* genus, the role of RV infections, especially RV-induced wheezing, in the development of asthma has been studied abundantly, whereas the role of species A-D EVs is less studied. The next

chapters provide an overview of the current data on EVs and atopy concentrating on EV species A-D and RVs.

2.4.1 Enterovirus species A-D

Gastrointestinal tract is important in the induction of tolerance and it has been suggested that gastrointestinal pathogens might be especially important with regard to atopy. In adults, seropositivity against hepatitis A virus (HAV) was observed to be inversely associated with atopy (107). Subsequent studies reported the risk of atopy to reduce with a gradient of exposure to HAV, *Helicobacter pylori* (*H. pylori*) and *Toxoplasma gondii* (*T. gondii*) (108,109), whereas another study observed no association between seropositivity to HAV, *H. pylori* or *T. gondii* and atopy (110). Similarly, gastrointerstinal noro- and rotavirus infections were not associated with protection from atopy in a prospective study including young children (111). In addition, seropositivity to intestinal bacterial pathogens causing typically acute infections (*Clostridium difficile*, *Campylobacter jejuni*, *Yersinia enterocolitica*) was associated with a higher prevalence of atopy (109). Altogether, it seems that some gastrointestinal pathogens might be inversely associated with atopy but the data are inconclusive.

Consequently, also EVs might be interesting microbes with regard to the development of atopy; EVs can be transmitted orofecally and gastrointestinal tract is one of the primary replication sites of EVs. Seiskari *et al* observed in a cross-sectional study setting that EV seropositivity at school age was inversely associated with IgE sensitization in Russian Karelia but not in Finland (7). They speculated that the difference between the countries might be explained by the assumption that Russian children are infected at a younger age, or that EVs are transmitted through different routes, for instance respiratory transmission predominating in Finland (7). They also found EV types to differ in their relation with IgE sensitization; CV-Bs were not associated with atopy, whereas certain other EV types, especially E11, were (10). However, E30 was associated with an increased risk of IgE sensitization, and the authors discuss that this might result from E30 infecting older children than other Es (10). In another study, the prevalence of E30 IgG antibodies was found to be inversely associated with total IgE levels and severe childhood asthma exacerbations (11) but the children included were younger than in the study by Seiskari *et al.*

In addition to orofecal transmission, EVs can transmit through the respiratory route and EVs are commonly isolated from nasal samples in children with severe wheezing illness; EVs were detected in 12 - 20% of nasopharyngeal aspirates taken from children hospitalized for acute wheezing (112,113). Thereby, EVs appear to be one of the major pathogens causing acute wheezing in children but data about the relation between EV-induced wheezing and atopic disease are inconclusive. Regarding IgE sensitization, EV-induced wheezing requiring hospitalization was found not to associate with IgE sensitization in children, in contrast to RV-induced wheezing (114). For asthma, EV-induced wheezing was not associated with the development of asthma (112) or impaired lung function (115). On the contrary, in a large register-based study children with laboratory-confirmed symptomatic EV infections had a higher risk of asthma (116). In that study, most children had herpangina or hand-foot-mouth disease (HFMD) but also children with more severe forms of EV infection, such as meningitis, were included (116). Furthermore, another study from the same database concentrating only on EV-induced herpangina and HFMD showed that children with HFMD had a decreased risk of asthma, whereas children with herpangina had an increased risk of allergic rhinitis and atopic eczema (117). The authors speculated that HFMD, generally causing more severe symptoms than herpangina, might induce a more intense inflammatory cytokine production and thus decrease the risk of atopic disease (117).

In summary, both gastrointestinal and respiratory EVs are frequent pathogens in childhood but data about the associations between EVs and the development of atopic disease or sensitization are inconclusive and there might be differences related *e.g.* to EV type and the route of infection.

2.4.2 Rhinoviruses

RVs and respiratory syncytial viruses (RSVs) predominate as pathogens in childhood wheezing episodes (118). Therefore, research about a possible relation between RVs and atopic disease has focused mostly on asthma. Both RV-induced and RSV-induced wheezing have been linked to the subsequent development of asthma with RV showing an especially strong correlation in some studies (119). For example, 47% of children who had RV-induced wheezing illness during the first year of life had asthma at the age of 6 years in comparison with 24% of children with non-RV wheezing illness (119). Accordingly, in a recent meta-analysis RV-induced wheezing

during the first 3 years of life was found to associate with subsequent development of wheezing/asthma (12). As also discussed in the meta-analysis, most data about the relation of RV-induced wheezing and asthma are derived from cohorts known to be at high risk for developing asthma, *i.e.* children with atopic background or children with severe wheezing requiring hospitalization (12).

However, it is possible that asymptomatic and symptomatic RV infections differ in their relation with asthma. Asymptomatic RV positivity in nasal samples during the first year of life was not associated with subsequent development of asthma, whereas RV episodes associated with wheezing were (119). Accordingly, in another study, the presence of RVs in nose and throat swabs collected regardless of symptoms was not associated with wheezing at the age of 4 years but wheezing RV-episodes during the first year of life increased the risk of having wheezing at the age of 4 years (120).

Genetic factors contribute extensively to the development of asthma and there is data linking this genetic susceptibility and RV infections (49,121). CDHR 3 is a transmembrane protein expressed on the airway epithelium and polymorphism of the CDHR3 gene locus has been identified important in susceptibility for severe childhood asthma (121). Interestingly, CDHR3 also mediates RV-C binding and replication (49). These findings suggest that RV-C infections could contribute to the development of childhood asthma.

Several studies have shown that concomitant IgE sensitization further increases the risk of asthma in children with RV-induced wheezing (118,119). The results from a study aiming to define the temporal relationship between RV-induced wheezing and aeroallergen sensitization suggested that atopic sensitization preceded RV wheezing (122). In that study, an opposite relation was not true, *i.e.* RV-induced wheezing was not associated with subsequent aeroallergen sensitization (122).

To conclude, RV-induced wheezing has been shown to be a risk factor for the development of asthma but data about the association between non-wheezing RV exposure and subsequent IgE sensitization or atopic disease are scarce.

2.5 Prenatal factors and atopy in the offspring

A number of phenotypic differences are present already at birth between children who develop atopic diseases and those who do not. For instance, reduced suppressor functions of Tregs and increased TLR-mediated innate immune responses were

observed in cord blood of newborns developing allergies in later life (123,124). As these differences were present already in newborns, it has been suggested that the factors influencing the risk of atopy might begin to act already *in utero* (125).

2.5.1 Maternal environment

A wide range of environmental exposures during pregnancy has been associated with atopy in the offspring. Among others, maternal smoking has been shown to associate with asthma (126), and maternal adverse life events increased the likelihood of asthma and eczema in the offspring (127). On the other hand, maternal exposure to farm environment has been shown to reduce the risk of atopy (89,90,128). In cross-sectional studies, maternal contact with farm animals during pregnancy was inversely associated with IgE sensitization (89) and atopic disease (128) at school age. Later, in a prospective birth cohort, maternal contact with farm animals during pregnancy was inversely associated with atopic eczema in the offspring (90).

2.5.2 Maternal infections

In studies relying on questionnaires or medical records in determining infections, maternal febrile infections during pregnancy have been reported to associate with childhood eczema (129,130), rhinitis (129) and asthma (131-133) but this association has not been seen in all studies (134). Some studies also suggest that timing of the maternal infection might affect the risk, but the results are inconclusive, as both first trimester (129-131,133) and third trimester (132) have been suggested to be especially important.

Data based on laboratory-confirmed gestational infections are less abundant. For bacteria, a Finnish study reported that adolescents born to mothers with an intrauterine growth of bacteria at the time of cesarean section were at an increased risk of asthma but not of atopic sensitization (135). For helminth infections, a Ugandan study observed maternal hookworm infection to be inversely associated with eczema in the offspring (136). They also found maternal asymptomatic malaria infection to have an inverse association with childhood eczema (136). In contrast, a study by Cooper *et al* observed no association between gestational hookworm infection and childhood atopic disease in rural Ecuador (137). However, they did

observe an inverse association between maternal helminth infections and inhalation allergen sensitization (137). With respect to viruses, there is an Australian study reporting more asthma and eczema in one-year-old children born to mothers with laboratory-confirmed upper respiratory tract viral infections during pregnancy as compared to children whose mothers had common cold symptoms without laboratory-confirmed viral infection (138). However, the study did not include a control group of mothers without respiratory infections, which might reduce the generalizability of the results. Combining the existing data, a meta-analysis concluded that maternal infections are positively associated with both asthma and eczema in the offspring (139).

In summary, most data about the relation of maternal infections during pregnancy and atopic disease in the offspring are based on questionnaires and medical records. There are observations that gestational febrile infections might increase the risk of atopic disease, perhaps especially asthma, in the offspring. However, microbiological identification of the pathogen has been infrequent with only one previous study concentrating on virus infections.

3 AIMS OF THE STUDY

The principal aim of this thesis study was to evaluate the association between EV infections in early life and atopy. The detailed aims were:

1. To evaluate the association between EV infections during the first two years of life and atopic disease.

2. To study the association between gestational EV infections and atopic disease in the offspring.

3. To investigate the association between EVs, including RVs, detected in stool samples during the first year of life and IgE sensitization.

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 DIPP study (Reports I and II)

The Type 1 Diabetes Prediction and Prevention (DIPP) study is an ongoing prospective birth cohort study initiated in Finland in 1994 (140). In the DIPP study, children at moderate or high human leukocyte (HLA) conferred genetic risk for T1D are followed from birth until the onset of T1D or the age of 15 years. After informed parental consent, cord blood of infants born in the University Hospitals of Oulu, Tampere or Turku is screened for T1D-associated HLA-DQB1 alleles: DQB1*02, *0301, *0302 and *0602/3 (141). Infants carrying the high-risk genotype (DQB1*02/*0302) or the moderate-risk genotype (DQB1*0302/x, with x ≠ DQB1*02, *0301 or *0602) are invited to enter the study follow-up. During the follow-up, study subjects visit the study clinic and biological samples are collected every 3-6 months until the age of 2 years and thereafter every 6-12 months. During the follow-up children are screened for the presence of T1D-associated islet cell antibodies and, if applicable, for other T1D-related autoantibodies.

4.1.2 Diabimmune study (Report III)

The Diabimmune study is an international multicenter study that was carried out in three countries during the years 2008-2014. It included a birth cohort arm where T1D-related HLA-DR-DQ alleles were analyzed after informed parental consent from cord blood of infants born in Estonia, Finland and Russian Karelia. Children with high (DR3-DQ2/DR4-DQ8), moderate (DR4-DQ8/x, x=non-protective allele, not DR3-DQ2) or slightly increased (DR3-DQ2/y, y=non-protective allele, not DR4-DQ8) genetic risk for T1D were invited to enter the follow-up study lasting

for 3 years. Serum samples were drawn at 3, 6, 12, 18, 24 and 36 months and the families collected monthly stool samples.

4.1.3 Subjects in Report I

Report I was a nested case-control study within the DIPP study. For this study, the DIPP database was screened for children born in the Tampere University Hospital between 1.1.1998 - 31.12.2003 for a physicians' diagnosis of asthma, atopic eczema or allergic rhinitis. Children with missing serum samples during the first 2 years of follow-up, discontinuing the follow-up before the age of 5 years and children with T1D-associated autoantibodies or T1D were excluded. As a result, 183 eligible children were identified. The serum samples drawn from the children at the age of 5 years were screened for IgE antibodies against an aeroallergen mixture. Altogether 71 children were IgE positive and they comprised the case group. Next, 142 non-atopic control children, *i.e.* having neither a diagnosis of an atopic disease nor IgE sensitization at the age of 5 years, were selected. These control children were matched for age (median age difference 9 days, range 0-64 days), sex and HLA-DQB1 genotype. Demographics of the study population are presented in Table 2 in chapter 5.1.

4.1.4 Subjects in Report II

Report II was a nested case-control study within the DIPP study. In addition to the DIPP samples, serum samples drawn from the mothers of the participating DIPP children were analyzed. Maternal serum samples were drawn at the end of the first trimester of pregnancy as part of the national screening protocol of infectious diseases in prenatal clinics. In Report II, the DIPP database was searched for children fulfilling the following case child criteria: Diagnosis of asthma, atopic eczema and/or allergic rhinitis recorded in the DIPP database and a positive serum IgE level against an aeroallergen mixture at the age of 5 years. Children with T1D or T1D-associated autoantibodies were excluded. Only children with maternal first trimester serum samples and cord blood samples were included. Altogether, 202 children fulfilling these criteria were identified. These case children were born between June 1996 and September 2004 in the cities of Tampere (n=91) and Oulu (n=111).

Next, 1-2 non-atopic control children were selected for each case child (*n*=333). Case and control children were matched for sex, T1D-related HLA-DQB1 alleles, place of birth and time of birth (± 3 months). The median age difference between cases and controls was 43 days (range 0-91 days). The demographics of the study population are presented in Table 4 in chapter 5.2.

4.1.5 Subjects in Report III

Report III was a nested case-control study within the birth cohort arm of the Diabimmune study. The study cohort was selected among the 1139 children born in Finland (Espoo) and Estonia (Tartu) and carrying T1D-associated HLA-DR-DQ genotypes. Altogether 717 of these 1139 children participated in the Diabimmune birth cohort study and 563 continued in the study until the age of 3 years. Data about the IgE results from these 563 children were drawn from the Diabimmune database. Altogether, 244 children with at least one allergen-specific IgE level ≥ 0.35 kilounit per liter (kU/L) at the age of 18 and/or 36 months were identified. These 244 children comprised the case group. Then 244 non-sensitized, *i.e.* all specific IgE values < 0.35 kU/L at 6, 18 and 36 months, control children were selected. The control children were matched for the country of birth but otherwise they were selected randomly.

There were 246 boys, including 140 (57%) cases and 106 (43%) controls, and 242 girls, including 104 cases (43%) and 138 controls (57%). T1D-related HLA types were divided as follows: 25 (10%) cases and 20 (8%) controls had DR3-DQ2/DR4-DQ8 genotype, 98 (40%) cases and 110 (45%) controls had DR4-DQ8/x (x=not DR3-DQ2 or a haplotype associated with protection against T1D) genotype and 121 (50%) cases and 114 (47%) controls had DR3-DQ2/y (y=not DR4-DQ8 or a haplotype associated with protection against T1D) genotype. Case and control children were born between September 2008 and May 2010. Out of the 488 children included, 304 (62%) were born in Finland and 184 (38%) in Estonia. The demographics of the study population are presented for the whole cohort (Table 5) and after stratification by sex (Table 6) in chapter 5.3.

Case children were further categorized according to their IgE sensitization profiles. Based on their age at the time of sensitization, case children were divided into two groups: "early sensitized" (*n*=57) and "late sensitized" (*n*=162) (IgE analyses at 6 months were missing from 25 children). Early sensitized children had

at least one specific IgE value ≥ 0.35 kU/L already at 6 months of age, and late sensitized children were IgE negative at 6 months but turned IgE positive later during the follow-up. In addition, case children were classified according to allergens; children sensitized against at least one aeroallergen (cat, dog, dust mite, birch, timothy) comprised the group "aeroallergen sensitized" (*n*=104) and children sensitized against at least one food (egg, milk, wheat, peanut) comprised to group "dietary sensitized" (*n*=214). When applicable, children could belong to both of these groups.

4.2 Methods

4.2.1 Neutralizing virus antibody assay (Report I)

Serum samples taken at the age of 1 and 2 years were analyzed with a standard plaque reduction neutralization assay as described earlier (142). In the plaque neutralization assay, EV antibodies in the serum prevent the virus from infecting cells *in vitro* and this is detected by a reduction in cell death in cell culture. The 2-year samples were analyzed for the presence of NABs against 12 EV types: CV-B1, CV-B2, CV-B3, CV-B6, CV-A9, E3, E5, E9, E11, E21, E26, E30, 1-year samples were analyzed for E3, E5, E9, E11 and E30. Both American Type Culture Collection (ATCC) prototype virus strains (CV-B2, CV-B6, E9, E11, E30) and wild-type EV isolates from Finland (CV-B1, CV-B3, CV-A9, E3, E5, E21, E26) were used. A fourfold dilution of serum was applied and the serum was considered antibody positive when it reduced the number of virus-induced plaques by 80% or more. The analyses were carried out blind to the case-control status of the child.

4.2.2 EIA virus antibody assay (Report II)

In this study, the presence of IgG class antibodies against EV, influenza virus A (IAV), cytomegalovirus (CMV), *H.pylori* and *M. pneumoniae* was analyzed from cord blood and/or maternal first trimester serum samples. Antibodies were measured by EIA, except CMV antibodies, which were measured either by EIA or by chemiluminescent immunoassay (CLIA) due to changes in laboratory equipment

during the study period. EV, IAV and *M. pneumoniae* antibodies were analyzed from maternal first trimester samples and cord blood, *H. pylori* antibodies from cord blood and CMV antibodies from first trimester sample (n=146) or cord blood (n=381) according to availability. Case-control pairs as well as related first trimester and cord blood samples were analyzed parallel in the same test run. The samples were analyzed blind to clinical information.

Following the manufacturer's instructions, acute gestational *M. pneumoniae* infection was defined as a 1.6-fold or higher rise in antibody levels between first trimester serum sample and cord blood. For cohesion, the same criteria were applied for EV and IAV when detecting acute infections during pregnancy. The prevalence of gestational *H. pylori* and CMV infections in the Finnish population was estimated low and thereby the presence of *H. pylori* and CMV antibodies was analyzed only from a single serum sample. These microbes cause chronic or latent infections, which can be detected using a single sample.

For EV, a synthetic peptide antigen KEVPALTAVETGAT-C was applied. This antigen is derived from the immunodominant region (VP1) of the EV virus capsid and it is known to detect several EVs (143,144). For IAV, IAV strain Beijing (BA1231VS, Virion Serion, Germany) was employed (145). Commercial kits were used for *H. pylori* (Enzygnost® Anti-Helicobacter pylori, Siemens, Germany) and *M. pneumoniae* (Mycoplasma pneumoniae, Labsystems Diagnostics Ltd, Finland) according to the manufacturers' instructions. CMV antibodies were analyzed with Enzygnost® Anti-CMV EIA kit (Siemens, Germany) or with LIAISON® CMV IgG II CLIA kit (DiaSorin S.p.A, Italy) according to the manufacturer's instructions.

4.2.3 Detection of viruses by RT-PCR (Report III)

The presence of RV, EV, norovirus (NoV) and parechovirus (HPeV) RNA in stool samples was analyzed by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). Stool samples were collected monthly during the first year of life. Viral RNA was extracted from 10% stool suspension with Qiagen Viral RNA kit according to manufacturer's protocol (Qiagen, Germany). Viral RNA was reverse-transcribed and amplified using the QuantiTect Probe kit (Qiagen, Germany). RT-qPCR was performed according to the manufacturer's instructions with QuantiTect Probe kit using TaqMan® (Thermo Fisher Scientific, USA) chemistry labelled primers and probes. For RV, forward primer 5'-CYA* GCC

T*GC GTG GC -3' (A* and T* locked nucleic acid primer by Exiqon, Denmark); reverse primer GAA ACA CGG ACA CCC AAA GTA and probe VIC-TCC TCC GGC CCC TGA ATG YGG C –TAMRA were applied, as described previously (46). For EV, forward primer: CGG CCC CTG AAT GCG GCT AA, reverse primer: GAA ACA CGG ACA CCC AAA GTA, probe 1: FAM-TCT GTG GCG GAA CCG ACT A-TAMRA, probe 2: FAM-TCT GCA GCG GAA CCG ACT A-TAMRA were used (146). NoV and HPeV were detected with primers and probes as described (147). Every sample was analyzed in three parallel runs and the sample was interpreted positive if at least one of the parallel runs positive. The samples were analyzed blind to the case-control status of the child.

4.2.4 Measurement of IgE antibodies

In Reports I and II, IgE class antibodies against a mixture of aeroallergens (Phadiatop®, Phadia AB, Sweden) were measured by ImmunoCAP® enzyme immunoassay (Phadia AB, Sweden) from serum samples taken at 5 years of age. In Report III, specific IgE antibodies against cat, dust mite, birch, timothy, egg, milk and wheat were analyzed from serum samples taken at 6, 18 and 36 months, antibodies against peanut from samples taken at 18 and 36 months and antibodies against dog from 36-month sample by ImmunoCAP® (Phadia AB, Sweden). Analyses were carried out blind to clinical information and values $\geq$ 0.35 kU/L were considered positive.

4.2.5 HLA typing

In Reports I and II, T1D-associated HLA-DQB1 alleles were determined from cord blood as previously described (141). In Report III, HLA-DQ-DR analyses were performed as described (148).

4.2.6 Statistical methods

In Report I, the association between EV seropositivity and atopic outcome was determined by conditional logistic regression analysis. The number of seropositivity against different EV types was calculated and used to estimate how much each seropositivity affects the child's risk of having an atopic outcome. The results are presented as odds ratio (OR) and 95% confidence interval (CI) for the atopic outcome.

In Report II, conditional logistic regression analysis was applied to determine the association between an acute infection during pregnancy (EV, IAV, *M. pneumoniae*) or seropositivity (CMV, *H.pylori*) and the atopic outcome. The results are expressed as OR and 95% CI for the atopic outcome.

In the unmatched study population in Report III, logistic regression analysis was used to estimate ORs and 95% CIs for IgE sensitization. Each stool sample positive for viral RNA was included in the analyses as an independent positive value. Association between the number of virus-positive samples and the atopic outcome was evaluated by calculating the number of positive stool samples in each child during the 12-month follow-up (6-month follow-up in case categories "early sensitized" and "late sensitized", see chapter 4.1.5.) and estimating how much each virus-positive sample affects the child's risk to become IgE sensitized.

Data about possible confounding factors are presented in Table 2 (Report I) and Table 4 (Report II). In Reports I and II, the possible confounding effects of sex, age and T1D-related HLA-DQB1 were controlled by matching on study inclusion. For other possible confounders, the effect of each factor on the study outcome was estimated by logistic regression analysis (univariate analysis). When a statistically significant association was observed, logistic regression analysis was used to adjust for this confounder. In Report I, none of the possible confounding factors was statistically significantly associated with atopic outcome. In Report II, furry animals at home and having older siblings were associated with atopic outcome and the results were adjusted for these confounders. Possible confounding factors in Report III are presented in Tables 5 and 6, and the possible effect of each factor on the study outcome was estimated by logistic regression analysis (univariate analysis). Prevalence of paternal atopy differed statistically significantly in the whole cohort and in girls and logistic regression analysis was applied to adjust for this confounder.

In Report II, the Bonferroni correction was applied to counteract the problem of multiple comparisons. As the associations between gestational infections and atopic outcome were determined for three microbes (EV, IAV, *M. pneumoniae*), Bonferroni

correction by 3 was used. For seropositivity analyses (*H. pylori* and CMV), Bonferroni correction by 2 was applied. The corrected thresholds for statistical significance were $p < 0.017$ and $p < 0.025$, respectively. In Report III, four different viruses were analyzed and after applying Bonferroni correction, $p < 0.013$ was regarded as statistically significant. Unadjusted p values are presented in the text.

Analyses were performed using STATA 8.2. (Statacorp, USA) (Report I) and R version 3.3.3 (2017-03-06, The R Foundation for Statistical Computing, https://www.R-project.org) (Reports II and III).

4.2.7 Ethical aspects

The DIPP study protocol has been approved by the Ethical Committees of the participating University Hospitals of Oulu, Tampere and Turku. The Diabimmune study has been approved by the local Ethics Committees of the participating hospitals. The Finnish Maternity Cohort Steering Group at the National Institute for Health and Welfare (THL) approved the use of maternal first trimester serum samples in Report II. Written informed consent was obtained from parents of participating children.

5 RESULTS

5.1 Neutralizing enterovirus antibodies and atopic disease (Report I)

In Report I, the association between NABs against different EV types and the atopic outcome was investigated. The study comprised of 71 atopic case children and 142 non-atopic control children. The demographics of the study population and their associations with the study outcome are presented in Table 2. NABs were measured against 12 different EV types from the serum samples drawn at the age of 2 years.

EV infections were common in early life, as 61 (86%) case children and 119 (84%) control children had NABs against at least one EV type. Control children were seropositive against a higher number of different EV types than case children were and this accumulation of EV seropositivity was associated with protection from the atopic outcome (OR: 0.73; 95% CI: 0.56-0.96; p=0.025).

Based on previously commonly used classification, CVs and Es were analyzed as separate groups. The number of children seropositive against at least one E or CV type did not differ between case and control children (75% *vs* 78% and 35% *vs* 32%, respectively). However, control children had NABs against a higher number of Es, which was inversely associated with the atopic outcome (OR: 0.63; 95% CI: 0.46-0.88; p=0.006) (Figure 1). This association was not detected with CVs (OR: 1.06; 95% CI: 0.67-1.66; p=0.82). None of the individual EV types showed statistically significant associations with atopy. For Es, the results showed a similar trend when children with asthma (n=29) and atopic eczema/allergic rhinitis (n=42) were analyzed separately (OR: 0.64; 95% CI: 0.39-1.04; p=0.069 and OR: 0.63; 95% CI: 0.40-0.98; p=0.039, respectively), even though in asthmatic children the result was not statistically significant.

In addition, NABs against E3, E6, E9, E11 and E30 were measured from serum samples drawn at the age of 1 year. The seroprevalence of these Es at the age of 1 year and 2 years is shown in Figure 2. At this early age, 98 (69%) control children and 39 (55%) case children were seropositive against one or more E type, and this was inversely associated with atopic outcome (OR: 0.52; 95% CI: 0.28-0.96; p=0.038).

Table 2. Demographic characteristics of the study population (Report I).

	Case *n*=71 (%)	Control *n*=142 (%)	OR (95% CI)	*p* value
Gender (male) §	43 (61)	86 (61)		
HLA-DQB1 genotype §				
DQB1 *02/*0302	10 (14)	20 (14)		
DQB1 *0302/x ¤	61 (86)	122 (86)		
Atopic disease				
Asthma	29 (41)			
Atopic eczema	37 (52)			
Allergic rhinitis	5 (7)			
Older siblings (yes)	35 (51)	85 (61)	0.64 (0.36-1.15)	0.14
Smoking during pregnancy (yes)	5 (8)	9 (7)	1.06 (0.34-3.31)	0.92
Indoor pet at home (yes)	18 (26)	39 (28)	0.91 (0.47-1.75)	0.78
Maternal higher education #	39 (57)	64 (46)	1.45 (0.81-2.58)	0.21
Paternal higher education #	30 (45)	53 (41)	1.11 (0.62-1.96)	0.73
Entry into daycare † ¶	27 (15)	25 (16)	0.99 (0.96-1.01)	0.41

OR, odds ratio; CI, confidence interval; §, case and control children were matched for sex and HLA-DQB1 genotype; ¤, x refers to other alleles that *02, *0301 or *0602; #, higher secondary or tertiary education; †, child's age in months; ¶, values are means (standard deviation in parenthesis). Associations with the atopic outcome (diagnosis of asthma, atopic eczema and/or allergic rhinitis and aeroallergen IgE positivity) are presented as OR and 95% CI.

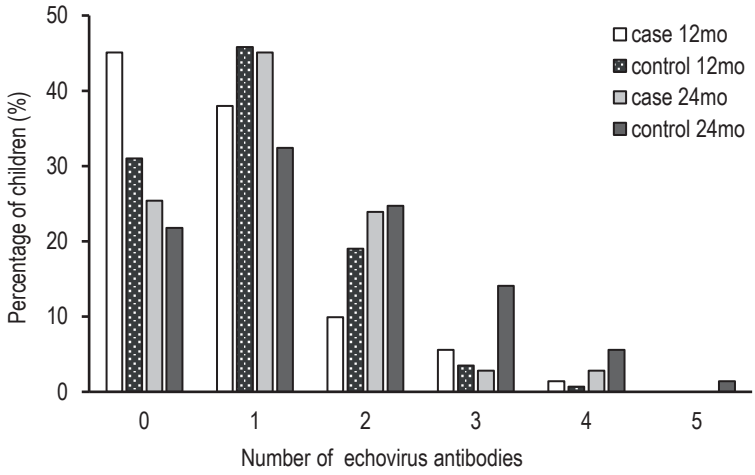

Figure 1. The percentage of children having neutralizing antibodies against 1-5 different echovirus types at the age of 12 and 24 months (Report I).

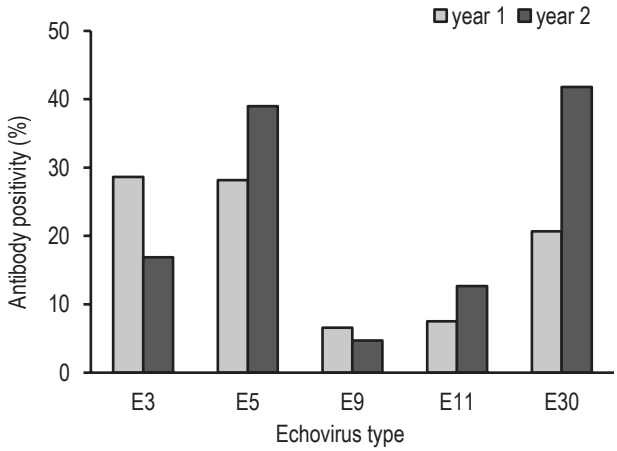

Figure 2. The percentage of children having neutralizing antibodies against echovirus 3 (E3), echovirus 5 (E5), echovirus 9 (E9), echovirus 11 (E11) and echovirus 30 (E30) at the age of 1 year and 2 years (Report I).

5.2 Gestational enterovirus infection and atopic disease in the offspring (Report II)

Report II addressed the possible associations between gestational infections and atopic disease in the offspring. An increase in IgG levels against EV, IAV or *M. pneumoniae* during pregnancy was interpreted as an acute infection during pregnancy. In addition, seropositivity against CMV and *H. pylori* was analyzed from either cord blood or first trimester serum sample taken from the mother.

The results indicated that out of the 5 microbes included, only gestational EV infections were statistically significantly associated with atopic outcome in the offspring. A gestational EV infection was detected in 17 (8%) mothers of the case children and in 53 (16%) mothers of the control children and it was inversely associated with atopic outcome in the offspring (OR: 0.43; 95% CI: 0.23-0.8; p=0.008) (Table 3). The result held true after adjusting for older siblings and furry pets, *i.e.* confounding factors found to associate with study outcome in univariate analyses (Table 4) (OR: 0.43; 95% CI: 0.23-0.81; p=0.009). Gestational IAV or *M. pneumoniae* infections or maternal seropositivity against CMV or *H. pylori* were not associated with atopic outcome in the offspring (Table 3).

Table 3. Prevalence (percentage in parenthesis) of maternal infections during pregnancy (enterovirus, *Mycoplasma pneumoniae*, influenza virus A) and cord blood seropositivity (*Helicobacter pylori*, cytomegalovirus) in Report II.

	Case *n*=202	Control *n*=333	OR (95% CI)	*p* value
Enterovirus	17 (8)	53 (16)	0.43 (0.23-0.80)	0.008
Mycoplasma pneumoniae	14 (7)	37 (11)	0.65 (0.34-1.22)	0.18
Influenza virus A	26 (13)	43 (13)	1.05 (0.62-1.79)	0.86
Helicobacter pylori	14 (7)	37 (11)	0.58 (0.30-1.12)	0.11
Cytomegalovirus §	148 (74)	243 (74)	0.99 (0.66-1.47)	0.95

CI, confidence interval; OR, odds ratio; § seropositivity in cord blood or first trimester serum sample. Associations with atopic outcome in the offspring are presented as OR and 95% CI.

Table 4. Demographic characteristics of the study population in Report II.

	Case *n*=202 (%)	Control *n*=333 (%)	OR (95% CI)	*p* value
Gender (male) §	128 (63)	207 (62)		
HLA-DQB1 genotype §				
DQB1 *02/*0302	18 (9)	30 (9)		
DQB1 *0302/x ¤	184 (91)	303 (91)		
Atopic disease				
Asthma	52 (26)			
Atopic eczema and/or rhinitis	126 (62)			
Both #	24 (12)			
Older siblings (yes)	95 (47)	201 (60)	0.60 (0.42-0.86)	0.006
Smoking in pregnancy (yes)	17 (9)	28 (8)	1.00 (0.53-1.89)	1.00
Furry pets (yes)	58 (29)	146 (44)	0.53 (0.36-0.78)	0.001
Maternal education				
No secondary	50 (27)	98 (31)	*ref*	
Lower secondary	91 (48)	151 (48)	1.01 (0.65-1.59)	0.96
Higher secondary	48 (25)	63 (20)	1.26 (0.76-2.11)	0.37
Paternal education				
No secondary	86 (46)	144 (48)	*ref*	
Lower secondary	46 (25)	88 (30)	0.92 (0.59-1.44)	0.71
Higher secondary	56 (30)	66 (22)	1.34 (0.85-2.11)	0.21
Duration of pregnancy † ¶	280 (224-300)	280 (209-300)	1.01 (0.99-1.02)	0.53
Birth weight † ~	3650 (1910-4830)	3610 (1660-5540)	1.00 (1.00-1.00)	0.27

OR, odds ratio; CI, confidence interval; §, case and control children were matched for sex and HLA-DQB1 genotype; ¤, x refers to other alleles that *02, *0301 or *0602; #, both asthma and atopic eczema and/or allergic rhinitis; †, values are medians (minimum and maximum); ¶, presented in days; ~, presented in grams. Associations with the atopic outcome (diagnosis of asthma, atopic eczema and/or allergic rhinitis and aeroallergen IgE positivity) are presented as OR and 95% CI.

5.3 Enteroviruses detected in stool and atopic sensitization (Report III)

In Report III, the possible association between EVs, and selected other viruses detected in stool, and IgE sensitization was investigated. Serial stool samples (*n*=4576) collected monthly from 244 children developing IgE sensitization and 244 children remaining IgE negative were screened for the presence of RV, EV, NoV and HPeV RNA. The demographics of the study population are shown for the whole study cohort (Table 5) and separately for both sexes (Table 6).

The results showed that RV was the most common virus detected with 921 (20%) of all samples positive for RV (Table 7). Altogether 371 (76%) children had at least one RV positive sample during the follow-up. The number of RV positive stool samples was inversely associated with IgE sensitization, but this phenomenon was observed only in boys. In other words, in the full cohort (both sexes) none of the viruses (RV, EV, NoV, HPeV) was statistically significantly associated with the atopic outcome. However, when the study cohort was stratified by sex, an inverse association between the number of RV positive samples and the atopic outcome was observed in boys; male controls had a significantly higher number of RV positive samples than male cases (Table 7). Sex did not influence the overall prevalence of RV infection, as the number of RV positive samples did not differ between boys and girls: 485 (21%) *vs* 436 (19%), respectively.

Table 5. Demographic characteristics of the study population in Report III.

	Case	Control		
	n=244 (%)	*n*=244 (%)	OR (95% CI)	*p* value
Maternal atopy (yes)	72 (30)	72 (30)	1 (0.68-1.48)	1
Paternal atopy (yes)	77 (32)	57 (23)	1.53 (1.02-2.29)	0.04
Parental atopy				
Neither	125 (52)	136 (56)	*ref*	
Either or both	117 (48)	108 (44)	1.18 (0.82-1.68)	0.37
Older siblings (yes)	156 (64)	168 (69)	0.8 (0.55-1.17)	0.25
Smoking inside (yes)	16 (7)	11 (5)	1.51 (0.69-3.34)	0.30
Place of Residence				
Urban	188 (79)	194 (81)	*ref*	
Rural	41 (17)	39 (16)	1.08 (0.67-1.76)	0.74
Both	9 (4)	8 (3)	1.16 (0.44-3.07)	0.76
Type of Housing				
Apartment	82 (34)	93 (38)	*ref*	
House or row house	118 (49)	127 (53)	1.05 (0.71-1.55)	0.79
Farm	16 (7)	8 (3)	2.27 (0.92-5.57)	0.07
More than one type	25 (10)	14 (6)	2.03 (0.99-4.15)	0.05
Pets at home (yes)	106 (44)	98 (41)	1.14 (0.79-1.63)	0.49
Entry in daycare § ¤	612 (463-799)	601 (441-836)	1	0.97
Cessation of breastfeeding § ¤	313 (154-392)	310 (148-382)	1	0.47
Vaginal delivery (yes)	224 (92)	222 (91)	0.9 (0.48-1.7)	0.75
Duration of pregnancy § #	281 (274-286)	282 (274-287)	0.99 (0.97-1)	0.10
Birth weight § †	3610 (3228-3910)	3570 (3295-3884)	1	0.82

OR, odds ratio; CI, confidence interval; §, values are medians (interquartile range); ¤, age of the child in days; #, presented in days; †, presented in grams. Associations with IgE sensitization are presented as OR and 95% CI.

Table 6. Demographic characteristics of boys and girls separately (Report III)

	Boys		Girls	
	Case	Control	Case	Control
	n=140 (%)	*n*=106 (%)	*n*=104 (%)	*n*=138 (%)
HLA risk class				
DR3-DQ2/DR4-DQ8	14 (10)	9 (9)	11 (11)	11 (8)
DR4-DQ8/x**	55 (39)	49 (46)	43 (41)	61 (44)
DR3-DQ2/y**	71 (51)	48 (45)	50 (48)	66 (48)
Maternal atopy (yes)	42 (30)	30 (28)	30 (29)	42 (30)
Paternal atopy (yes) §	41 (30)	26 (25)	36 (35)	31 (23)
Parental atopy				
Neither	72 (52)	60 (57)	53 (52)	76 (55)
Either or both	67 (48)	46 (43)	50 (49)	62 (45)
Older siblings (yes)	89 (64)	73 (69)	67 (64)	95 (69)
Smoking inside (yes)	9 (7)	5 (5)	7 (7)	6 (4)
Place of Residence				
Urban	112 (82)	78 (74)	76 (75)	116 (85)
Rural	19 (14)	22 (21)	22 (22)	17 (13)
Both	5 (4)	5 (5)	4 (4)	3 (2)
Type of Housing				
Farm	7 (5)	3 (3)	9 (9)	5 (4)
House or row house	70 (50)	55 (52)	48 (47)	72 (53)
Apartment	48 (35)	41 (39)	34 (33)	52 (38)
More than one type	14 (10)	7 (7)	11 (11)	7 (5)
Pets at home (yes)	61 (44)	39 (38)	45 (44)	59 (43)
Entry in daycare ¤ #	606 (458-814)	583 (423-828)	621 (475-783)	616 (449-841)
Cessation of breastfeeding ¤ #	318 (154-398)	309 (147-370)	301 (154-379)	312 (150-390)
Vaginal delivery (yes)	128 (91)	96 (91)	96 (92)	126 (91)
Duration of pregnancy ¤ †	280 (272-286)	282 (274-286)	282 (275-286)	282 (275-288)
Birth weight ¤ ¶	3680 (3296-3966)	3655 (3341-3979)	3470 (3194-3788)	3512 (3265-3828)

x** not DR3-DQ2 or a haplotype associated with protection against type 1 diabetes (T1D), y** not DR4-DQ8 or a haplotype associated with protection against T1D, § in girls: odds ratio for IgE sensitization: 1.85 (95% confidence interval: 1.05-3.28) *p*=0.03. ¤ values are medians (interquartile range), # age of the child in days, † presented in days, ¶ presented in grams.

Table 7. Number of virus positive stool samples in boys and girls (Report III).

	Case	Control	OR (95% CI)	*p* value
Rhinovirus total	429 (19)	492 (21)	0.91 (0.82-1.01)	0.09
Rhinovirus in boys	239 (19)	246 (24)	0.81 (0.69-0.94)	0.006
Rhinovirus in girls	190 (19)	246 (19)	1.02 (0.87-1.19)	0.83
Enterovirus total	103 (5)	100 (4)	1.01 (0.80-1.20)	0.89
Enterovirus in boys	52 (4)	42 (4)	0.97 (0.70-1.30)	0.83
Enterovirus in girls	51 (5)	58 (5)	1.07 (0.80-1.40)	0.60
Norovirus total	111 (5)	132 (6)	0.90 (0.70-1.10)	0.29
Norovirus in boys	58 (5)	59 (6)	0.80 (0.60-1.10)	0.19
Norovirus in girls	53 (5)	73 (6)	1.00 (0.70-1.30)	0.88
Parechovirus total	92 (4)	102 (4)	0.90 (0.70-1.20)	0.55
Parechovirus in boys	56 (4)	51 (5)	0.90 (0.60-1.21)	0.43
Parechovirus in girls	36 (4)	51 (4)	1.00 (0.70-1.37)	0.80

OR, odds ratio; CI, confidence interval. Altogether 4576 stool samples were analyzed, 2258 from cases and 2318 from controls. The number of virus-positive samples is shown (percentage in parenthesis). The association between the number of virus-positive samples and IgE sensitization is presented as OR and 95% CI.

The observed association between RV and atopy was also analyzed in different sensitization groups (see chapter 4.1.5.). The sex-dependent effect was observed in all of these 4 groups (Figure 3). Importantly, the effect was also observed when analyzing the association between the number of RV positive samples during the first 6 months of life and IgE sensitization developing later, after the age of 6 months ("late sensitized"): OR: 0.79; 95% CI: 0.60-0.94; p=0.016.

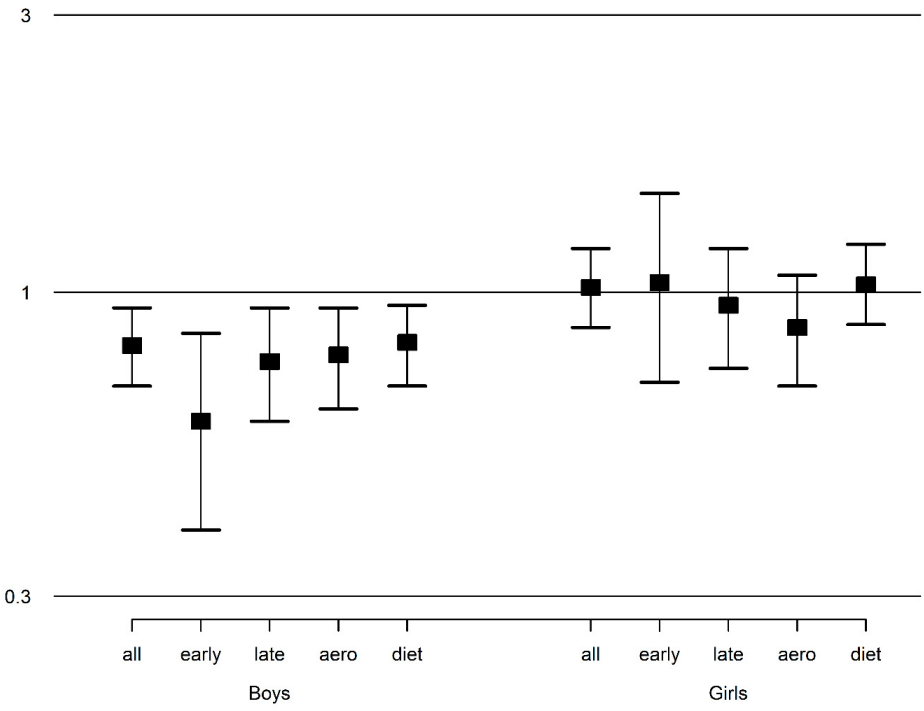

Figure 3. The association between the number of rhinovirus positive samples and IgE sensitization in groups with different IgE sensitization profiles (Report III). The results are shown as odds ratios and 95% confidence intervals. All, case children sensitized at 18 and/or 36 months; early, case children sensitized at 6, 18 and 36 months; late, case children sensitized at 18 and/or 36 months but not at 6 months; aero, case children sensitized against aeroallergens; diet, case children sensitized against dietary allergens.

6 DISCUSSION

All three reports in the current study provide new information about the relation between EVs and atopy in children. The results suggest that repeated exposure to different EV types as well as to RVs during early childhood is inversely associated with atopy. The study also suggests that EV infections may be important already *in utero*, as maternal EV infections during pregnancy were inversely associated with atopic disease in the offspring. As a whole, these results add new insight to the current knowledge about the role of EVs in the development of atopy.

6.1 Enterovirus infections in early childhood and atopy

The present study shows that cumulative exposure to different species A-D EV types during the first 2 years of life is inversely associated with atopic diseases. More specifically, this inverse association was related to cumulative seropositivity against different Es, whereas no association was observed with CVs.

Previous research has linked EVs to the pathogenesis of T1D (38), another disease with an immunological background and a rapid increase in incidence over the recent decades. In addition, species A-D EVs replicate in the intestinal mucosa, and gut-associated immune system has been considered an important immunological compartment in the regulation of immune responses and induction of tolerance. These factors contributed to the current hypothesis that EVs might play a role in the development of atopy. Yet, existing data about EVs and atopy are scarce (7,10,11) and prospective studies focusing on EVs and atopy have been missing.

In the present study, the inverse association between EVs and atopic illnesses varied according to the type of EV in question, which is in line with previous findings of our group. Seiskari *et al* detected that cumulative seropositivity against several non-CV-B types was inversely associated with IgE sensitization in Russian Karelia (10). They also detected E11 seroprevalence to be more frequent in non-sensitized children than in IgE sensitized children (10). In the current study, the overall cumulative exposure to different Es was inversely associated with atopic disease but

no statistically significant associations between individual EV types and atopic disease were detected. This could be due to lack of statistical power as the EV seroprevalence in Finnish children of the present study was lower than that in Russian Karelian children studied by Seiskari *et al*. In addition, children in the study by Seiskari *et al* were older and thereby had likely encountered more EV infections than the younger children included in the present study. In the current study, the atopic endpoint was defined as having a physician diagnosed atopic disease in addition to IgE sensitization. This strict endpoint enabled focusing only on clinically relevant atopy but might have also led to missing some interesting associations. Finally, children in the present study lived in Finland where the overall standard of hygiene is better and microbial exposure lower than in Russian Karelia, which might have influenced the timing of EV infections. This comparison demonstrates that studying multifactorial diseases, such as atopic diseases, is complex and the results inevitably reflect features of the study population, outcome definition, laboratory method applied *etc.*

However, similar to the results by Seiskari *et al*, this study showed that the protective association with atopy was especially related to Es and not to CVs (10). This finding has been further supported by Iwasaki *et al*, who reported the prevalence of E30 antibodies to be significantly lower in children with severe asthma than in non-asthmatic (11). It is known, that EVs differ from each other in their biological properties, such as tissue tropism, as well as their clinical manifestations. For instance, CV-Bs have been suggested to be especially important in myocarditis and pancreatic infections including a possible link to T1D (38), whereas EV-A71 and CV-A16 are important pathogens in HFMD (36). It is also possible that Es and CVs differ in terms of *e.g.* cell entry mechanisms, target cells, evasion from the host immune system or prevalence in the infant's environment. In fact, a recent study identified neonatal Fc receptor as a pan-echovirus receptor that did not bind CVs or PVs (149). This Fc receptor is expressed on the placenta, but also throughout life *e.g.* in the small intestine, hepatocytes and endothelium of the blood-brain barrier (149). The protective effect associated with some EV types might also be related to possible immunomodulatory effects of these viruses. Es have been shown to infect DCs *in vitro*, unlike CV-Bs (150). In another study, different CV-B1 strains varied considerably in their interaction with plasmacytoid DCs and in the immune activation profile they induced (151). However, currently the biological basis of the observed difference between Es and CVs in relation to atopy remains speculative.

6.2 Gestational enterovirus infection and atopy

In the present study, a novel finding of maternal EV infection during pregnancy associating with a reduced risk of atopic disease in the offspring was observed. There are only a few previous studies evaluating the possible role of gestational infections in the development of atopic disease by analyzing laboratory-confirmed infections and these few studies have focused mainly on bacterial or helminth infections (135-137,139). Laboratory-confirmed viral infections during pregnancy have been analyzed only in one prior study (138). In that study, samples from asthmatic mothers with respiratory infectious symptoms were analyzed by PCR against a panel of common respiratory viruses. The authors report that the risk of asthma and eczema at the age of 1 year was increased in children born to mothers with PCR-confirmed viral infection when compared to mothers with common cold symptoms without laboratory-confirmed infection (138). EVs were also included in the microbial panel but they were detected in only 3 of the 42 samples taken (138). Therefore, reliable conclusions about the role of EVs in the development of atopy in the offspring cannot be drawn from that study.

In contrast to the current study, most previous studies have relied on questionnaires or medical records in defining infections. This approach does not allow the distinction of possible microbe-specific differences, and questionnaire-based studies are also susceptible to recall bias. In addition, only symptomatic infections are recorded and thereby a major part of microbial exposures might go unrecognized. This is particularly true for EVs since the majority of EV infections are subclinical. Serological assays, such as the ones applied in this study, capture both symptomatic and silent infections and are thus more sensitive tools in evaluating microbial exposure. On the other hand, with no information about maternal infectious symptoms, the current study was unable to address possible differences between symptomatic and asymptomatic infections and their relation with atopy.

In addition to EVs, selected other microbes, *i.e.* IAV, *M. pneumoniae*, CMV and *H. pylori*, were also included in the microbial panel in the present study. However, gestational EV infection was the only one with a statistically significant association with atopy in the offspring. One explanation for this difference might lie in the route of infection. EVs are transmitted mainly orofecally and gastrointestinal pathogens have been reported to inversely relate with atopy (108,109), but the results are not uniform (110,111) leaving the issue unresolved. Further, *H. pylori* is also a gastrointestinal microbe and postnatal *H. pylori* infection has been shown to inversely associate with atopy in some studies (152). However, in the current study no

association between maternal *H. pylori* infection and atopy in the offspring was observed. This could partly be due to the low number of *H. pylori* positive mothers, which might have resulted in insufficient statistical power to detect subtle associations. Nevertheless, the results suggest that microbes differ in their effect on the development of atopy and EVs appear to be especially interesting in this respect. The possibly special nature of gestational EV infections is further supported by the findings of a recent meta-analysis reporting EV infections during pregnancy to increase the risk of T1D in the offspring (153).

The mechanisms of how gestational EV infections might influence the offspring's susceptibility to atopic disease are not known. In general, maternal viral infections during pregnancy can affect the fetus in several ways. Several viruses can cross the placental barrier and infection of the fetus can have severe consequences (154). Maternal EV infection during the first trimester has been associated with early fetal loss and infection in late pregnancy has resulted in clinical EV disease in the newborn infant (155). EVs are also capable of infecting the placenta, as shown by Satosar *et al* who detected EVs in a marked proportion of placentas obtained from pregnancies with severe neonatal morbidity (156). Thus, also in the current study an EV infection in the placenta-fetal unit is possible. However, in such case the infections did not cause severe fetal manifestations as neither the duration of pregnancy nor the birth weight of the newborn infant differed between pregnancies with and without maternal EV infection (data not shown).

In addition to infecting the fetus or the placenta, microbes could also affect the fetus through a local inflammatory response in the placenta or activation of maternal systemic immune responses. These immunological effects could modify the fetus' cytokine balance and influence the maturation of the immune system. As an illustration, there are data suggesting that prenatal environmental factors might affect the offspring's risk of atopy through changes in innate immunity. Maternal exposure to farming has been shown to increase gene expression of certain TLRs in cord blood cells (88), and increased expression of TLR genes at birth has in turn been associated with a reduced risk of atopic eczema in the offspring (90). Additionally, different microbes are known to activate different types of TLRs (84).

Recent observations about the neonatal microbiota have opened an intriguing new area in atopy research. Although speculatively, one might hypothesize that also gestational infections could have an impact on the microbiota of the newborn. It is becoming increasingly evident that the intrauterine environment is not sterile, as bacteria have been detected from placenta, amniotic fluid and the newborn infant's first intestinal discharge (meconium) (104-106,157). Fetuses continuously swallow

amniotic fluid, and meconium has been shown to harbor a microbial composition similar to amniotic fluid and placenta (105) and thus, the meconium microbiota has been suggested to reflect the *in utero* environment (106). Maternal bacterial infections during the first trimester have been shown to associate with distinct shapes of placental microbiome that might further influence the microbial composition of meconium (104). Most data have focused on bacterial microbiota but there are some emerging data about human enteric virome and its role in host health and disease (158). EVs might be potent pathogens in contributing to the enteric virome, as they often replicate in the gut for a relatively long time after an infection. Currently it seems that viruses constitute only a minority of the human gastrointestinal microbiota but there is some data indicating that intestinal viruses and bacteria might interact with each other (159,160). For instance, results from mouse studies suggest that optimal infectivity of poliovirus, a member of the EV C species, depends on the presence of concomitant bacteria in the gut (161).

6.3 Rhinovirus exposure and atopic sensitization

The results from this study suggest a sex-based difference in the effects of early RV exposure; RV positive stool samples during the first year of life were found to inversely associate with IgE sensitization in boys. In this study, RVs were analyzed from stools, which is an unconventional method in measuring RV exposure in allergy research. The results offer novel insights into the role of RVs in the development of atopy.

In addition to RVs, selected other viruses were included in the analysis, *i.e.* species A-D EVs, NoVs and HPeVs, but they were not associated with atopic sensitization. Reports I and II showed an inverse association between species A-D EVs and atopic disease, which was not observed in the present study (Report III). This is most likely due to methodologic differences. RT-qPCR is a direct virus detection method and due to the transient nature of EV RNA in feces, the results are highly dependent on the timing of sampling. In contrast, serology is less dependent on timing, as virus antibodies are detectable in the serum for months or years after the infection. Additionally, the RT-qPCR test applied did not enable distinction between Es and CVs, which might have had an impact on the results as suggested by Report I and previous studies (10,11). For NoVs, the current study reinforces previous results

where no association between NoV seropositivity and IgE sensitization was detected (111).

Although RVs have not been analyzed from stool samples in allergy studies previously, there are prior data about RV detection in stool. Harvala *et al* were able to detect all three RV species in stool, and they also observed that RV positive samples were most frequent in children under 2 years of age (162). Species C RVs have also been detected in stool in children hospitalized for gastroenteritis, also in the absence of respiratory symptoms (163). Honkanen *et al* found RVs to be frequent in stool samples collected prospectively from young children by applying the same RT-qPCR method as in the current study (164).

In the present study, RVs were frequent in stool samples during the first year of life with 20% of samples positive for RV RNA. Honkanen *et al* detected a slightly lower prevalence with 14% of stool samples positive for RVs (164, oral communication). However, in that study fecal sample collection started at the age of 3 months, whereas in this study collection started already at the age of 1 month. In the present study, RV positive samples were most frequent during the first 6 months of life and this might partly confer to the slightly higher prevalence observed. RVs have been shown to be abundant in stool in children also previously, as RVs were detected in 35% of fecal samples taken during acute infections (165).

The reasons for the high frequency of RVs in stool are not known. RVs are respiratory viruses replicating in the nasal and nasopharyngeal epithelia and they are commonly detected in nasal samples during upper respiratory tract infections (42,166). RVs are generally considered not to infect the gastrointestinal tract, as they degrade in the low pH of the stomach due to their acid sensitive capsid (167). In addition, RVs grow best on cell cultures at temperatures of 33-35ºC, equaling that of the intranasal temperature. However, the acid sensitivity of RVs might not be particularly stable, as only a single amino acid change in the RV capsid protein has been shown to be sufficient to confer resistance to inactivation in low pH (168). Furthermore, the pH of the stomach in young infants is higher than in older children or adults (169), which might enable the preservation of RVs in the gastrointestinal tract in this age group and explain the high frequency of RV positive samples among the youngest subjects in the present study. There is also an *in vitro* study showing that, unlike previously thought, the replication of some RVs was only minimally decreased at 37ºC as compared to replication at 33ºC (51). Taken together, detection of RVs in stool can reflect either a passive drift of RVs from the respiratory tract to the gut without marked degradation in the stomach, or active replication of RVs in the gastrointestinal tract, or perhaps both. Nevertheless, gastrointestinal tract

replication of RVs has not been proved and the passive drift phenomenon seems at present a more plausible explanation for detecting RVs in stool.

In the current study, the inverse association between RV positive samples and IgE sensitization was detected only in boys. This is interesting since sex can affect susceptibility to a wide range of infections and immune-mediated diseases. In general, the severity and prevalence of many infections, including viral infections, is higher in males (170,171). For instance, boys are more susceptible to severe forms of EV infections, such as myocarditis (172). In a study by Uekert *et al*, boys were shown to have more moderate-to-severe RV illnesses during early childhood, as well as higher IFN-γ responses (173). They also observed boys to be more often IgE sensitized, which is in line with results from other studies (174). These data do not provide direct explanations to the sex-based difference in the present study but rather suggest that sex might have a diverse effect on immune functions. For instance, the effect of parental atopy on the offspring's risk of atopy was shown to depend on the sex of the child; maternal atopic history increased the risk of atopic diseases in girls and paternal history in boys, whereas the effect of the parent from the opposite sex was insignificant (175). Another study reported that dog exposure during the first year of life was inversely associated with IgE sensitization against dogs only in males (176). There are also indications that sex-based differences might be present already at birth, since boys were shown to have lower Treg numbers as well as TLR1 and TLR2 gene expression in cord blood than girls (88,177). In conclusion, the biological mechanism behind the sex-based effect observed in the present study remain to be elucidated but this finding together with previous results suggests that sex might be one important factor in the pathogenesis of atopic diseases.

6.4 Enteroviruses and atopy – protection or predisposition?

The data from the current study suggests that species A-D EV infections in early childhood and during pregnancy are inversely associated with atopic disease, which supports their role as possible protective factors. This is in line with the concept of hygiene hypothesis. However, it is possible that the effect is not limited to these specific pathogens but instead a broader insight should be adapted when interpreting the results. For example, in the present study, one possibility is that EV infections might in fact be a surrogate marker for some other known or unknown

environmental factor, either predisposing to EV infections or occurring concomitantly.

For RVs, abundant data show that RV-induced wheezing predisposes to the development of asthma (178). However, most previous research has focused on RV-induced wheezing and asthma, and data about RVs and other atopic diseases or IgE sensitization *per se* are scarce. Jartti *et al* observed in a cross-sectional setting an association between wheezing RV illness and IgE sensitization (114) but later, a prospective study reported that RV-induced wheezing did not predispose to aeroallergen sensitization (122). Th2-type cytokines IL-4 and IL-13 have been shown to impair the innate immune response to RVs suggesting that children with atopic diseases might have altered RV immunity (179). In addition, polymorphism in the CDHR3 locus might predispose some children to more severe RV-C infections and asthma (49,121). The complex interplay between IgE sensitization and RV infection is also illustrated in a study where IgE-mediated reactions were blocked by omalizumab (an anti-IgE antibody), which resulted in fewer and milder respiratory RV infections (180). Taken together, it has been demonstrated that IgE sensitization, and wheezing RV infections predispose to childhood asthma both independently and synergistically, but the nature of the interaction between IgE sensitization and RV infection remains less known.

RV-induced wheezing represents just one form of RV infection and some data suggest that asymptomatic or mild RV infections might differ from severe ones in relation to atopy (119). Jackson *et al* reported that severe wheezing RV infections increased the risk of asthma but no association between asthma and RVs detected from nasal samples during prescheduled visits was detected (119). However, they included data only from a few clinic visits per child, whereas in the current study monthly analysis of stool samples was performed. In the light of previous and present data, it could be hypothesized that asymptomatic or mild RV infections might serve a beneficial function in driving postnatal immune maturation, thereby reducing the risk of allergic sensitization, whereas severe RV infections resulting in wheezing might increase the risk of asthma.

6.5 Genetic aspects

The study subjects in the present study were selected among the participants of ongoing studies with a primary focus on T1D. Therefore, the cohorts in the current

studies comprise of children with T1D-associated HLA genotypes, which could have influenced the results. In fact, some studies have linked HLA-DQ region with asthma and HLA-DRB1 region with IgE sensitization (181,182). However, as the genetic background of atopy is highly polymorphic and it has been estimated that HLA-DRB1 locus only accounts for 2-3% of the variation in specific IgE titers (181), it is unlikely that the HLA selection would have substantially influenced our results. Furthermore, the case and control groups were matched for the T1D-associated HLA genotypes (Reports I and II).

6.6 Methodological aspects

In this study, EVs were analyzed by two different serological methods; detection of NABs in Report I and detection of IgG class antibodies by EIA in Report II. NABs are long lasting and serotype-specific, which makes analyzing NABs especially useful in seroprevalence studies on a population level. In Report II, acute maternal EV infections were diagnosed by an increase in IgG antibody levels between maternal first trimester serum sample and cord blood. The EV antigen applied has been shown to detect antibodies against a wide range of EV types (143,144). The interval between paired serum samples was longer than usually applied in clinical diagnostics but since IgG responses usually last for several month or years, it is probable that the current method was able to detect infections reliably.

RVs were analyzed from stool samples by RT-qPCR. As with all PCRs, avoiding contamination is crucial for the reliability of results. In the present study, contamination of the samples is unlikely since virus negative control samples (sterile water) were included in every PCR run and they remained negative. The qPCR method applied is highly sensitive, which was also confirmed in the blinded test runs of external quality control samples provided by Quality Control for Molecular Diagnostics (QCMD). As some RV PCR methods have been shown to detect also EVs (162), the possibility of false positive reactions due to cross-detection of EVs by RV PCR must also be considered. However, in a study comparing the performance of different PCR primers in the detection of RVs, the primers used in the PCR analysis in Report III did not amplify any EVs included (183). In Report III, the specificity of the PCR reaction was further increased by applying a RV specific probe. In addition, the QCMD blind tests showed that the RT-qPCR

method used in the present study, did not amplify EV-D68, one of the closest phylogenetical relatives of RV.

6.7 Limitations of the study

Even though the prospective study setting enabled the identification of temporal relations between viral exposure and atopic outcome, interpretation of a possible causal relationship should be made with caution. This is true especially in Report I, where data about the exact age of the child at the time of IgE sensitization or diagnosis of the atopic disease were not available and therefore the time order of the events could not be addressed. In other words, some children might have developed atopy already before EV infections. In addition, the current study did not address the biological mechanisms behind the associations observed, which further hinders from drawing strong conclusions about causality.

The background information was limited to the data collected within the routine DIPP and Diabimmune study protocols. Thereby some potentially interesting and relevant information might have been missed. Especially in Reports I and II, data about the parental history of atopy would have allowed studying the associations between EVs and atopy with respect to different genetic backgrounds. In addition, the lack of nasal samples in Report III prevented the possibly interesting comparison between RVs in simultaneously collected stool and nasal samples.

The study subjects were selected for T1D-related HLA genotypes, which could have influenced the generalizability of the results. Other possible limitations concerning the HLA selection in this study are discussed in chapter 6.5. "Genetic aspects". In addition, there might be inherent weaknesses in laboratory methods applied and even though the laboratory methods were carefully optimized and controlled, this possibility cannot be completely excluded. The limitations of the methods are discussed further in chapter 6.6. "Methodological aspects".

7 CONCLUSION AND FUTURE PROSPECTS

The main aim of the present study was to evaluate the possible relation between EV infections and atopy. This was addressed by studying different aspects of species A-D EV and RV infections in early life. The study utilized serum and stool samples collected in two different prospective birth cohorts and EVs were tested by serology (neutralization assay or EIA) or by detecting viral RNA (RT-qPCR).

Taken together, this study showed that EV infections in early life are inversely associated with atopy. More specifically, this was observed when studying cumulative seropositivity against different EV types in early childhood and maternal EV infections during pregnancy, both of which were inversely associated with atopic disease. In addition, the number of RV positive stool samples during infancy was inversely associated with subsequent IgE sensitization in boys. Thereby, the results from this study support the hygiene hypothesis and reinforce it with new data about some of the less-studied pathogens. As the prevalence of atopic diseases is high and continues to increase in many parts of the world, providing new data that might eventually contribute to the prevention of these common illnesses is of value.

It seems clear that atopic diseases develop as a result from a complex interplay between cumulative environmental factors and genetic susceptibility. Studying such multifactorial diseases is challenging since it is important to both adequately control for the known confounders and to discover new relevant connections. Prospective follow-up studies enabling the identification of causal relationships are ideal but demand a considerable amount of resources. However, it is of equal value to gain new data about the biological mechanisms behind the associations as it enhances the more in-depth understanding of the pathogenesis of atopic diseases. In addition, emerging new data about the role of microbiota, both individual and environmental, special characteristics of the *in utero* period, pheno- and genotyping of atopic diseases and uncovering gene-environment interactions *etc*, offer numerous new areas for future research.

The results from this study provide further support to the earlier observations that the nature of early microbial exposure is important and EVs continue to be interesting pathogens in the development of atopy. In the future, it would be of value to combine data about EV infection during prenatal and early postnatal period and

study the combined effect in more detail. The results from this and previous studies also emphasize the possible serotype-specific differences of EVs, and possibly also RVs, and addressing this question further would be of interest. Especially with regard to RVs, analyzing prospectively collected nasal and stool samples, and ideally genotyping the viruses, would provide important new data. Further, novel molecular methods, such as next-generation sequencing, could be applied to try to identify features of the microbiota that might, independently or together with EV infections, confer to the development of atopy. In the future, a more detailed knowledge about the role of viruses and other microbes in atopic diseases might enable the development of interventions conferring the immunomodulatory effects of these microbes, ideally resulting in prevention of these common diseases.

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9 PUBLICATIONS

PUBLICATION I

Enterovirus infections in early childhood and the risk of atopic disease – a nested case-control study

Korhonen L, Kondrashova A, Tauriainen S, Haapala AM, Huhtala H, Ilonen J, Simell O, Knip M, Lönnrot M, Hyöty H.

Clin Exp Allergy 2013;43:625-32

doi: 10.1111/cea.12068 *Clinical & Experimental Allergy*, **43**, 625–632

ORIGINAL ARTICLE **Clinical Mechanisms in Allergic Disease**

Enterovirus infections in early childhood and the risk of atopic disease – a nested case-control study

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Clinical & Experimental Allergy

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Cite this as: L. Korhonen, A. Kondrashova, S. Tauriainen, A. M. Haapala, H. Huhtala, J. Ilonen, O. Simell, M. Knip, M. Lönnrot and H. Hyöty, *Clinical & Experimental Allergy*, 2013 (43) 625–632.

Summary

Background Enterovirus infections in childhood have been associated with a reduced risk of atopy in cross-sectional studies.

Objective To study the relation between enterovirus infections in the first 2 years of life and atopic disease with IgE sensitization in a prospective study setting.

Methods This was a nested case-control study among children who had been followed from birth. Neutralizing antibodies against 12 enterovirus serotypes were analysed at the age of 2 years from 71 atopic children and 142 non-atopic control children. Atopy was defined as having an atopic disease and IgE antibodies against at least one aeroallergen by the age of 5 years.

Results Cumulative exposure to different enterovirus serotypes was inversely associated with atopy [odds ratio (OR) 0.73; 95% confidence interval (CI): 0.56-0.96]. The most pronounced protection was seen when echoviruses were analysed as a separate group (OR 0.63; 95%CI: 0.46–0.88).

Conclusions and Clinical Relevance We propose that exposure to several different enteroviruses in early childhood is inversely associated with atopic diseases. Our results support the hypothesis that repeated microbial infections in early life may protect from atopic sensitization and atopic diseases.

Keywords allergy, atopic disease, atopic sensitization, atopy, enterovirus, hygiene hypothesis, neutralizing antibodies
Submitted 15 May 2012; revised 27 October 2012; accepted 26 November 2012

Introduction

The prevalence of atopic diseases in childhood is high in most Western industrialized countries and the increasing trend is still continuing [1]. Also in Finland, the prevalence of atopic diseases, such as asthma and allergic rhinitis, as well as IgE sensitization has increased manifold in recent decades [2, 3]. According to the "hygiene hypothesis" this increase is related to a reduced microbial load in early childhood [4]. Numerous studies have provided indirect evidence to support the hypothesis, as growing up on a farm [5, 6], having one or more older siblings [4, 5, 7] and early attendance in daycare [8] have been shown to be associated with protection from allergy. It has also been reported that geographically adjacent but socioeconomically contrasting countries differ markedly in the prevalence of allergic sensitization [9–11]. However, direct observations of the role of microbial infections in the development of atopic diseases have been somewhat controversial [12].

Viruses could theoretically be highly effective microbes in preventing atopy, as they induce a strong T helper (Th) 1 type immune response, which might suppress the excessive Th2 immune response often seen in atopic diseases. In fact, certain viral infections, such as

hepatitis A virus, Epstein Barr virus, varicella zoster virus, and measles [13–16], have been associated with lower risk of atopic diseases and allergic sensitization. However, there are also studies reporting conflicting results [17–20].

The possible protective effect conferred by infections may depend on their timing [12]. Early life has been proposed as an especially favourable period for protective microbial contacts [6, 14, 15, 21]. The route of exposure to microbial antigens may also be critical. The gut is believed to be an important organ in the early development of immunological tolerance and accordingly, previous studies suggest that orofecally transmitted infections are especially potent in protecting from allergic sensitization [22–24].

Enteroviruses are small RNA viruses comprising almost 100 serotypes. They are traditionally classified into echoviruses, coxsackie A and B viruses, and polioviruses. Vaccination against polio serotypes has almost eradicated wild-type polioviruses, while other enterovirus serotypes are common world-wide. Enterovirus infections are frequent from early infancy onwards. They are mainly subclinical or cause only mild upper respiratory tract infections, gastroenteritis or non-specific febrile illnesses, although severe manifestations, such as meningitis and myocarditis, also occasionally occur. Enteroviruses are transmitted mainly orofecally and replicate primarily in the gut. They have also been linked to the pathogenesis of type 1 diabetes [25], another disease with a rapidly increasing incidence over recent decades [26]. As the concept of hygiene hypothesis has recently been extended from allergies to other immune-mediated disorders including autoimmune diseases such as type 1 diabetes [27], we hypothesized that enteroviruses might also modulate the risk of atopic diseases. This presumption has recently been reinforced by our own studies showing an association between enterovirus infections and a lower prevalence of atopy in a cross-sectional setting [11, 28].

In this study, we wanted to investigate the relation between enteroviruses and atopic diseases in a nested case-control setting based on the prospective Diabetes Prediction and Prevention (DIPP) Study. Furthermore, we set out to study the importance of different enterovirus serotypes in atopic diseases.

Methods

Study population

The study population was derived from the DIPP Study, initiated in Finland in 1994. The basic design of the DIPP study has been described in detail elsewhere [29]. Briefly, newborn infants at the University Hospitals in Turku, Oulu and Tampere were screened at birth with parental consent for type 1 diabetes associated HLA-DQB1 alleles [30]. The designated HLA-DQB1 risk genotypes are present in about 15% of the Finnish population [31]. Infants with these designated HLA-DQB1 risk alleles were invited to enter the DIPP study follow-up with regular visits to the DIPP study clinic with an interval of 3–6 months for the first 2 years of life and thereafter with an interval of 6–12 months. At each visit, the children underwent a comprehensive interview and a clinical examination, and a venous blood sample was taken. Serum samples were stored at −70°C in the DIPP study biobank. Data about diagnosed atopic diseases, parental education, attendance in daycare, pets at home, and siblings were asked regularly and recorded in the DIPP database.

For this study, a cohort of children was selected as follows. In spring 2009, we screened all DIPP children born in Tampere University Hospital between January 1, 1998 and December 31, 2003 for the physician-set diagnosis of bronchial asthma, atopic dermatitis, or allergic rhinitis. Age of the children at the time of the screening ranged from 5 to 11 years. The diagnoses had been made according to national guidelines and they had been recorded in the DIPP database. Only children with serum samples taken during the DIPP follow-up at the ages of 1, 2, and 5 years were eligible for the study. Children with type 1 diabetes associated autoantibodies and/or type 1 diabetes were excluded. Eligible children were then screened for IgE antibodies against a mixture of aeroallergens from the serum samples taken at the age of 5 years. The serum samples for IgE analyses were obtained from the DIPP study biobank. Children who had both an atopic disease and IgE antibodies comprised the group of case children. For each case child, two control children having neither a diagnosis of an atopic disease or IgE antibodies against aeroallergens were chosen. The case and control children were matched for the time of birth, gender, and HLA-DQB1 alleles.

Information about parental education, pets at home, number of older siblings, and child's age at the beginning of daycare was obtained from the DIPP database. Data about maternal smoking during pregnancy were received from the Finnish birth register.

This study was carried out within the DIPP cohort born in Tampere University Hospital. The DIPP study protocol has been approved by the ethical committee of the Tampere University Hospital (Tampere University Hospital approval number ETL 97193M).

IgE antibodies

IgE measurements were made from serum samples taken at the age of 5 years. IgE antibodies against a mixture of common airborne allergens [a multi-allergen

test (Phadiatop®, Thermo Fisher Scientific, Uppsala, Sweden) containing allergens of birch, timothy, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus, Cladosporium herbarum*] were measured using the ImmunoCAP® enzyme immunoassay (Thermo Fisher Scientific, Uppsala, Sweden) according to manufacturer's protocol. Values of 0.35 kU/L or more against the allergen mixture were considered positive.

Virus strains

The ATCC (American Type Culture Collection) reference strains of enteroviruses [coxsackievirus B2 (CBV2), coxsackievirus B6 (CBV6), echovirus 9 (EV9), echovirus 11 (EV11), echovirus 30 (EV30)] as well as wild-type enterovirus isolates from Finland [coxsackievirus A9 (CAV9), coxsackievirus B1 (CBV1), coxsackievirus B3 (CBV3), echovirus 3 (EV3), echovirus 5 (EV5), echovirus 21 (EV21), echovirus 26 (EV26)] were used in this study.

Cell lines

A continuous cell line from African green monkey kidneys (GMK cells) was used for virus propagation. Cells were grown in minimal essential medium supplemented with foetal bovine serum (10% for cell growth, 1% for virus propagation), 15 mM Hepes, pH 7.4 and 15 mM $MgCl_2$. A549 cell line from human alveolar adenocarcinoma was used for EV30 virus propagation.

Plaque neutralization assay

Serum samples taken at 1 and 2 years of age were available from each case and control child to be used for the classical plaque neutralization assay [32]. This method measures antibodies in a serotype-specific manner against the enterovirus serotypes included in the assay. Neutralizing antibodies remain elevated for several years or decades reflecting the past infection history of the child. The serum samples taken at 2 years of age were analysed for the presence of neutralizing antibodies against each of the 12 enterovirus serotypes studied. The 1-year samples were analysed for echovirus serotypes 3, 5, 9, 11, and 30. In the assay, a fourfold dilution of serum was mixed with an equal volume of pretitrated virus (~100 plaque-forming units in 6 μL) and allowed to react for 1 h at 36°C followed by an overnight incubation at room temperature. After addition of the virus propagation medium, the sample was placed on monolayers of GMK cells or A549 cells on six well plates, density of $\sim 0.8 \times 10^6$ cells per well. After 30 min incubation at 36°C, 2 mL of plaquing overlay (cellulose in the virus propagation medium) was added. The amount of infectious virus was detected by counting the plaques after 2 days of incubation at 36°C. The serum was considered antibody positive if it blocked more than 80% of the virus infectivity.

Statistical methods

Conditional logistic regression analysis was used to determine the association between enterovirus infections and atopic diseases in the matched data. The results are presented as odds ratios (OR) and 95% confidence intervals (CI) for atopic diseases. Conditional logistic regression analysis was applied when comparing the association for each serotype individually, for having antibodies against at least one enterovirus serotype, for the cumulative prevalence of enterovirus antibodies and for confounding factors. Categorized variables were used for confounding factors: pets at home (no pets vs. at least one indoor pet of any kind), maternal education (no professional training or lower secondary training vs. higher secondary or tertiary training), paternal education (no professional training or lower secondary training vs. higher secondary or tertiary training), maternal tobacco smoking during pregnancy (no vs. yes), and having older siblings (no older siblings vs. at least one older sibling). A continuous variable was used for the attendance in daycare (number of months in daycare before the diagnosis of an atopic disease was recorded in the DIPP database). Conditional logistic regression analysis was applied when determining the association between individual confounders and atopic diseases. All analyses were performed using STATA 8.2 (Statacorp, College Station, TX, USA).

Results

A total of 2942 infants born in Tampere between January 1, 1998 and December 31, 2003 entered the DIPP study and 1852 (63%) stayed in the follow-up for at least until the age of 5 years. One hundred eighty-three children had a diagnosis code for an atopic disease and blood samples taken at the ages of 1, 2, and 5 years. 71 (39%) of them were IgE positive for aeroallergens at the age of 5 years. The case children having both a diagnosed atopic disease and IgE sensitization ($n = 71$) included 28 (39%) girls and 43 (61%) boys. Thirty-seven (52%) of the case children had atopic dermatitis, 29 (41%) bronchial asthma, and 5 (7%) allergic rhinitis. The median age when the atopic disease was recorded in the DIPP database was 22 months (range 2–102 month). There were 142 control children matched for age, gender, and HLA-DQB1 genotype. The median age difference between cases and controls was 9 days (range 0–64 days) and 14% of them had the HLA-DQB1 *02/*0302 genotype and 86% had the *0302/x genotype (x referring to other alleles than *02, *0301 or *0602).

Data about pets were available from 209 (98%), maternal tobacco smoking from 204 (96%), maternal education from 206 (97%), paternal education from 194 (91%), older siblings from 209 (98%), and attendance in daycare from 194 (91%) children. There were no statistically significant associations between the confounders analysed and atopy.

Neutralizing antibodies against 12 different enterovirus serotypes were measured from the serum samples taken at the age of 2 years. The prevalence of neutralizing antibodies against different enterovirus serotypes varied greatly (Fig. 1). Sixty-one (85.9%) case children and 119 (83.8%) control children had neutralizing antibodies against one or more enterovirus serotypes (p = ns). However, the control children were seropositive against a higher number of different enterovirus serotypes as compared with the cases (OR: 0.73; 95%CI: 0.56-0.96; p = 0.025) (Fig. 2a).

For subgroup analyses, the enteroviruses were divided into echovirus and coxsackievirus groups. Fifty-three (74.7%) case children and 111 (78.2%) control children had neutralizing antibodies against one or more echovirus serotypes (p = ns). The control children were seropositive against a higher number of different echovirus serotypes as compared with cases (OR: 0.63; 95%CI: 0.46–0.88; p = 0.006) (Fig. 2b). In the coxsackievirus group, 25 (35.2%) and 45 (31.7%) of case and control children, respectively, had antibodies against at least one serotype (p = ns). The number of neutralizing antibodies against different coxsackievirus serotypes did not differ between cases and controls.

To study the early encounters with enteroviruses, additional measurements of neutralizing antibodies against five echovirus serotypes (EV3, 5, 9, 11 and 30) were carried out from serum samples obtained at the age of 1 year. For most of these serotypes, antibody positivity was slightly more frequent among the control children, but no statistically significant difference was seen in any individual serotype (Fig. 3a). When the number of neutralizing antibodies in each child was analysed at the age of 1 year, we did not observe any association between the accumulation of infections by different echovirus serotypes and protection from atopy (OR: 0.77; 95%CI: 0.54–1.10; p = ns), although this protective effect was seen at 2 years of age (Fig. 2b). However, 98 (69.0%) control children but only 39 (54.9%) case children had neutralizing antibodies against any echovirus serotype (OR: 0.52; 95%CI: 0.28–0.96; p = 0.038)(Fig. 3b).

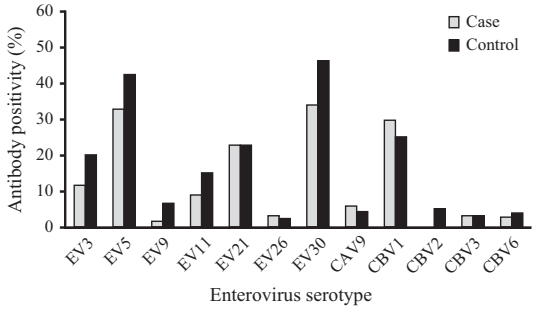

Fig. 1. The prevalence of neutralizing antibodies against different enterovirus serotypes. The percentage of case children (n = 71) and control children (n = 142) having neutralizing antibodies against individual enterovirus serotypes at the age of 2 years is shown.

Discussion

In this study, we found an inverse association between the cumulative exposure to enteroviruses and atopic diseases with IgE sensitization. More specifically, this finding seemed to be related to echoviruses only. Already at the age of 1 year, case children had a lower prevalence of echovirus antibodies and by the age of 2 years they were seropositive against a lower number of echovirus serotypes. This suggests that exposure to a

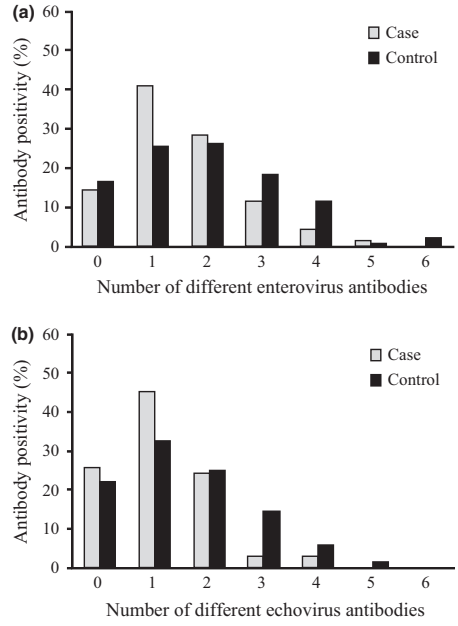

Fig. 2. The number of positive antibody test results against different enterovirus serotypes at the age of 2 years. The proportion of case (n = 71) and control (n = 142) children falling into each category (antibodies against 0–6 different serotypes) is presented as a percentage. Altogether 12 serotypes were analysed. Panel (a) shows the results for all enterovirus serotypes (echoviruses and coxsackieviruses) and panel (b) for the seven echovirus serotypes.

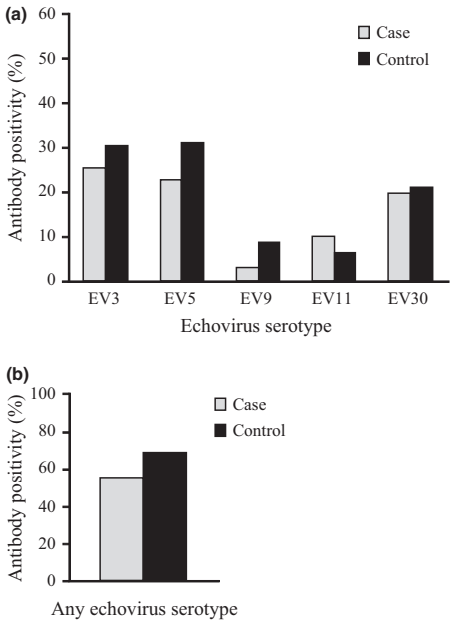

Fig. 3. The prevalence of neutralizing antibodies against echoviruses at the age of 1 year. The percentage of case (n = 71) and control children (n = 142) having neutralizing antibodies against individual echovirus serotypes is shown in the panel (a). Panel (b) shows the proportion of case and control children having neutralizing antibodies against one or more echovirus serotypes.

high number of different echoviruses during the first years of life might protect from atopic diseases.

There are only a few previous reports on the role of enteroviruses in atopy [11, 28, 33, 34], and to our knowledge, no earlier studies have been carried out in a prospective setting. In a cross-sectional study, seropositivity against enteroviruses protected from atopic sensitization in Russian Karelia but not in Finland [11]. However, in that study enterovirus antibodies were measured from older children using an EIA assay, a method known to lack serotype specificity. In a more recent study from the Russian Karelia, the presence of neutralizing antibodies against echoviruses was associated with a reduced risk of IgE sensitization in schoolchildren [28]. This is in line with our results from this study carried out among very young children, suggesting that enterovirus infections are associated with lower risk of atopy and that enterovirus serotypes differ in terms of their effect on atopic diseases. These findings further emphasize the advantages of serotype-specific assays in studies evaluating the possible role of enteroviruses in atopic sensitization and atopic diseases.

Enteroviruses are transmitted principally orofecally, a feature suggested to be an important determinant of the protection provided by certain microbes against atopic diseases. In adults, seropositivity to orofecal microbes has been shown to be associated with a reduced prevalence of atopic diseases and IgE sensitization [13, 22, 23]. Matricardi et al. reported that Italian atopic adults had a lower prevalence of hepatitis A virus antibodies than non-atopic controls, and that the risk of atopy decreased with a gradient of exposure to foodborne microbes but not with cumulative exposure to viruses transmitted via other routes [23]. However, in a nested case-control study from Scotland, seropositivity to hepatitis A was unrelated to the atopic status in adults [17]. In young children, it has been shown in a longitudinal study that children with salmonellosis had a lower prevalence of allergic rhinoconjunctivitis and asthma than children with enteritis due to other microbes [24]. It was also recently reported from a prospective birth cohort study, that seropositivity to rota- or norovirus did not protect from IgE sensitization [20].

In our study, the risk of childhood atopy decreased along with the gradient of exposure to different enterovirus serotypes, especially to echoviruses. This is in line with the results from some previous reports showing the importance of cumulative exposure to microbes [11, 23, 28]. In addition, atopic diseases have quite consistently been observed to be less frequent in children having several siblings, especially older siblings, most likely reflecting the importance of repeated early microbial contacts [4, 7, 35]. It has also been shown for enteroviruses that infants with one or more siblings had stronger T cell responses to coxsackievirus B4 and polioviruses than single infants [36].

As the immunological events resulting in the Th2-skewed immune response characteristic for atopic diseases are thought to begin already in neonates or even *in utero*, microbial contacts very early in life can be assumed to be of special importance. In a longitudinal birth cohort study, children with recurrent episodes of runny nose before the age of 1 year were less likely to develop asthma and IgE sensitization than children experiencing viral infections later in childhood [21]. The protective effect of early viral infections has been reported from other studies as well [14, 15] but there is also contradictory data suggesting that early viral infections might in fact increase the risk for atopy [18, 37, 38]. These conflicting data might partly be due to the heterogeneous nature of atopic diseases, perhaps modulated by different environmental factors [39, 40].

Previous studies have often relied on parental reported or physician diagnosed infections, thus detecting only those microbial contacts resulting in a clinical disease. However, viruses frequently cause subclinical infections and there are some reports implying a poor correlation between parental reported infections and virus serology [7]. It is likely that focusing on clinically

manifested infections misses a substantial proportion of microbial contacts. Serological assays, such as the plaque neutralization assay, have the advantage of detecting both subclinical and clinically overt infections and therefore provide a more comprehensive picture of microbial contacts. We were also able to measure neutralizing antibodies at two distinct time points, thus providing reliable information about the accumulation of antibodies to different enterovirus serotypes over time. In addition, atopy was defined as having both IgE antibodies against common aeroallergens and a clinically relevant atopic disease, a definition often used also in clinical settings. This strict definition reduces heterogeneity within the case group, thus facilitating a more reliable evaluation of risk factors. However, this also limits our conclusions to IgE positive atopic patients.

The genetic background of atopic diseases is complex and a significant number of susceptibility genes may be involved [41]. Our study population was selected from the cohort of DIPP study children carrying designated HLA-DQB1 alleles. Sporadic studies have reported some association between HLA-DQB1 alleles and atopic diseases or IgE sensitization [42, 43] but in other reports, no association has been observed [44, 45]. A recent Finnish study found no association between type 1 diabetes associated HLA genotypes and atopic sensitization [46]. Therefore, we do not suspect any bias due to the genetic selection of the population in this study. Most importantly, to further rule out the possibility of bias, the case and control children were matched for their HLA-DQB1 genotypes on study inclusion. They were also matched on study inclusion for age and gender. Other confounding factors analysed and possibly influencing the infectious environment of a child showed no independent association with atopic diseases.

The precise age of the case child at the time of the diagnosis of the atopic disease or IgE sensitization was not available and therefore any conclusions about causality should be made with caution. However, we did show a dose-dependent like effect with exposure to different enterovirus serotypes, thus supporting true causality. Further studies are needed to find out whether this effect is specific for enteroviruses or if they are merely surrogate markers for the total burden of infections or some other environmental factors.

In conclusion, this study shows that cumulative exposure to enterovirus infections in early childhood is associated with a reduced risk of atopic diseases. As the factors influencing the atopic outcome are still largely unknown, measures aimed at eliciting the role of different environmental factors are urgently needed. Enteroviruses are suspected to play a role in the pathogenesis of type 1 diabetes and our research brings new insight into their possible role in atopic diseases as well. However, larger prospective studies are needed to further define their role in the pathogenesis of atopic diseases.

Acknowledgements

This study was supported by the Academy of Finland (PREVALLER consortium of the Salve program), the Päivikki and Sakari Sohlberg Foundation, Tampere Tuberculosis Foundation and the University Hospital of Tampere. We thank Ms. Anne Karjalainen for her skilful technical assistance. We also thank the families participating in the DIPP study.

Conflict of interest

Professor Hyöty and Professor Knip are minor shareholders of Vactech Ltd, which develops vaccines against picornaviruses. Other authors declare no conflict of interest.

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PUBLICATION II

Enterovirus infection during pregnancy is inversely associated with atopic disease in the offspring

Korhonen L, Seiskari T, Lehtonen J, Puustinen L, Surcel HM, Haapala AM, Niemelä O, Virtanen S, Honkanen H, Karjalainen M, Ilonen J, Veijola R, Knip M, Lönnrot M, Hyöty H.

Clin Exp Allergy 2018;48:1698-1704

Received: 24 January 2018 | Revised: 12 August 2018 | Accepted: 1 September 2018

DOI: 10.1111/cea.13280

ORIGINAL ARTICLE

Epidemiology of Allergic Disease

WILEY

Enterovirus infection during pregnancy is inversely associated with atopic disease in the offspring

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Funding information
This work was funded by the European Comission Persistent Virus Infection in Diabetes Network [PEVNET] Frame Programme 7Contract No. 261441, Tampere Tuberculosis Foundation, Sigrid Juselius Foundation, Academy of Finland [PREVALLER consortium, Grant 129448-2008; Research Grant 132362-2009; Centre of Excellence in Molecular Systems Immunology and Physiology, Grant 250114 2012–2017], The National Technology Agency in Finland (TEKES), Päivikki and

Summary

Background: Prenatal environment has been shown to influence child's risk of atopic diseases. Laboratory-confirmed data about the role of maternal infections during pregnancy is scarce.

Objective: The aim of this study was to determine the associations between serologically confirmed maternal infections during pregnancy and atopic disease in the offspring.

Methods: This was a nested case-control study within a prospective birth cohort study. Altogether 202 atopic case children and 333 matched non-atopic control children were included. Atopic outcome was defined as having an atopic disease and IgE sensitization by the age of 5 years. We analysed serologically acute enterovirus (EV), influenza virus A (IAV) and *Mycoplasma pneumoniae* (*M. pneumoniae*) infections

Sakari Sohlberg Foundation, The Finnish Medical Foundation and Finnish Cultural Foundation.

during pregnancy, and mother's seropositivity against human cytomegalovirus (CMV) and *Helicobacter pylori*.

Results: Maternal EV infection during pregnancy was inversely associated with atopic outcome in the offspring (odds ratio 0.43; 95% confidence interval: 0.23-0.80, $P = 0.008$). Acute IAV or *M. pneumoniae* infections or seropositivity against CMV or *Helicobacter pylori* were not associated with the atopic outcome.

Conclusions and Clinical Relevance: Our results suggest that maternal EV infections during pregnancy are inversely associated with atopic disease in the offspring. Our finding provides further support to the previous studies suggesting an important role of the *in utero* environment in the development of atopic diseases.

1 | INTRODUCTION

Genetic factors regulate the susceptibility to atopic diseases but the environment has a significant impact on the disease development. Atopic diseases usually manifest in childhood stressing the importance of environmental factors present in very early life.[1] Accordingly, extensive data suggest that a variety of environmental exposures in early childhood can influence the development of atopic diseases.[2] Postnatal microbial infections have been among the most studied factors and several reports have suggested a modulation of the risk of atopic diseases by childhood infections.[3-11]

Recently, it has become increasingly evident that already the *in utero* environment may be important in the development of atopic diseases.[2] A number of prenatal factors, for example, maternal smoking,[12] adverse life events[13] and pre-pregnancy overweight[14] have been associated with increased risk of atopic diseases in the offspring. In contrast, maternal exposure to farming environment has been shown to protect the child from atopy.[15-17] These findings, consistent with the framework of hygiene hypothesis, raise the question about the role of prenatal microbial infections in atopy.

The majority of previous data on prenatal infections are derived from questionnaire-based studies, most of which report an increased risk of atopic disease in children born to mothers with febrile or flu-like infections during pregnancy.[18] However, questionnaire-based studies do not enable the identification of culprit microbes and they may be affected by recall bias. Applying laboratory assay-based methods can overcome these shortcomings but such studies are scarce.[19-22] Maternal gastrointestinal helminth infections have been reported both to inversely associate with eczema in the offspring[20] and not to associate with atopy.[19] Intrauterine bacterial growth at birth as well as chorioamnionitis has been shown to increase the risk of asthma in the offspring.[21,23]

We wanted to investigate whether laboratory-confirmed prenatal infections are associated with atopic outcome in the offspring. In addition, we aimed to determine whether microbes with different infectious and immunological reaction patterns differ in their effect. Five microbes, featuring gastrointestinal and respiratory pathogens, as well as microbes causing acute and chronic infections were included in the current study.

Enteroviruses (EV) can replicate in the gastrointestinal tract, and they have been linked to atopy in some studies[6-8] but not all.[4,5] *Helicobacter pylori* (*H. pylori*), causing chronic gastric infections and inflammation, and also considered to be a marker of the general hygiene level, has been shown to associate with atopy.[24] Influenza virus A (IAV) causes a strong acute systemic infection, whereas cytomegalovirus (CMV) persists as a lifelong latent infection. *Mycoplasma pneumoniae* (*M. pneumoniae*) in turn causes often subclinical, chronic lower respiratory tract infections. We analysed acute gestational EV, IAV and *M. pneumoniae* infections by serology from paired serum samples taken during pregnancy. We estimated the incidence of acute *H. pylori* and CMV infections during pregnancy to be very low in the Finnish population and therefore chose to analyse the presence of these chronic/latent infections from a single serum sample.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study cohort was derived from the prospective Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland.[25] In the DIPP study, newborn infants with HLA conferred susceptibility to type 1 diabetes are invited to enter a prospective follow-up with visits at the study clinic every 3-12 months. At each visit, children undergo a comprehensive interview and clinical examination. Biological samples, including venous blood samples, are collected according to the study protocol.

In the present study, we first searched the DIPP database for children fulfilling the atopic outcome criteria applied: Diagnosis of bronchial asthma, atopic dermatitis and/or allergic rhinitis and a positive serum IgE level against aeroallergens at the age of 5 years. Altogether, 202 atopic case children were identified. They were born between June 1996 and September 2004 in the regions of Tampere and Oulu cities. Next, we selected 1-2 non-atopic control children for each case child having neither specific IgE against aeroallergens nor diagnosis of any atopic disease (n = 333). Case and control children were matched for gender, region of birth, time of birth (± 3 months) and type 1 diabetes-related HLA-DQB1 alleles. In total,

128 (63%) case children and 207 (62%) control children were boys. The mean age difference between cases and controls was 45 days (SD 34 days, range 0-91 days), and the percentages of case and control children born each season of the year were equal. Altogether, 9% of children carried the HLA-DQB1 *02/*03:02 genotype and 91% had the *03:02/x genotype with x referring to other alleles than *02, *03:01 or *06:02.

Paired serum samples were available from each mother of participating children. First sample was taken in prenatal clinics at the end of the first trimester of pregnancy as a part of a national screening for infectious diseases. These samples are stored in the nationwide Finnish Maternity Cohort biobank. The second sample was cord blood serum from the newborn infant collected according to DIPP study protocol. The Finnish Maternity Cohort Steering Group at the National Institute for Health and Welfare approved the use of biobank samples in this study. The DIPP study protocol has been approved by the ethical committees of the participating university hospitals (ETL 97193M), and parents have given written informed consent.

2.2 | IgE antibodies

IgE antibodies against a mixture of common aeroallergens were analysed from the serum samples taken at the age of 5 years with ImmunoCAP® enzyme immunoassay (Phadia AB, Uppsala, Sweden). The multi-allergen test used (Phadiatop®, Phadia AB, Uppsala, Sweden) contains allergens of common pollens, moulds and animals. Values of ≥0.35 kU/L were considered positive.

2.3 | Microbial analyses

IgG class antibodies against EV, IAV, *H. pylori* and *M. pneumoniae* were measured by applying enzyme immune assays (EIA). IgG class antibodies against CMV were measured either by applying EIA or by chemiluminescent immunoassay (CLIA). For CMV, two different assays were used due to changes in laboratory equipment during the study period but each case-control pair was always analysed with the same method.

Enterovirus, IAV and *M. pneumoniae* antibodies were analysed from both first trimester serum samples and cord blood, *H. pylori* antibodies from cord blood and CMV antibodies from first trimester serum samples (n = 146) or cord blood (n = 381), whichever was available. Samples from case-control pairs and related first trimester and cord blood samples were analysed in parallel in the same test run. All analyses were carried out blind to clinical information.

The assay for EV antibodies employed a synthetic EV peptide that carries an immunodominant epitope of the viral VP1 protein (sequence KEVPALTAVETGAT-C) as an antigen, as described.[26] This epitope is highly conserved among EVs detecting antibodies against a wide range of different EV types. For IAV, we used influenza A virus strain Beijing (BA1231VS, Virion Serion, Würzburg, Germany) as an antigen, as previously described.[27] In brief, microtiter plates (Nunc Immuno™ plate, Maxisorb, Thermo Fisher Scientific, Waltham, MA, USA) were coated by the antigens at concentrations 1 μg/mL for EV and 3 μg/mL for IAV in carbonate buffer (pH 9.4). Serum samples were analysed diluted 1:1000 in PBS supplemented with 1% bovine serum albumin, 2% NaCl and 0.05% Tween 20. Serial dilutions for strong positive serum samples were used to reach standard curve range. Binding of antibodies was documented by using peroxidase-conjugated anti-human IgG (P214, Dako, Glostrup, Denmark) and measuring the absorbance at 490 nm. The results were given in enzyme immunoassay units (EIU) with reference to negative and positive control samples. An EIU value of 15 was applied as a cut-off level for seropositivity as described.[26]

Antibodies against *H. pylori* (Enzygnost® Anti-Helicobacter pylori/IgG, Siemens, Marburg, Germany) and antibodies against *M. pneumoniae* (Mycoplasma pneumonia IgG, Labsystems Diagnostics Ltd, Helsinki, Finland) were measured using commercial kits according to the manufacturers' instructions. For the analysis of antibodies against CMV from the first trimester samples, we applied Enzygnost® Anti-CMV/IgG EIA kit (Siemens), and for cord blood samples, we used LIAISON® CMV IgG II CLIA (DiaSorin S.p.A., Saluggia (VC), Italy) according to the manufacturers' instructions. We used Siemens BEP III (Siemens) for processing and calculation of the antibody levels for the EIAs and LIAISON® XL (DiaSorin S.p.A.) for CLIA.

2.4 | Definition of acute infection

According to the manufacturer's instructions, we defined an acute *M. pneumoniae* infection as a 1.6-fold or higher increase in antibody level between the maternal first trimester sample and the child's cord blood sample. For cohesion, we applied the same criteria also for EV and IAV to indicate an acute infection during pregnancy.

2.5 | Statistical methods

We applied conditional logistic regression analysis to determine the association between an acute infection during pregnancy (EV, IAV, *M. pneumoniae*) or seropositivity (CMV, *H. pylori*) and atopic disease. The results are presented as odds ratios (OR) and 95% confidence intervals (CI) for atopic disease.

Demographic factors are presented in Table 1. We applied conditional regression analysis to estimate the individual association of each variable with atopic outcome. When an association was observed, conditional regression analysis was applied to adjust for these factors. If a value was missing from the case child or all controls in the case-control group, that case-control pair/triplet was excluded from the analyses.

We applied the Bonferroni correction to counteract the problem of multiple comparisons. As we analysed the association between acute infection during pregnancy and atopic disease for three microbes, the Bonferroni correction to control type I error was justified. After applying the Bonferroni correction, that is, dividing $P = 0.05$ by 3, P-values < 0.017 were regarded as statistically significant. Similarly, as seropositivity in a single serum sample was analysed for two microbes, after applying Bonferroni correction, P-

TABLE 1 Frequencies of potential confounders in case and control children and associations with the study outcome

	Case n = 202 (%)	Control n = 333 (%)	OR (95% CI)	*P* value
Older siblings (yes)	95 (47)	201 (60)	0.60 (0.42-0.86)	**0.006**
Furry pets (yes)	58 (29)	146 (44)	0.53 (0.36-0.78)	**0.001**
Smoking in pregnancy (yes)	17 (9)	28 (8)	1.00 (0.53-1.89)	1.00
Maternal education				
No secondary	50 (27)	98 (31)	ref	
Lower secondary	91 (48)	151 (48)	1.01 (0.65-1.59)	0.96
Higher secondary	48 (25)	63 (20)	1.26 (0.76-2.11)	0.37
Paternal education				
No secondary	86 (46)	144 (48)	ref	
Lower secondary	46 (25)	88 (30)	0.92 (0.59-1.44)	0.71
Higher secondary	56 (30)	66 (22)	1.34 (0.85-2.11)	0.21
Duration of pregnancy[ab]	280 (224-300)	280 (209-300)	1.01 (0.99-1.02)	0.53
Birth weight[ac]	3650 (1910-4830)	3610 (1660-5540)	1.00 (1.00-1.00)	0.27

OR and 95% CI were estimated using conditional logistic analysis. *P* values below 0.05 are marked in bold.
[a]Values are medians (minimum and maximum).
[b]Presented in days.
[c]Presented in grams.

values < 0.025 were statistically significant. Unadjusted *P*-values are presented in the text.

Analyses were performed by using R version 3.3.3 (2017-03-06, The R Foundation for Statistical Computing, https://www.R-project.org).

3 | RESULTS

Out of the 202 case children, 52 (26%) had asthma, 126 (62%) had atopic eczema and/or allergic rhinitis, and 24 (12%) had both asthma and eczema/rhinitis. Specific IgE values varied between 0.36 and 101 kU/L (mean 22.9, median 11). Demographics and their associations with atopic outcome are presented in Table 1.

A gestational EV infection was observed in 17 (8%) mothers of the case children and in 53 (16%) mothers of the control children (Figure 1). *M. pneumoniae* infection was detected in 14 (7%) vs 37 (11%), and IAV in 26 (13%) vs 43 (13%) mothers of case and control children, respectively. Seropositivity against CMV was observed in 148 (74%) vs 243 (74%), and *H. pylori* in 14 (7%) vs 37 (11%) mothers of the case and control children, respectively.

Maternal EV infection during pregnancy was inversely associated with atopic outcome in the offspring (OR: 0.43; 95% CI: 0.23-0.80; *P* = 0.008). The result remained statistically significant after adjusting for the relevant confounding factors, that is, older siblings and furry pets (OR: 0.43; 95% CI: 0.23-0.81; *P* = 0.009) (Figure 2). Maternal IAV or *M. pneumoniae* infections during pregnancy did not associate with atopic outcome (OR: 1.05; 95% CI: 0.62-1.79 and OR: 0.65; 95% CI: 0.34-1.22, respectively). When all the three microbes were analysed together, 49 (24%) case mothers and 115 (35%) control mothers had experienced at least one acute infection during pregnancy (OR: 0.60; 95% CI: 0.39-0.90; *P* = 0.015).

Maternal seropositivity against CMV or *H. pylori* was not associated with atopic outcome in the offspring (OR: 0.99; 95% CI: 0.66-1.47 and OR: 0.58; 95% CI: 0.30-1.12, respectively).

4 | DISCUSSION

The current study suggests that maternal EV infections during pregnancy are inversely associated with atopic disease in the offspring. To our knowledge, EVs or other microbes included have not been studied in a similar laboratory-based study setting previously.

We did not find any association between prenatal IAV and *M. pneumoniae* infections and atopic outcome. One reason may lie in the route of infection; IAV and *M. pneumoniae* are respiratory microbes, whereas EVs can replicate also in the gut. There are indications that exposures to enteric pathogens might be particularly important in the development of atopy and they have been reported to inversely associate with atopy in some studies[28,29] but not in all.[3,11] Our group has previously observed an inverse association between neutralizing antibodies against echoviruses, belonging to the enterovirus genus, and atopy.[6,7] A recent study showed reduced anti-echovirus 30 antibody titres in asthmatic children as compared with non-asthmatics,[8] which was contrary to a previous rhinovirus antibody finding.[9] In addition, some EVs replicate for relatively long periods in the gut-associated lymphoid tissue that is believed to be important in maintaining immunological tolerance, and EV infections have been shown to associate with tolerogenic immune responses, for example, production of IL-10.[30]

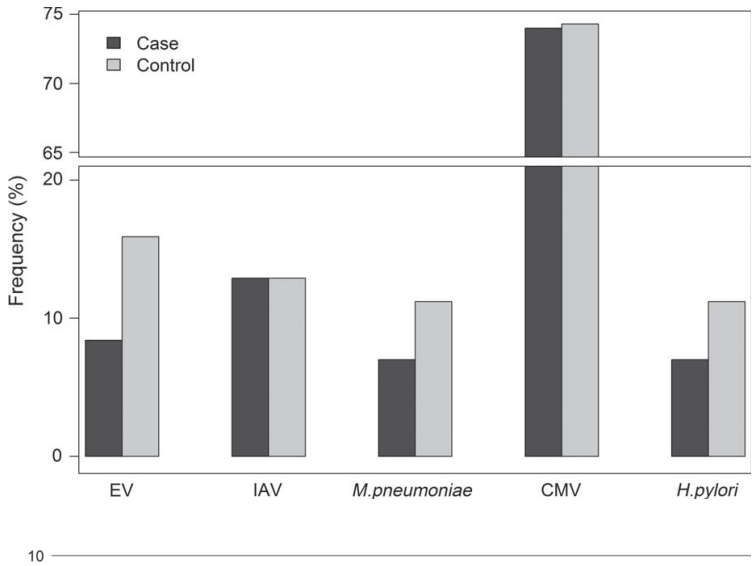

FIGURE 1 The proportion (%) of case and control mothers with an acute EV, IAV or *M. pneumoniae* infection during pregnancy or seropositivity against CMV or *H. pylori*. CMV, cytomegalovirus; EV, enterovirus; *H. pylori*, *Helicobacter pylori*; IAV, influenza A virus; *M. pneumoniae*, *Mycoplasma pneumoniae*

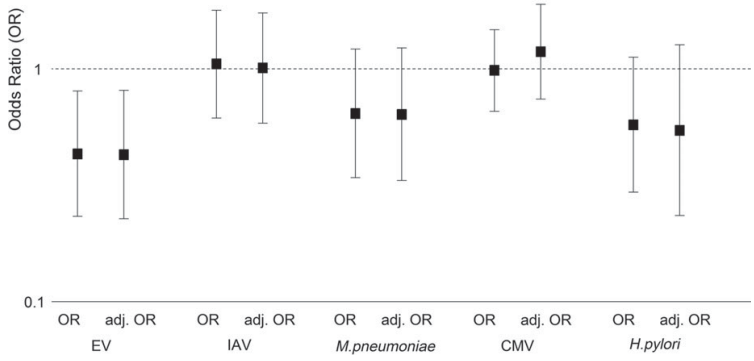

FIGURE 2 The associations between acute infections during pregnancy (EV, IAV and *M. pneumoniae*) or mother's seropositivity (CMV and *H. pylori*) and atopic disease in the offspring. Results are presented as OR and 95% CI for each microbe before and after adjusting for relevant confounding factors, that is, having older siblings and contact with furry animals (adj. OR). CMV, cytomegalovirus; EV, enterovirus; *H. pylori*, *Helicobacter pylori*; IAV, influenza A virus; *M. pneumoniae*, *Mycoplasma pneumoniae*

Maternal microbial infections during pregnancy can affect the fetus in several ways. Viral infections can cross the placental barrier or infect the placenta, which can have severe consequences for the fetus.[31-33] Infections can also affect the fetus through a local inflammatory response in the placenta or activation of the maternal systemic immune response.[31] For instance, toll-like receptors (TLRs) are an important part of innate immunity against viruses and the type of TLR activated is suggested to depend on the mechanism of replication used by the virus.[34] Maternal exposure to farming has been shown to increase gene expression of certain TLRs in cord blood,[17] and increased expression of some TLR genes at birth has been associated with a reduced risk of atopy in the offspring.[16] There are also some indications that maternal infections during pregnancy might affect the placental microbiome,[35] that may in turn influence the microbial composition of the newborn infant's first intestinal discharge (meconium).[36] Differential activation of innate immunity or changes in the composition of early microbiota might be possible mechanisms behind the findings in our study and offer an interesting field for future research.

The strengths of this study include the prospective study setting, availability of paired serum samples taken during pregnancy and the strict definition of atopic outcome including both IgE sensitization and a clinically relevant atopic disease. Most importantly, we relied on serologically confirmed infections. Many infections, including EV infections, are asymptomatic or cause only mild clinical presentations, but serological assays are able to capture both symptomatic and silent infections. There are only a few previous studies with laboratory-confirmed infections during pregnancy, and to our

knowledge, the microbes included in the present study have not been studied previously in detail.[19-21] In a study by Murphy *et al*,[22] they used PCR to detect viruses (including EVs and IAVs) from nasal swabs taken from pregnant asthmatic mothers during a symptomatic upper respiratory infection. They reported that infants born to mothers with nasal swab positive for any of the tested viruses had an increased risk of atopy as compared to virus negative mothers. However, numbers of detected viruses were relatively small; for example, only 3 EVs detected in 42 samples, and therefore, no conclusions could be drawn about the role of individual viruses. In the present study, we used systematic serological screening to detect both symptomatic and asymptomatic infections as well as infections that did not coincide with the sample draws.

Acute gestational infections were diagnosed by increases in IgG levels between maternal serum samples taken at the end of the first trimester and cord blood serum. The time interval between the two samples was longer than that usually applied in clinical diagnostics, but it enabled us to capture acute gestational infections as extensively as possible. IgG responses last usually for several months or years, but it is possible that some infections may have remained undetectable due to low or short IgG responses. However, this should have occurred similarly in both case and control groups. We used cord blood serum as the second sample, since no maternal serum was taken at delivery. We have previously shown that EV IgG levels in cord blood sera correlate well with IgG levels in maternal sera at the time of delivery.[37] We did not measure IgM antibodies, since IgM antibodies do not cross the placenta and IgM responses do not develop in all EV infections leading to diminished sensitivity.[38]

There are some limitations of our study. First, lack of information on parental history of atopy prevented us from analysing the results with respect to different genetic backgrounds. Therefore, the possibility that atopic mothers are genetically less susceptible to EV infections or that there is another immunological or environmental factor affected by maternal atopy status, cannot be excluded. Secondly, our study population was selected for type 1 diabetes-associated HLA genotypes, which could affect the generalizability of the results. Although HLA-DQ region has been linked to asthma, the genetic backgrounds of IgE sensitization and atopic diseases are highly polymorphic with no strong association to HLA-DQ region.[39] Therefore, we find it unlikely that the HLA selection would substantially influence our results. Furthermore, the case and control groups were matched for the type 1 diabetes-associated HLA genotypes. It should also be noted that the prevalence of acute EV infections during pregnancy was found to be relatively low, being in line with previous studies.[37,40] As atopic diseases are common in childhood, it is likely that gestational EV infections are not a major risk-modifying factor in atopy. However, the observational design of the present study makes it difficult to estimate the potential size of this effect on population level reliably. One should also note that even though the specificity of the current EV antibody assay has been well documented,[41] we cannot completely exclude the possibility that in some cases, it could have detected antibodies against other viruses than EVs. Finally, we have not addressed the mechanisms behind the inverse association between gestational EV infections and atopy, and therefore, it is possible that EVs are merely a surrogate marker for a certain kind of environment mediating the effect observed.

In conclusion, our study suggests that maternal EV infections during pregnancy are inversely associated with atopic disease in the offspring. Other included microbes causing acute or chronic infections lacked this association suggesting that microbes differ in their capability to affect the processes involved in the development of atopy. This stresses the importance of microbe-specific identification of infections when evaluating their role in the pathogenesis of atopy. Previous research has shown that postnatal infections play a role in the development of atopy, and our study adds to this knowledge by suggesting that microbial exposure already *in utero* might also be of importance. Thus, addressing the overall effect of gestational and postnatal infections on the development of atopy is an important objective for future studies.

ACKNOWLEDGEMENTS

We thank the DIPP study group and study nurses. We are deeply grateful to Prof. Olli Simell for his valuable contribution to the DIPP study and to Prof. Jorma Toppari as the current principal investigator of the Turku clinical DIPP centre. We are thankful to Eveliina Jalonen, Aaro Piirainen, Tuula Ruuti, Irma Valtonen and Sini Wallenius for their skilful technical assistance. We also thank the local study personnel and the participating families for their collaboration.

CONFLICT OF INTEREST

Professors Hyöty and Knip are minor shareholders of Vactech Ltd developing vaccines against picornaviruses. Other authors declare no conflict of interest.

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How to cite this article: Korhonen L, Seiskari T, Lehtonen J, et al. Enterovirus infection during pregnancy is inversely associated with atopic disease in the offspring. *Clin Exp Allergy*. 2018;48:1698–1704. https://doi.org/10.1111/cea.13280

PUBLICATION III

Rhinoviruses in infancy and risk of immunoglobulin E sensitization

Korhonen L, Oikarinen S, Lehtonen J, Mustonen N, Tyni I, Niemelä O, Honkanen H, Huhtala H, Ilonen J, Hämäläinen AM, Peet A, Tillmann V, Siljander H, Knip M, Lönnrot M, Hyöty H.

J Med Virol 2019 (Epub ahead of print)

Received: 26 November 2018 | Revised: 20 February 2019 | Accepted: 9 March 2019

DOI: 10.1002/jmv.25455

RESEARCH ARTICLE

WILEY JOURNAL OF MEDICAL VIROLOGY

Rhinoviruses in infancy and risk of immunoglobulin E sensitization

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Funding information
Tampere Tuberculosis Foundation; Estonia Research Council, Grant/Award Number: PUT 1382; Suomen Akatemia, Grant/Award Numbers: 250114 2012-2017, 132362-2009, 129448-2008; Sigrid Juselius Foundation; The National Technology Agency in Finland (TEKES); Orion-Farmos Research Foundation; Päivikki ja Sakari Sohlbergin Säätiö; European Union Seventh Framework Program FP7/2007-2013, Grant/Award Number: 202063

Abstract

Previous data about the role of viruses in the development of allergic immunoglobulin E (IgE) sensitization are contradictory. The aim of this study was to determine the possible associations between exposure to different viruses (rhinovirus, enterovirus, norovirus, and parechovirus) during the first year of life and IgE sensitization. Viruses were analyzed from stool samples collected monthly from infants participating in a prospective birth cohort study. From that study, 244 IgE sensitized case children and 244 nonsensitized control children were identified based on their allergen-specific IgE antibody levels at the age of 6, 18, and 36 months. Stool samples (n = 4576) from the case and control children were screened for the presence of rhinovirus, enterovirus, norovirus, and parechovirus RNA by reverse transcription quantitative polymerase chain reaction. The study showed that rhinovirus was the most prevalent virus detected, present in 921 (20%) samples. None of the viruses were associated with IgE sensitization in the full cohort but after stratifying by sex, the number of rhinovirus positive samples was inversely associated with IgE sensitization in boys

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(odds ratio [OR]: 0.81; 95% confidence interval [CI]: 0.69-0.94; $P = 0.006$). There was also a temporal relation between rhinoviruses and IgE sensitization, as rhinovirus exposure during the first 6 months of life was associated with a reduced risk of subsequent IgE sensitization in boys (OR: 0.76; 95% CI: 0.6-0.94; $P = 0.016$). In conclusion, early exposure to rhinoviruses was inversely associated with IgE sensitization but this protective association was restricted to boys.

KEYWORDS
allergy, atopy, sex, stool, virus

1 | INTRODUCTION

The rapid increase in the prevalence of atopic diseases during the last decades highlights the role of environmental factors in the pathogenesis of these diseases.[1] IgE sensitization is a marker of atopic constitution and is often associated with atopic diseases, such as asthma, allergic rhinoconjuctivitis, and atopic eczema. Viral infections are among the potentially interesting environmental exposures affecting IgE sensitization but current data on the associations between viruses and IgE sensitization are conflicting. Some studies have shown virus infections to associate with a lower risk of IgE sensitization,[2,3] whereas others have observed no associations[4,5] or an increased risk.[6-8] There are also indications that viruses may differ in their ability to modulate the risk of IgE sensitization.[3,6,7]

The aim of this study was to evaluate the associations between viral infections in infancy and IgE sensitization. The gut immune system is important in the development of immunological tolerance in early childhood and gastrointestinal viruses have been suggested to be of special significance with regard to atopic sensitization.[9-11] Therefore, viruses known to replicate in the gastrointestinal tract were selected for the present study: Enteroviruses (EVs) (family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus A-D*), noroviruses (NoVs) (family *Caliciviridae*, genus *Norovirus*) and parechoviruses (HPeVs) (family *Picornaviridae*, genus *Parechovirus*, species *Parechovirus A*). Rhinoviruses (RVs) belong to the *Enterovirus* genus of the *Picornaviridae* family, and they are classified into three species: *Rhinovirus A-C*. Wheezing RV-infections have been shown to increase the risk of childhood asthma,[12,13] and they have been reported to be present in fecal samples.[14-17] Thereby, also RVs were included in the present study, even though there is no evidence that they could replicate in the intestinal mucosa, but are rather passively transmitted from their replication site in the respiratory mucosa into the gastrointestinal tract. In this study, RV, EV, NoV, and HPeV RNA was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) from stool samples collected monthly during the first year of life from IgE sensitized and nonsensitized children. To our knowledge, there are no previous studies about the associations between viruses detected in stool and risk of IgE sensitization.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study was carried out as a part of the DIABIMMUNE study aimed at delineating environmental factors predisposing to immune-mediated diseases such as allergies and type 1 diabetes (T1D).[18] Human leukocyte antigen (HLA) DR-DQ genotypes associated with T1D were screened from the cord blood of 5819 newborns in Finland and Estonia, as described earlier.[18] All children with designated T1D-related HLA DR-DQ genotypes ($n = 1139$) were invited to participate in the DIABIMMUNE birth cohort study. Infants were categorized as having high (DR3-DQ2/DR4-DQ8 genotype), moderate (DR4-DQ8/X genotype; X = nonprotective allele but not DR3-DQ2) or slightly increased (DR3-DQ2/Y genotype; Y = nonprotective allele but not DR4-DQ8) risk for T1D. The study was approved by the local Ethics Committees (228/13/03/03/2008 and 172/T-15; 20.08.2008) and the parents gave their written informed consent.

Altogether 717 out of the 1139 HLA-eligible newborns participated in the birth cohort study and 563 children continued until the end of the follow-up at 36 months. For the present study, children were selected according to their IgE levels among these 563 children. In the DIABIMMUNE study, allergen-specific IgE levels were measured at 6, 18, and 36 months of age. Altogether 244 (43%) children had positive IgE values against at least one allergen at the age of 18 and/or 36 months. These 244 children comprised the case group. Next, 244 non-IgE sensitized control children were selected, that is, control children had negative allergen-specific IgE levels at 6, 18, and 36 months. Case and control children were matched for the country of birth, 304 children from Finland, and 184 children from Estonia, but otherwise, control children were selected randomly. All children were born between September 2008 and May 2010. Demographic characteristics of the case and control groups are presented in Table 1.

2.2 | Virus analyses

Stool samples collected during the first 12 months of life were systematically screened for the presence of RV, EV, NoV, and HPeV RNA using RT-qPCR. Viral RNA was extracted from 10% stool suspension in HANK's solution with Qiagen Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral

TABLE 1 Demographic characteristics of the study population

	Case[a] *n* = 244 (%)	Control[b] *n* = 244 (%)	OR (95% CI)
Sex (male)	140 (57)	106 (43)	1.75 (1.22-2.51)
HLA risk class			
DR3-DQ2/DR4-DQ8	25 (10)	20 (8)	*Ref*
DR4-DQ8/x[c]	98 (40)	110 (45)	0.71 (0.37-1.36)
DR3-DQ2/y[d]	121 (50)	114 (47)	0.85 (0.45-1.61)
Maternal atopy (yes)	72 (30)	72 (30)	1 (0.68-1.48)
Paternal atopy (yes)	77 (32)	57 (23)	1.53 (1.02-2.29)
Parental atopy			
Neither	125 (52)	136 (56)	*Ref*
Either or both	117 (48)	108 (44)	1.18 (0.82-1.68)
Older siblings (yes)	156 (64)	168 (69)	0.8 (0.55-1.17)
Smoking inside (yes)	16 (7)	11 (5)	1.51 (0.69-3.34)
Place of Residence			
Urban	188 (79)	194 (81)	*Ref*
Rural	41 (17)	39 (16)	1.08 (0.67-1.76)
Both	9 (4)	8 (3)	1.16 (0.44-3.07)
Type of Housing			
Apartment	82 (34)	93 (38)	*Ref*
Farm	16 (7)	8 (3)	2.27 (0.92-5.57)
House or row house	118 (49)	127 (53)	1.05 (0.71-1.55)
More than one type	25 (10)	14 (6)	2.03 (0.99-4.15)
Pets at home (yes)	106 (44)	98 (41)	1.14 (0.79-1.63)
Entry in daycare, median (IQR)[e]	612 (463-799)	601 (441-836)	1
Cessation of breastfeeding, median (IQR)[e]	313 (154-392)	310 (148-382)	1
Vaginal delivery (yes)	224 (92)	222 (91)	0.9 (0.48-1.7)
Duration of pregnancy, d, median (IQR)	281 (274-286)	282 (274-287)	0.99 (0.97-1)
Birth weight, g, median (IQR)	3610 (3228-3910)	3570 (3295-3884)	1

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; IgE, immunoglobulin E; IQR, interquartile range; OR, odds ratio.
The association between each factor and IgE sensitization was estimated by logistic regression analysis (univariate analysis). The results are presented as OR and 95% CI for IgE sensitization.
[a]Case, a child with at least one positive allergen specific IgE level at 18 and/or 36 months.
[b]Control, a child with negative allergen specific IgE levels at 6, 18, and 36 months.
[c]x, not DR3-DQ2 or a haplotype associated with protection against type 1 diabetes (T1D).
[d]y, not DR4-DQ8 or a haplotype associated with protection against T1D.
[e]Age of the child in days.

RNA was reverse-transcribed and amplified according to the manufacturer's protocol with QuantiTect Probe Kit (Qiagen) using primers and labeled probes (Thermo Fisher Scientific, Waltham, MA). RV was detected by forward primer 5′-CYA* GCC T*GC GTG GC-3′ (A* and T* locked nucleic acid primer by Exiqon, Vedbaek, Denmark); reverse primer GAA ACA CGG ACA CCC AAA GTA and probe VIC-TCC TCC GGC CCC TGA ATG YGG C-TAMRA.[19] For EV, the primers and probes applied were: forward primer: CGG CCC CTG AAT GCG GCT AA, reverse primer: GAA ACA CGG ACA CCC AAA GTA, probe 1: FAM-TCT GTG GCG GAA CCG ACT A-TAMRA, probe 2: FAM-TCT GCA GCG GAA CCG ACT A-TAMRA.[20] Primers and probes for NoV and HPeV detection were applied as described earlier.[21] All samples were tested in three reactions and if any of the reactions were positive that sample was interpreted as positive for the tested virus. The samples were analyzed blind to case-control status of the child.

2.3 | IgE antibodies

Serum samples taken at the age of 6, 18, and 36 months were analyzed by ImmunoCAP enzyme immunoassay (Thermo Fisher Scientific, Uppsala, Sweden) for IgE antibodies against cat, dust mite, birch, timothy, egg, milk, and wheat. IgE antibodies against peanut were analyzed at the age of 18 and 36 months and antibodies against dog at 36 months. All analyses were carried out blind to clinical

information. Values greater than or equal to 0.35 kU/L were considered positive.

2.4 | Statistical methods

Logistic regression analysis was applied to estimate odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for IgE sensitization. The association between virus exposure and IgE sensitization was evaluated by calculating the number of virus-positive stool samples in each child during the follow-up and comparing the number of positive samples in case and control children. After stratification by sex, this comparison was made between male cases and controls and accordingly, between female cases and controls. OR value expresses an estimation of how much each virus-positive sample affects the risk to become IgE sensitized. This estimate was calculated for each virus separately. Additionally, the number of case and control children having at least one virus positive sample during the follow-up was calculated for each of the four viruses and logistic regression analysis was applied to estimate the ORs and 95% CIs for IgE sensitization.

IgE values were measured at 6, 18, and 36 months of age and viruses were analyzed from stool samples collected monthly during the first year of life (Figure 1). To observe temporal relations between virus exposure and IgE sensitization, case children were divided into two groups; late sensitized (n = 162) and early sensitized (n = 57) (IgE values at 6 months were missing from 25 cases and 10 controls). The late sensitized group comprised of case children who were IgE negative at 6 months but became IgE positive later during the follow-up, and the early sensitized children were case children who were IgE positive already at 6 months. In the late and early sensitized groups, the associations between virus exposure and IgE sensitization were determined by calculating the number of virus-positive samples during the first 6 months of follow-up.

Children were also classified according to the nature of their IgE sensitization: children sensitized against at least one aeroallergen (cat, dog, dust mite, birch, timothy) were classified as "aeroallergen sensitized" (n = 104) and children sensitized against at least one dietary allergen (egg, milk, wheat, peanut) comprised the group of "dietary sensitized" (n = 214). Depending on their sensitization profile, case children could belong to one or both of these groups (Table S1).

Demographic characteristics of the study population are shown in Table 1. These data included: T1D-related HLA risk classes, parental atopy (having at least one of the following: asthma, hay fever/allergic rhinitis, or atopic dermatitis), having older siblings, smoking inside, place of residence ("urban": city center or residential areas, "rural": population centers/sparsely populated areas, and "both": change between the categories during the follow-up), type of housing, having furry pets at home, child's age at the beginning of daycare, duration of breastfeeding (including partial breastfeeding), route of delivery, duration of pregnancy, and birth weight. These factors were also addressed as possible confounding factors. Univariate logistic

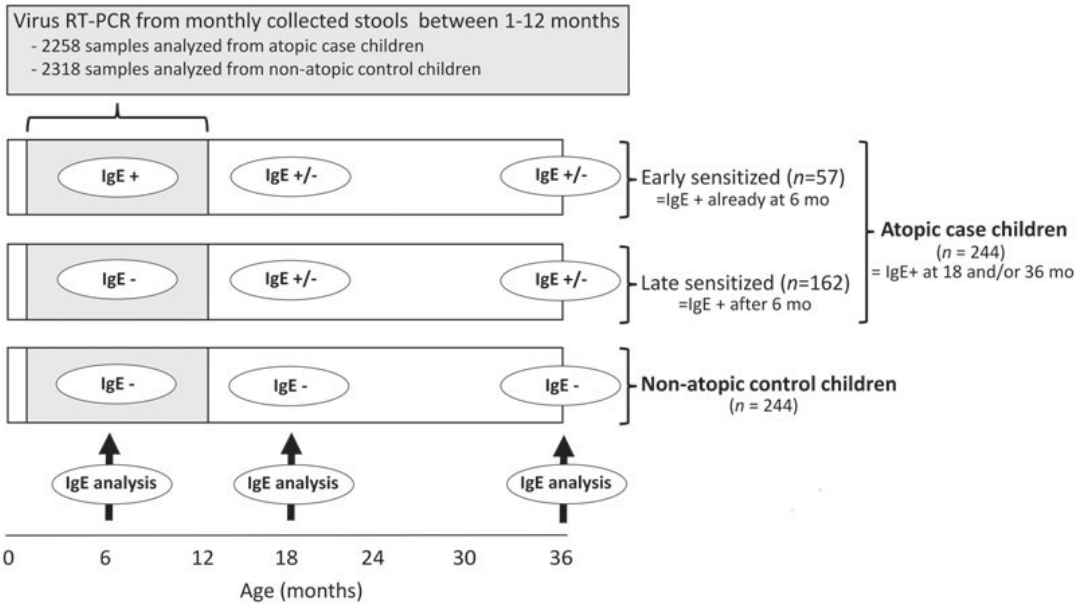

FIGURE 1 Timeline of the study. Allergen-specific IgE levels were measured at 6, 18, and 36 months. Stool samples were collected monthly during the first year of follow-up. Definition of an atopic case child was IgE sensitization at 18 and/or 36 months. These case children (n = 244) were divided into early sensitized and late sensitized according to their IgE levels at 6 months (data was missing from 25 case children). Control children were IgE negative at 6, 18, and 36 months (data at 6 months was missing from 10 control children). IgE, immunoglobulin E; RT-PCR, reverse transcription polymerase chain reaction

regression analysis was applied to estimate the ORs and 95% CIs for each factor (Table 1). When a significant effect on IgE sensitization was observed, logistic regression analysis was used to adjust for this factor. Unadjusted *P* values are presented in the text.

The Bonferroni correction was applied to counteract the problem of multiple comparisons. As the association between virus exposure and IgE sensitization was analyzed for four different viruses, the Bonferroni correction to control type I error was justified. After Bonferroni correction, $P < 0.013$ was considered statistically significant.

Analyses were performed by using R version 3.3.3 (2017-03-06, The R Foundation for Statistical Computing, https://www.R-project.org).

3 | RESULTS

Altogether 4576 stool samples were analyzed, 2258 from case children and 2318 from control children. The number of stool samples available for analysis each month is presented in Table S2. RV was the most prevalent virus detected during the first year of life, with 921 of 4576 (20%) positive samples. The number of positive samples for EV, NoV, and HPeV was 203 (4%), 243 (5%), and 194 (4%), respectively. RVs were prominent already during the first 6 months of life, whereas the prevalence of EVs, NoVs, and HPeVs started to increase later (Figure 2).

The number of children having at least one virus-positive sample during the follow-up did not differ between case and control children for any of the viruses. For RVs: 184 (75%) case children vs 187 (77%) control children had at least one positive sample (OR: 0.93; 95% CI: 0.62-1.42; $P = 0.75$), for EVs: 57 (23%) case children vs 51 (21%) control children (OR: 1.15; 95% CI: 0.75-1.77; $P = 0.51$), for NoVs: 69 (28%) case children vs 83 (34%) control children (OR: 0.76; 95% CI: 0.52-1.12; $P = 0.17$), and for HPeVs: 62 (25%) case children vs 66 (27%) control children (OR: 0.92; 95% CI: 0.61-1.38; $P = 0.68$).

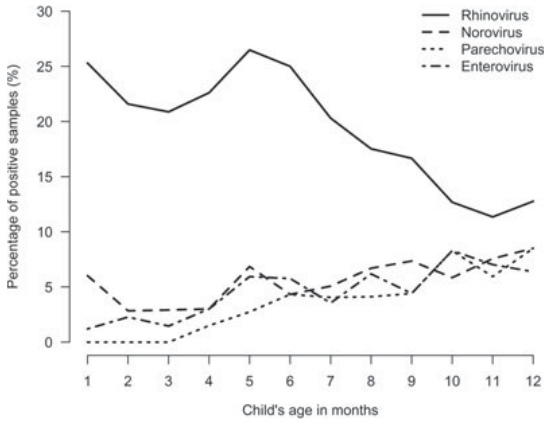

FIGURE 2 The percentage of reverse transcription quantitative polymerase chain reaction (RT-qPCR)-positive stool samples for each virus during the 12-month follow-up. The number of stool samples available for analysis each month is presented in Table S2. RT-qPCR, quntitative reverse-transcription polymerase chain reaction

The number of virus-positive stool samples during the follow-up did not differ between case and control children for any of the viruses in the full cohort (Table 2). However, after stratifying the study cohort by sex, the number of RV-positive samples was inversely associated with IgE sensitization in boys (OR: 0.81; 95% CI: 0.69-0.94; $P = 0.006$) but not in girls (OR: 1.02; 95% CI: 0.87-1.19; $P = 0.83$). In boys, 104 (74%) case children had at least one RV-positive stool sample vs 89 (84%) control children. In girls, 80 (77%) case children had at least one RV-positive sample vs 98 (71%) in control children. Sex did not influence the overall prevalence of RV infections, as the number of RV-positive samples did not differ between boys and girls: 485 (21%) vs 436 (19%), respectively. Stratification by sex did not have an effect on the associations observed with EV, NoV, or HPeV (Table 2). The demographic characteristics stratified by sex are presented in Tables S3 and S4. The number of case and control children with the positive paternal history of atopy differed statistically significantly in the full cohort and among girls. Adjusting for this confounder did not affect the results.

The sex-based difference in the association between RV exposure and IgE sensitization was observed also in the late and early sensitized groups (definitions in Table S1). In boys, the number of RV-positive samples during the first 6 months of life was inversely associated with subsequent IgE sensitization (late sensitized group): OR: 0.76; 95% CI: 0.6-0.94; $P = 0.016$, and simultaneous IgE sensitization (early sensitized group): OR: 0.6; 95% CI: 0.39-0.85; $P = 0.008$. In girls, no associations were observed. In addition, an inverse association between RV positivity and IgE sensitization was

TABLE 2 The number of virus-positive samples and associations with IgE sensitization

	Case *n* (%)	Control *n* (%)	OR (95% CI)	*P* value
Rhinovirus				
Full cohort	429 (19)	492 (21)	0.91 (0.82-1.01)	0.09
Boys	239 (19)	246 (24)	0.81 (0.69-0.94)	**0.006**
Girls	190 (19)	246 (19)	1.02 (0.87-1.19)	0.83
Enterovirus				
Full cohort	103 (5)	100 (4)	1.01 (0.80-1.20)	0.89
Boys	52 (4)	42 (4)	0.97 (0.70-1.30)	0.83
Girls	51 (5)	58 (5)	1.07 (0.80-1.40)	0.60
Norovirus				
Full cohort	111 (5)	132 (6)	0.90 (0.70-1.10)	0.29
Boys	58 (5)	59 (6)	0.80 (0.60-1.10)	0.19
Girls	53 (5)	73 (6)	1.00 (0.70-1.30)	0.88
Parechovirus				
Full cohort	92 (4)	102 (4)	0.90 (0.70-1.20)	0.55
Boys	56 (4)	51 (5)	0.90 (0.60-1.21)	0.43
Girls	36 (4)	51 (4)	1.00 (0.70-1.37)	0.80

Abbreviations: CI, confidence interval; IgE, immunoglobulin E; OR, odds ratio.

Each virus was analyzed from stool samples collected during the first year of life ($n = 4576$). The association between the number of virus-positive samples and IgE sensitization is presented as OR and 95% CI.

P values below 0.05 are marked bold.

detected in both aeroallergen and dietary sensitized boys (OR: 0.78; 95% CI: 0.63-0.94; $P = 0.014$ and OR: 0.82; 95% CI: 0.69-0.95; $P = 0.012$, respectively), whereas no association was observed in girls (Figure 3). The number of samples positive for EV, NoV, or HPeV was not associated with IgE sensitization in any of the case categories (data are not shown).

4 | DISCUSSION

The results from this prospective birth cohort study reinforce previous data that RVs are frequent in stool in infancy. The study demonstrates for the first time that RVs detected in stool are inversely associated with IgE sensitization. The study also reports a novel sex-based difference, as the inverse association between RV exposure and IgE sensitization was only observed in boys. Furthermore, RV detection during the first 6 months of life was inversely associated with subsequent IgE sensitization in boys. This temporal order, that is, RV exposure preceding IgE sensitization, suggests that RV infections in early life may act as protective environmental factors against IgE sensitization, but the effect seems to be associated with sex.

Previous studies have shown that RVs, although considered respiratory pathogens are detected frequently in stool in childhood.[14-17] The reasons for this phenomenon are not clear. RVs infect primarily nasopharyngeal epithelia, and RVs passing through the gastrointestinal tract is the most plausible explanation for detecting RV in the stool. RVs are also acid sensitive and thereby thought to degrade in the acidic gastric environment. One explanation for the high detection rate of RVs in stool in early life might lie in the relatively high gastric pH in infancy that might lead to diminished degradation of RV RNA in the stomach.[22] The prospective study setting in the present study allowed also detecting temporal changes in the virus prevalence; the number of RV-positive stool samples was high in early infancy and gradually decreased during the first year of life. The observed decreasing RV prevalence with age may well be

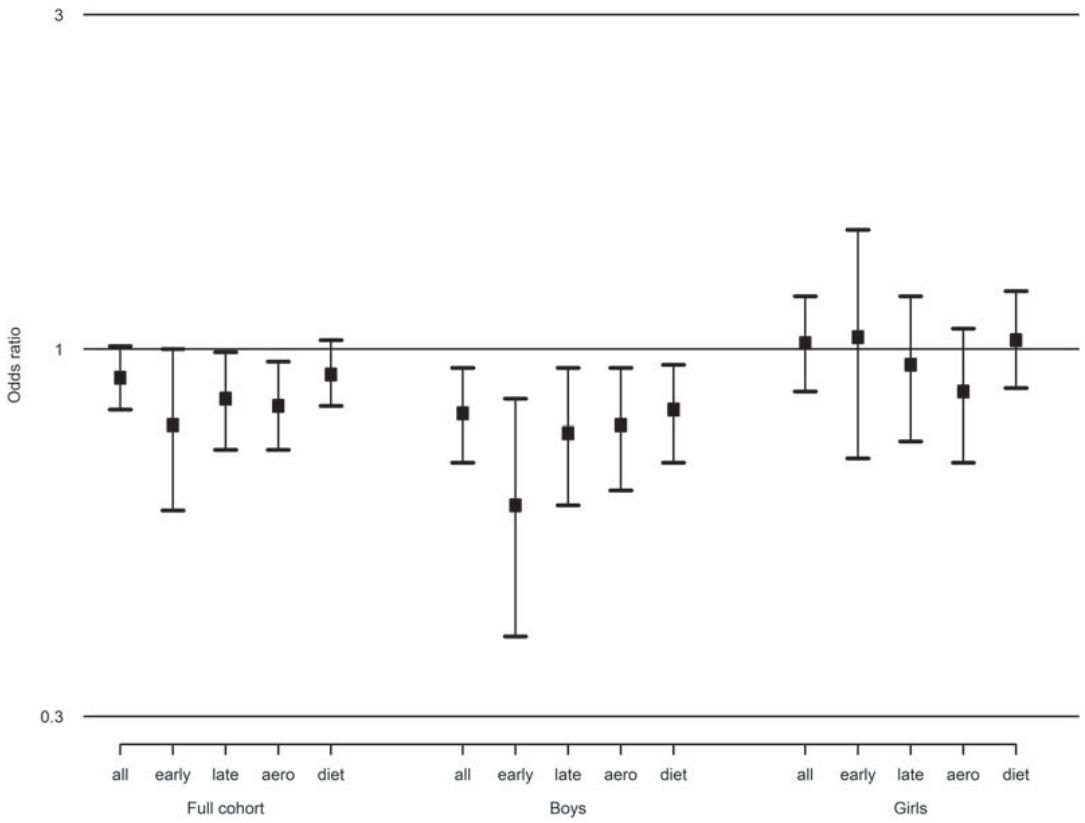

FIGURE 3 Associations between rhinovirus-positive stool samples and IgE sensitization in the full cohort and among boys and girls. Results are expressed as odds ratios (ORs) with 95% confidence intervals (CIs) for IgE sensitization. Aero, case children sensitized against aeroallergens; All, all case children, that is, children IgE sensitized at 18 and/or 36 months, ($n = 244$, 140 boys and 104 girls); Diet, case children sensitized against dietary allergens; Early, case children sensitized already 6 months; Late, case children sensitized at 18 and/or 36 months but not at 6 months

explained by a steep decrease in gastric pH during the first year of life, observed by Nagita et al.[22] There is also an in vitro study showing that acidic environment selects rapidly RV strains carrying a point mutation in the capsid protein conferring resistance to inactivation in low pH.[23] In addition to acid sensitivity, RVs have been thought to replicate optimally at temperatures of 33°C to 34°C equaling that of the upper airways. However, Papadopoulos et al[24] reported only minimal differences in the replication capacity of some RVs between temperatures of 33°C and 37°C. Even though not addressed in the current study, it may, therefore, be that some RV types also retain their viability in the gut, as suggested by the results showing RVs replicating upon cultivation after isolation from stool.[15,16]

RV-induced wheezing has been shown to increase the risk of childhood asthma[25] but there are fewer data about the role of RVs in IgE sensitization.[4,7,26] Jartti et al[7] reported a positive association between wheezing RV infection and IgE sensitization in a cross-sectional study setting. Later, a prospective study showed that aeroallergen sensitization predisposed children to RV-induced wheezing, but the opposite was not seen.[4] It might also be important to notice, that most previous studies have relied on samples taken during symptomatic RV infections, especially RV-induced wheezing, with few data derived from systematic sample collection regardless of symptoms.[13,27] In fact, there are indications that asymptomatic or mild RV infections might differ from severe ones in relation to atopy. Jackson et al[13] reported severe wheezing RV infections to increase the risk of asthma but found no association between asthma and RVs detected from nasal samples during prescheduled visits. In accordance, picornaviruses, including RVs, detected from routine nasal samples taken at four distinct time points during the first year of life, were shown not to associate with atopic sensitization.[27] Thus, it is possible that the association between RVs and wheezing episodes is characteristic for children who already have airway hyperreactivity, while RV infections that occur in early infancy may reduce the risk of IgE sensitization. The latter association may be more difficult to detect since it requires prospective birth cohort studies and depends on the sex of the child.

The sex-based difference observed is interesting, since susceptibility to a wide range of infections and immune-mediated diseases is influenced by sex. In general, boys have increased susceptibility to many virus infections whereas girls often develop stronger immune responses resulting in lower prevalence and intensity of infections.[28,29] There are not much prior data about possible sex-based differences in RV infections. Boys were shown to have more moderate-to-severe RV illnesses during early childhood as well as higher interferon-γ (IFN-γ) responses than girls did.[30] In addition, an animal study showed that early RV infections contributed to the development of allergen-induced lung disease in the female, but not in male mice.[31] In contrast, there is a well-known sex-based difference in the prevalence of IgE sensitization and childhood asthma with male sex predisposing to both.[30,32,33] The reasons for the male dominance in susceptibility to virus infections and atopy are not known but diverse and widespread effects of sex hormones probably play a role.[34]

Most previous birth cohorts on atopy have included children genetically at high risk for atopy. There is a strong and heterogeneous genetic component in childhood asthma[35] and maternal atopy has also been reported to increase the risk of severe RV bronchiolitis.[36] Furthermore, variants at the 17q21 gene locus have been shown to associate with asthma especially in children presenting with RV-associated wheezing illness.[37] It is, therefore, possible that in genetically selected cohorts, children are not only at higher risk for asthma but also for severe RV infections, which could contribute to the previously observed positive associations between RV infections and asthma. In contrast, the study population in the present study was selected for T1D-associated HLA types. There are some data linking HLA-DQ region to asthma, and HLA-DRB1 has also been linked to IgE sensitization.[38] However, as the genetic background of atopy is highly polymorphic and it has been estimated that HLA-DRB1 locus only accounts for 2% to 3% of the variation in specific IgE titers,[38] it is unlikely that the HLA selection would have substantially influenced our results. Furthermore, the distribution of HLA-DR/DQ types was similar in case and control groups.

RV infections are generally diagnosed by RT-PCR assays targeting the 5′ noncoding region (5′-NCR) of the RV genome.[39] Due to sequence similarities in the 5′NCR between RVs and EVs, it is known that some RV PCR assays detect also EVs thereby reducing the specificity of the assays. In a study by Lu et al,[19] the RV RT-PCR assay applied in the present study was shown to amplify all of the 100 RV prototype strains and 85 field isolate strains included in their sensitivity and specificity analysis. The assay did not amplify 34 of the 48 EV types included, while 14 EV types gave weakly positive reactions when present in the sample in high titers.[19] In addition, the RV RT-PCR primers and probes applied in the present study were included in the study by Faux et al[40] comparing the usefulness of different PCR primers in detecting RV infections. The RV RT-PCR assay in question, amplified 20 out of the 29 RV types including types from all RV species (*Rhinovirus A-C)* and most importantly, the assay did not amplify any of the EVs included.[40] These studies confirm that the RV RT-PCR primers and probes applied in the current study are both sensitive and specific. However, the possibility that some RVs might have been missed or that there might be some low-degree cross-detection between RVs and EVs cannot be excluded.

In the current study, no genotyping of RVs was performed. Thereby, the results do not indicate whether consecutive RV-positive stool samples represent a single prolonged infection or separate independent infections. However, in a former study by our group, 43 different RV types were identified among the 63 RV-positive stool samples sequenced and only once was the same genotype detected in two sequential samples.[15] This finding by Honkanen et al is an illustration of the high genetic diversity characteristic for RVs and suggests that shedding of RVs in the stool is short-term. These previous results support the current interpretation that each RV-positive sample represents individual exposure and the high

frequency of RVs in stool probably reflects the diversity of circulating RVs.

The allergen panel applied in the IgE analysis was defined according to local sensitization patterns against dietary and aeroallergens.[41] It includes the majority of the relevant allergens and is thereby likely to detect most IgE sensitized children in this age group. However, some children sensitized only to some of the more uncommon allergens might have been missed but the number of these children is estimated to be low.

In conclusion, this study provides new insights into the role of different viruses, especially RVs, in atopic sensitization. Data based on monthly collection of stool samples demonstrated that RVs are commonly present in young infants' gut and that this RV exposure was associated with a reduced risk of IgE sensitization in boys. The findings suggest that RVs might have a more versatile role in the development of atopy than previously perceived. We hypothesize that early RV exposure could serve a beneficial function in driving postnatal maturation of the immune competence, thereby reducing the risk of allergic sensitization. In addition, the clear sex-based difference in the effect of RVs observed emphasizes the importance of exploring the impact of sex also in future studies aimed at elucidating the mechanisms of atopic disease.

ACKNOWLEDGMENTS

The authors would like to thank the DIABIMMUNE study group. The authors are grateful to Katriina Koski and Matti Koski for their valuable assistance in database work and to Pekka Keränen, Tanja Kuusela, and Maria Ovaskainen for their skillful technical assistance. The authors would also like to thank the local study personnel and the participating families for their collaboration. This study was funded by the European Union Seventh Framework Program FP7/2007-2013 (grant agreement no. 202063), Tampere Tuberculosis Foundation, Sigrid Juselius Foundation, Academy of Finland (Prevaller Consortium, grant no. 129448-2008; Research grant no. 132362-2009; Center of Excellence in Molecular Systems Immunology and Physiology, grant no. 250114 2012-2017), The National Technology Agency in Finland (TEKES), Päivikki and Sakari Sohlberg Foundation, Orion-Farmos Research Foundation, and the Estonia Research Council grant PUT 1382.

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CONFLICT OF INTERESTS

Professors Hyöty and Knip are minor shareholders of Vactech Ltd developing vaccines against picornaviruses. Remaining authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Korhonen L, Oikarinen S, Lehtonen J, et al. Rhinoviruses in infancy and risk of immunoglobulin E sensitization. *J Med Virol*. 2019;1-9.
https://doi.org/10.1002/jmv.25455