Helminth exposure and its association with allergies and lung function in Northern European cohorts

Nils Oskar Jõgi

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The work in this thesis was conducted at the Department of Clinical Science at the University of Bergen and at the Department of Occupational Medicine Haukeland University Hospital. It was financed by the University of Bergen (UiB PhD Scholarship). During the research work I have been in collaboration with the RHINE, ECRHS and RHINESSA consortium and a member of the BRuSH research group.

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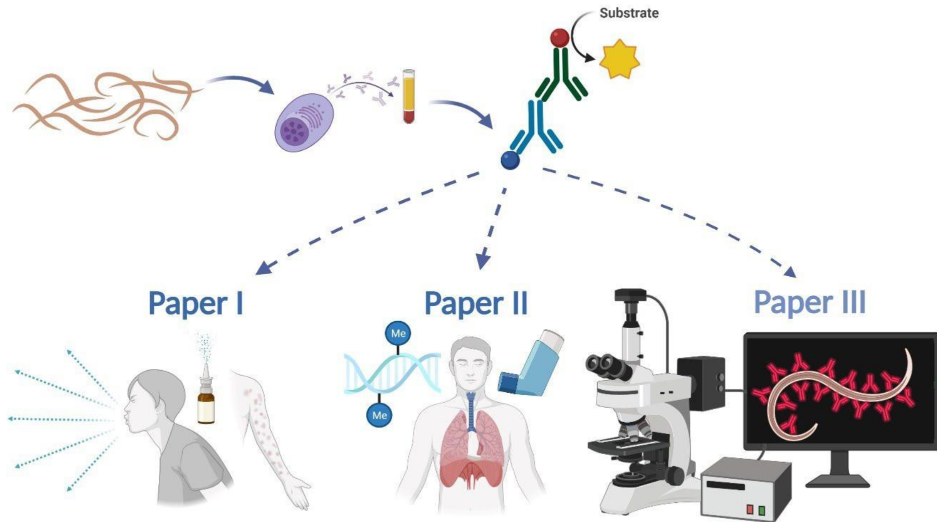
Abbreviations

AD	Atopic dermatitis
ASC	*Ascaris*
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary disease
DNMA	DNA methyltransferase
dPCR	Digital polymerase chain reaction
ECRHS	European Community Respiratory Health Survey
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory-secretory
EV	extracellular vesicle
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GLI	Global Lung Initiative
GSTA	*Ascaris* glutathione transferase
GWAS	Genome wide association studies
HDM	House dust mite
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
ILC2	Type 2 innate lymphoid cells

NTU	NovaTec Units
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween
PCR	Polymerase chain reaction
PNPP	p-Nitrophenyl Phosphate
PRR	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RHINESSA	Respiratory Health in Northern-Europe, Spain and Australia
SPT	Skin prick test
STH	Soil-transmitted helminth
TGF-β	Transforming growth factor beta
Th1	T-helper 1
Th17	T-helper 17
Th2	T-helper 2
TNF-α	Tumour necrosis factor alpha
TSLP	Thymic stromal lymphopoietin

Thesis at a glance



Overview of the three papers included in this thesis:

Paper I: Helminth exposure (*Toxocara, Ascaris*) and allergies in two generations in Norway: exposure associated with increased risk of allergies in younger but not in older adults.

Paper II: Helminth exposure in three Northern European countries: *Ascaris* seropositivity associated with lower lung function and DNA methylation in men.

Paper III: Immunofluorescent imaging of measured IgG4 antibody binding with cultured *Ascaris* larvae: IgG4 response and binding to larvae is not driven by HDM sensitization.

Abstract in English

Background: The prevalence of asthma and allergies have increased all over the world during the past few decades. In countries where helminth infections are endemic, the prevalence of asthma and allergies appears to be lower. Therefore, it has been hypothesized that helminth infections might confer protection against allergic diseases. Results from studies linking helminth infections and allergies are still controversial with most studies addressing allergy in children in endemic regions.

The round worm, *Ascaris lumbricoides,* is a common helminth infection worldwide with an estimated prevalence of 800 million people. During its life cycle, *Ascaris* migrates through the lungs causing direct damage. The infection is also likely to cause long-term altered immune responses. Data addressing the prevalence of helminths infections and their association with allergies and asthma in Europe is scarce. High sanitary standards are believed to have broken the life cycle of many helminthiases.

Objectives: 1) Describe the prevalence of *Ascaris* and *Toxocara* seropositivity and test for association with allergies in two generations in Norway. 2) Investigate the association of lung function, asthma, and DNA methylation with helminth exposure in three Northern European cohorts. 3) Explore if serum anti-*Ascaris* IgG4 from European cohorts binds to *Ascaris* larvae, and test if house dust mite (HDM) sensitization leads to anti-*Ascaris* IgE and IgG4 responses in humans.

Materials and Methods:

1) Serum levels of IgG4 against *Ascaris spp.* and *Toxocara spp.* were established by end point titration ELISA in two cohorts: parents born 1945-1972 (n = 171) from the ECRHS III study and their offspring born 1969-2003 (n = 264) from the RHINESSA study. Serum samples were first pre-incubated on plates covered with *Ascaris* proteins before measuring antibody levels towards *Toxocara*, in order to reduce cross-reactivity with *Toxocara* spp. Allergic outcomes and covariates were recorded through interviews and clinical examinations including measurement of serum IgE levels and skin prick tests. Multivariate logistic regression models were used to measure the association between seropositivity and allergic outcomes.

2) Serum IgG antibodies against *Ascaris* were measured with ImmunoCAP (ThermoFisher) in 671 adults aged 18-47 years (46% women) from Aarhus, Bergen,

and Tartu RHINESSA study centres. Seropositivity was defined as IgG above the 90th percentile. Multivariate linear and logistic regression models were used to analyse *Ascaris* seropositivity as associated with lung function measured via spirometry, and asthma. All models were adjusted for age, height, smoking, and clustered by study centre. DNA methylation was measured from peripheral blood using Human-MethylationEPIC (Illumina, USA) chip, which measures 850k 5′-C-phosphate-G-3′ (CpG) sites. Additionally, in the Bergen study centre, IgG antibodies against five helminths were measured with commercially available ELISA kits (NovaTec Immunodiagnostica, Germany).
3) Men from the RHINESSA Bergen study centre were divided into groups, based on their HDM sensitization and *Ascaris* IgG4 seropositivity. Four different *Ascaris* antigens were used as coating antigens for the IgG4 and IgE ELISAs to confirm *Ascaris* exposure. Kruskal-Wallis test followed by Dunn's Multiple comparison test was used to identify significant differences ($p<0.05$) between the groups. The presence of serum IgG4 and IgE binding to the larvae was imaged using fluorescence microscopy.
Results: 1) Anti-*Ascaris* spp. IgG4 was detected in 29% of the parent generation and in 10% of the offspring, anti-*Toxocara* spp. IgG4 in 18% and 8% of parents and offspring, respectively. Among offspring, *Toxocara* seropositivity was associated with pet keeping before age the of 15 (OR = 6.15; 95%CI = 1.37-27.5) and increasing BMI (1.16 [1.06-1.25] per kg/m2). *Toxocara* seropositivity was associated with wheezing (adjusted odds ratio 2.97 [1.45- 7.76]), hay fever (4.03[1.63-9.95]), eczema (2.89 [1.08-7.76]) and sensitization towards cat allergens (5.65 [1.92-16.6]) among offspring, but was not associated with allergic outcomes among parents. Adjustment for childhood or current pet keeping did not alter associations with allergies. Parental *Toxocara* spp. seropositivity was associated with increased offspring allergies following a sex-specific pattern, although the exposure was measured years after the birth of the offspring. 2) Among the five helminths measured, *Ascaris* exposure was the most common. *Ascaris* seropositivity was associated with lower FEV_1 (-247 mL [-460, -34]) and higher odds for ever reporting asthma (aOR 5.84 [1.67, 20.37]) among men but not women, also after further adjusting for HDM sensitization. These results were consistent across study centres. At a genome-wide level, *Ascaris* exposure was associated with 23 differentially

methylated DNA sites in men and three in women. We identified hypermethylation of the MYBPC1 gene, which is involved with regulation of airway muscle contraction. We also identified genes linked to asthma pathogenesis such as CRHR1 and GRK1, as well as a differentially methylated region in the PRSS22 gene, previously linked to nematode infection, suggesting that we are measuring true helminth exposure. 3) Serum IgG4 levels against *Ascaris* were consistent using the different antigens. We detected IgG4 binding to *Ascaris* larvae only from sera that had raised *Ascaris* specific IgG4 levels measured with the ELISAs. HDM sensitization only (without concurrent *Ascaris* IgG4 seropositivity) did not facilitate serum IgG4 or IgE binding to *Ascaris* larva. Furthermore, no *Ascaris* specific IgE levels were detected in any of the samples, and no specific or non-specific IgE binding to larvae was detected.

Conclusion: 1) Helminth exposure in Norway was less frequent in younger than older adults. However, seropositivity was associated with increased risk of allergic manifestations in the younger generation, but not among their older parents. Potential changes in response to helminth exposure may provide insights into the increase in allergy incidence in affluent countries. 2) *Ascaris* exposure was associated with lower lung function and increased asthma risk among men. Seropositive participants had sex-specific differences in DNA methylation compared to the unexposed, thus, suggesting that *Ascaris* exposure may lead to sex-specific epigenetic changes that might contribute to effects on lung pathology. 3) Our results suggest that HDM sensitization does not lead to IgG4 or IgE cross-sensitization towards *Ascaris,* but that anti-*Ascaris* IgG4 appears to also bind to HDM allergens. Thus, IgG4 against Ascaris and HDM allergens appear to reflect *Ascaris* exposure and not HDM sensitization.

Implications: Results from this PhD project suggest that exposure to helminths might be an overlooked risk factor in the development of allergies, asthma and low lung function in Northern Europe. From a public health perspective, future research should focus on pinpointing the source of this neglected exposure. Overall, our findings identify a need to investigate the role of helminths on long-term lung health globally,

including in high- and middle-income countries, as well as in low-income countries, where helminth exposure is highly prevalent.

Abstract in Norwegian

Bakgrunn: De siste tiårene har forekomsten av astma og allergi økt over hele verden. I land hvor parasittinfeksjoner er endemiske, er forekomsten av astma og allergi lavere enn i land med lavere forekomst av parasitter. En hypotese er at parasittinfeksjoner kan beskytte mot allergiske sykdommer. Studier som har undersøkt dette er fremdeles kontroversielle, de fleste studier er fra endemiske områder og studiene gjelder oftest barn og ikke voksne. Rundormen *Ascaris lumbricoides* forårsaker en av de vanligste parasittinfeksjonene på verdensbasis med om lag 800 millioner smittetilfeller årlig. I løpet av livssyklusen, passerer *Ascaris* lungene. I tillegg til direkte skade på vev, kan infeksjonen føre til vedvarende immunologiske forandringer. I europeiske land antar man at denne typen infeksjoner begrenses av gode sanitære forhold, men forekomsten av parasitter som helminter (rundorm) og hvordan disse påvirker astma og allergi er lite studert i høyinntektsland.

Formålet med studien er å: 1) Beskrive forekomst av *Ascaris* og *Toxocara* seropositivitet og eventuell sammenheng med allergiske sykdommer i to generasjoner i Norge; 2) Undersøke sammenhengen mellom helmintеksponering og lungefunksjon, astma og DNA-metylering i tre kohorter fra Nord-Europa; 3) Undersøke om humant serum med *Ascaris* IgG4 binder seg til *Ascaris*-larver, og om husstøvmidd (HDM) sensibilisering bidrar til anti-*Ascaris* IgE- og IgG4-responser i humant serum.

Material og metode: 1) Konsentrasjon av serum IgG4 mot *Ascaris* spp. og *Toxocara* spp. ble målt med endepunkttitrering i to kohorter: Foreldre fra ECRHS III-studien født 1945-1972 (n=171), og barna til disse deltagerne født 1969-2003 (n=264) (RHINESSA-studien). Data om allergisk sykdom og symptomer og andre faktorer ble samlet inn gjennom intervju og kliniske undersøkelser, bl.a. med måling av IgE i serum og hudprikktest for allergisk sensibilisering. Multivariat logistisk regresjon ble benyttet for å analysere sammenheng mellom seropositivitet og allergiske utfall.

2) IgG-antistoffer mot *Ascaris* i serum ble kvantifisert med ImmunoCAP (ThermoFisher) hos 671 voksne i alderen 18-47 år (46% kvinner) fra RHINESSA sentre i Aarhus, Bergen og Tartu. Seropositivitet ble definert som IgG over 90 persentilen. Multivariat logistisk/lineær regresjon ble anvendt for å analysere sammenhengen mellom *Ascaris* seropositivitet og astma/lungefunksjon. Alle modeller ble justert for alder, høyde og røyking, og vektet i forhold til studiesenter. DNA-metylering ble målt med Human-MethylationEPIC-chip (Illumina, USA), som dekker 850 CpG sites. I tillegg ble IgG-antistoffer mot fem helminter målt med kommersielt tilgjengelig ELISA kits (NovaTec Immunodiagnostica, Tyskland) for deltakere fra Bergen RHINESSA.

3) ELISA med fire forskjellige *Ascaris*-antigener for IgG4 og IgE binding ble benyttet for å bekrefte *Ascaris*-eksponering, hos mannlige RHINESSA deltagere fra Bergen senter kategorisert på bakgrunn av HDM sensibilisering og *Ascaris* IgG4 serum positivitet. Kruskal-Wallis test og Dunns Multiple sammenligningstest ble bruk for å identifisere signifikante forskjeller ($p<0.05$) mellom gruppene. Fluorescensmikroskopi ble benyttet for å undersøke IgG i serum og IgE binding til *Ascaris*-larvene.

Resultater: 1) Anti-*Ascaris* spp. IgG4 påvist i serum hos 29% av deltakerne fra den eldre generasjonen (foreldre) og hos 10% av deres «barn», den yngre generasjonen. Anti-*Toxocara* spp. IgG4 ble påvist hos 18% av foreldrene og hos 8% av barna. Hos den yngre generasjonen var *Toxocara* seropositivitet assosiert med å ha hatt kjæledyr før 15 års alder (odds ratio (OR) = 6.15; 95%CI = 1.37-27.5) og økende BMI (1.16 [1.06-1.25] per kg/m2). *Toxocara* positivitet var assosiert med piping i brystet (justert OR 2.97 [1.45- 7.76]), høysnue (4.03[1.63-9.95]), eksem (2.89 [1.08-7.76]) og sensibilisering mot katt (5.65 [1.92-16.6]) for deltakere i den yngste generasjonen, men ikke for den eldre generasjonen. Selv når kjæledyrhold ble tatt med i betraktningen endret ikke dette sammenhengen mellom *Toxocara* seropositivitet og allergi. *Toxocara* spp. Seropositivitet i foreldre-generasjonen var assosiert med høyere forekomst av allergi hos deres barn (kjønns-spesifikke mønster); det bemerkes at eksponeringen ble målt flere år etter at barna var født.

2) Av de fem helmintene som ble målt med kommersielt ELISA kit, var positivt utslag på *Ascaris* mest hyppig. *Ascaris* seropositivitet var assosiert med lav FEV_1 (-247 mL [-

460, -34]) og høyere odds for noen gang å ha hatt astma (aOR 5.84 [1.67, 20.37]); dette gjaldt kun for menn. Funnene endret seg ikke etter å ha tatt høyde for HDM sensibilisering, og til stede for alle studiesentre (Aarhus, Bergen og Tartu). Epigenetikk analysene viste at *Ascaris* eksponering var forbundet med endret DNA metylering for 23 CpG lokasjoner hos menn og tre hos kvinner. Vi identifiserte hypermetylering i MYBPC1 gener, som er involvert i regulering av luftveiskontraksjon, samt endret metylering av gener som påvirker astma patogenese, slik som CRHR1 og GRK1, og av PRSS22 genet som er assosiert med nematode infeksjon. De epigenetiske analysene understøttet dermed helmint eksponeringsmålingene i populasjonene våre, sammenheng med lungehelseutfall samt kjønnsforskjell i sammenhengene.
3) Serum IgG4 binding mot *Ascaris* var konsistent, selv når vi benyttet forskjellige antigener. Vi påviste IgG4 binding til *Ascaris* larver kun for serum med forhøyet nivå av *Ascaris* spesifikk IgG4 (målt med ELISA), mens sensibilisering mot husstøvmidd alene (uten *Ascaris* IgG4 seropositivitet) ikke førte til IgG4 eller IgE binding mot *Ascaris* larver. Ingen av prøvene hadde påvisbare nivåer av IgE mot *Ascaris* og det ble heller ikke observert spesifikk eller uspesifikk IgE binding mot larvene.

Konklusjon: 1) I Norge var eksponering for parasittene *Ascaris* og *Toxocara* mindre vanlig hos yngre enn hos eldre generasjoner. Imidlertid var seropositivitet mot disse assosiert med økt risiko for allergisk sykdom og symptomer hos den yngre generasjonen, men ikke hos den eldre generasjonen. Våre funn tyder på at parasitt eksponering per i dag påvirker immunsystemet på en slik måte at det kan gi økt risiko for allergi også i høyinntektsland.
2) *Ascaris* eksponering var assosiert med lavere lungefunksjon og økt risiko for astma hos menn. Deltagere som var *Ascaris* seropositive hadde endringer i DNA-metylering, forskjellig for menn og kvinner. Dette kan tyde på at *Ascaris* eksponering kan føre til kjønnsspesifikke epigenetiske endringer i DNA metylering som kan påvirke lungepatologi.
3) Resultatene tyder på at sensibilisering mot husstøvmidd ikke gir IgG4 eller IgE kryss-sensibilisering mot *Ascaris*, men at IgG4 mot *Ascaris* også kan binde mot allergen fra husstøvmidd. Oppsummert er dette en indikasjon på at IgG4 mot *Ascaris* og HDM reflekterer *Ascaris* eksponering og ikke HDM sensibilisering.

Implikasjoner: Resultatene fra dette PhD-prosjektet tyder på at parasitteksponering i Nord-Europa kan være en risikofaktor for astma, allergi og nedsatt lungefunksjon som har vært oversett. I et folkehelseperspektiv bør det forskes mer på kilder til og eksponeringsveier for parasitter. Vi har vist i dette prosjektet at det er viktig å undersøke hvordan parasitt eksponering påvirker lungehelse i et globalt perspektiv, det gjelder både høy- og middelinntektsland, og ikke minst lavinntektsland hvor parasittinfeksjoner er svært vanlig.

List of Publications

This thesis is based on the following three original papers:

I. Jõgi NO, Svanes C, Siiak SP, Logan E, Holloway JW, Igland J, et al. Zoonotic helminth exposure and risk of allergic diseases: A study of two generations in Norway. Clin Exp Allergy 2018; 48:66-77..

II. Jõgi NO, Kitaba N, Storaas T, Schlünssen V, Triebner K, Holloway JW, Horsnell WGC, Svanes C, Bertelsen RJ. Ascaris exposure and its association with lung function, asthma, and DNA methylation in Northern Europe. J Allergy Clin Immunol. 2021 Dec 14:S0091-6749(21)01797-8. doi: 10.1016/j.jaci.2021.11.013. Epub ahead of print. PMID: 34996616.

III. Jogi NO, Murangi T, Amoa T, Darby M, Sebaa S, Levin M, Davies N, Storaas T, Bertelsen RJ, Svanes C, Falcone F, Horsnell WGH. IgE or IG4 response against *Ascaris* in Northern Europe is independent of house dust mite sensitization. (Submitted 2022)

1. Introduction

1.1 Asthma and allergies

According to the Global Initiative for Asthma (GINA), asthma is a heterogeneous disease defined by the history of respiratory symptoms (e.g., wheeze, shortness of breath, chest tightness and cough) that vary over time and in intensity, together with variable expiratory airflow limitation [1]. The prevalence of asthma is over 20% worldwide and is increasing according to a recent report from a study including almost 120 000 adolescents from 14 countries, ranging from 1% in India to nearly 30% in some parts of Spain. Although, the prevalence can be substantially heterogeneous also within countries. Substantially lower prevalence of asthma symptoms has been shown in low-income countries, while an increase (with urbanisation) in lower-middle-income countries has been observed, with no evidence of change in asthma symptoms in more affluent countries. Overall, severe asthma seems to be decreasing, while ever reporting to have asthma to be increasing [2]. This suggests that asthma prevalence is still on the rise, but due to the improved diagnostic and treatment accessibility, the reporting of asthma symptoms seems to be levelling out in more affluent countries. As the diagnosis is based mostly on symptoms, the cultural differences and language variations in describing these symptoms might influence the reporting of ever having asthma or wheeze in different regions. To be noted, there are also much fewer studies in low- and middle-income countries than in high income countries.

In Europe it has been argued that geographical variations in the prevalence of asthma are most likely due to environmental factors [3]. Our study population in this thesis involves participants from three European study centres from Denmark, Estonia and Norway. The prevalence of asthma in Norway is reported to be rising, with 7.3% reported in 1985 and 17.6% in 2008 [4]. In Denmark, however, the trend in prevalence of asthma has been stable for the last 20 years at around 3.4% [5]. In Estonia, the prevalence is reported to be very similar to Denmark with 3.6% [6]. Although, self-reported asthma attacks and asthma medication use have increased in the past the 20 years [7].

Multiple factors can be associated with the increased prevalence of asthma. In addition to the real increase of asthma [8], a higher awareness of asthma, increased availability of diagnostic tests and the change in the definition of asthma over time can explain this increase. Data collected over several decades in Europe indicate that although the prevalence is rising [9], symptoms of asthma are decreasing due to smoking cessation [9] and availability of effective treatments [2]. Nevertheless, mortality from asthma and the loss of quality of life from uncontrolled asthma [10] remain a heavy burden to society today [11].

Asthma is a multifactorial and heterogenous disease, and a full understanding of the pathogenesis is a challenge and yet to be explained [12]. There is a variety of definitions and categorizations of asthma phenotypes and subgroups. Asthma phenotyping initially focused on combinations of clinical characteristics but is now evolving to link biology to phenotype [13]. However, the overlap in characteristics in different phenotypes is substantial [14], making the differentiation of various subgroups very difficult. Allergic asthma and non-allergic asthma are the two most commonly defined phenotypes. Allergic asthma can be defined as asthma associated with sensitization to aeroallergens, which leads to asthma symptoms and airway inflammation. The onset of allergic asthma is often in childhood and can be accompanied by other comorbidities including atopic dermatitis (AD) and allergic rhinitis [15].The incidence of allergic asthma is highest in early childhood and steadily decreases with age, while the incidence of non-allergic asthma is lower until it peaks in late adulthood [16].

One of the plausible mechanisms for the development of allergic diseases is "the epithelial barrier hypothesis" [17]. The barrier hypothesis of type 2 inflammatory disease postulates that barrier dysfunction causes inappropriate exposure of the epithelial and epidermal tissue to the environment, and can result in allergic sensitization and development of type 2 inflammatory disease. Furthermore, it has been proposed that environmental changes caused by industrialization, urbanization and a westernized lifestyle affect the epithelial barrier of the skin, upper and lower airways, and gut mucosa. Such development of leaky epithelial barriers then leads to microbial dysbiosis. The translocation of bacteria to interepithelial and subepithelial areas, and

the development of tissue microinflammation. Moreover, it has been suggested that these processes underlie not only the development of allergy and autoimmune conditions in barrier-damaged tissues but also a wide range of diseases in which immune responses to translocated bacteria have systemic effects [18]. This is supported by research into the development of AD in infancy and subsequent allergic rhinitis and asthma in later childhood. Studies support the idea of a causal link between AD and the later onset atopic disorders. These studies suggest that a dysfunctional skin barrier serves as a site for allergic sensitization to antigens and colonization of bacterial superantigens whichinduces systemic Th2 immunity. This predisposes patients to allergic nasal responses and promotes airway hyperreactivity [19] and can manifest as allergic rhinitis, which is predominantly caused by environmental allergen exposure in genetically predisposed individuals. Common allergens implicated in allergic rhinitis are mainly proteins and glycoproteins found in airborne particles. Important allergens causing intermittent or persistent symptoms vary in different parts of the world, with around 25 % of world's population being affected by allergic rhinitis [20]. Sensitization to allergens can be measured by the presence of specific IgE antibodies in serum or with a skin prick test (SPT) in order to measure the skin's reactivity to allergens.

As for the complexity of different mechanisms in the development of asthma and manifestations as different phenotypes the research in the field of causes and risk factors for asthma and allergies are still of great interest to the scientific community. However, we did not distinguish between different asthma phenotypes in this thesis. Allergen sensitization will only be discussed in relation to aeroallergens; whereas food sensitization or other types of allergies will not be discussed.

1.2 Lung function

Spirometry is used to measure important aspects of lung function. There are three basic related measurements: volume, time and flow. Spirometry is objective, non-invasive, reproducible, and sensitive to early change. The main spirometry measurements are: forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and the ratio between these (FEV_1/FVC).

FVC responds to the maximum amount of air that can be exhaled when blowing out as fast as possible, in a "forced" manoeuvre. FEV_1 responds to the amount of air exhaled during the first second of the manoeuvre. FVC and FEV_1 should be measured from a series of at least three forced expiratory curves that have an acceptable start of test and are free from artefacts, such as a cough (e.g. "usable curves") [21]. These measurements are used to determine whether there is any obstruction (e.g. asthma; chronic obstructive pulmonary disease (COPD)) present, or they can be indicative of restriction (e.g. pulmonary fibrosis; extra pulmonary causes that limit the movement of the chest wall). Obstruction in asthmatics is usually reversible but not always present. Thus, a normal spirometry result does not exclude the diagnosis of asthma. During an asthma attack or exacerbation, the FEV_1/FVC ratio is usually reduced and reversible. Reversibility is measured by administering a bronchodilator, commonly a short acting $beta_2$-agonist, to relax the smooth muscles of airways and then spirometry is repeated. A positive bronchodilator test with an increase in FEV_1 of $\geq$ 12% or 200mL is indicative of asthma [1]. To assess and compare lung function to the "norm", reference values are established that take into account the sex, age, height and ethnicity of the participant. The Global Lung Initiative (GLI) reference values are currently the reference material that are considered the "norm". The GLI is led by six international societies, which describe normal spirometry values in people aged 3 to 95 years in 26 countries [22]. The data is mostly based on Caucasian subjects, thus, applicable for the study population reported on in this thesis. The range of values obtained from such a "healthy population" is assumed to represent normal [23]. The normal lung function trajectory from birth to death has three phases (see Figure 1). The growth phase, in which lung function increases as lungs mature (particularly in puberty), is followed by the plateau phase from around 20-25 years (but earlier in women) ending with the final decline phase due to physiological lung ageing. Numerous genetic and environmental factors (e.g. smoking) can alter one or more of these phases resulting in a range of lung function trajectories (Figure 1; reproduced from Agusti et al [24]) that can have important clinical consequences [24].

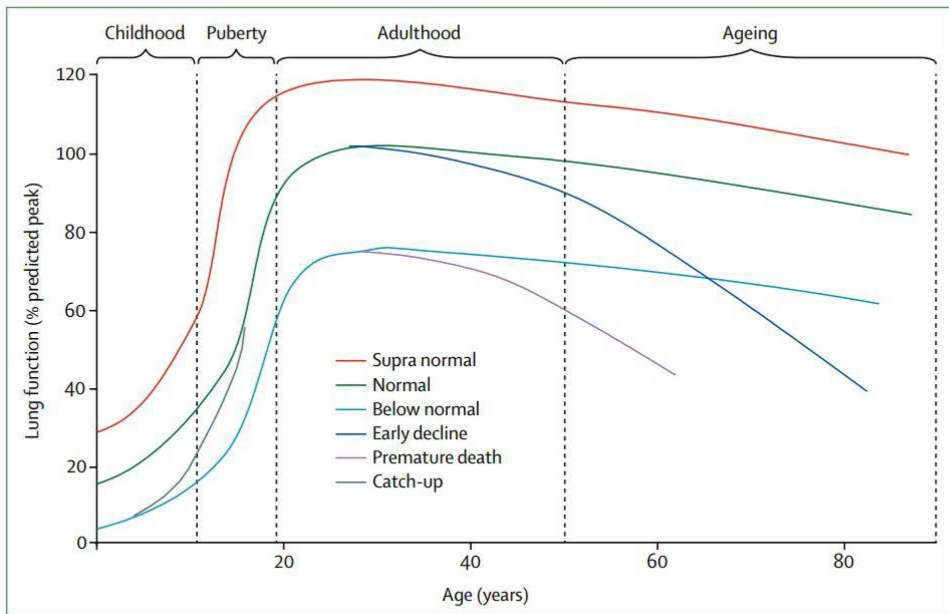


Figure 1: **Potential lung function trajectories throughout the life course**

1.3 Soil-transmitted helminth infections in Europe

Soil-transmitted helminth (STH) infections are among the most prevalent of chronic human infections worldwide [25]. More than a quarter of the world's population is at risk of infection with the soil-transmitted helminths *Ascaris lumbricoides*, hookworm (*Ancylostoma duodenale* and *Necator americanus*), *Trichuris trichiura*, and *Strongyloides stercoralis* [26]. *Ascaris* is considered to have the highest prevalence, with over 800 million people likely to be infected as of 2010 [27]. The prevalence of *Ascaris* infection can reach >50% in some regions in East-Asia and Sub-Saharan Africa. In endemic regions nearly all the population is likely to have been exposed at some point [28] [29]. Some regions, including Europe, are considered to be non-endemic, as the cycle of infection is believed to be broken through the implementation of advanced public health provisions, especially in relation to water and sewage treatment [30]. Helminth infections are not considered a public health threat and even recreational bathing spots are not considered as likely sources of helminth exposure [31]. However,

studies addressing helminth infections in Europe are relatively scarce. Recent studies have nonetheless reported increased detection of *Strongyloides* spp infections in some regions. This is attributed to immigration and travel from endemic areas [32] and is not considered as endemic in Europe [33]. Hookworm infections are extremely rare in Europe with only a few cases reported each year. However, several cases of local infection have been reported for hookworm (as well as *Strongyloides*) in Southern-France [34] and Spain [35], indicating that these infections could be relevant and should not be neglected in the future.

T. trichuria aka whipworm infection is rarely reported and mostly as an accidental finding [36]. However, recent studies from Poland indicate that the prevalence of this helminth infection might be much higher than previously expected. Here *Ascaris spp*., *Trichuris spp. and Toxocara spp.* eggs were found in sewage treatment plants with a prevalence of 95%, 96% and 60% respectively [37]. Additionally, eggs of geohelminths were detected in 88.5% of soil samples from conventional farms, *Ascaris* eggs in 87% of cultivated land samples and *Toxocara* eggs in 74% of home garden soil. This body of evidence suggests a potential route of infection from direct or indirect contact with soil, fertilizers or vegetables [38].

A small number of studies also suggest that *Ascaris* prevalence in Europe has remained around 20% for over a thousand years and is still of importance [39] [40]. The frequency of occurrence of ascariasis in Poland is estimated at 1–17% [41] [42]. Studies examining antibodies against *Ascaris* suggest unexpectedly high seroprevalence, with 42% reported in a study from the Netherlands [43], 13% reported in a general population in Estonia [44] and detectable levels in Belgian blood donors [45]. A recent study in Swedish pig farms showed 43% of the herds being infected [46]. The potential for pigs being a source of infection is supported by findings from Denmark, where *Ascaris* is considered a zoonosis [47].

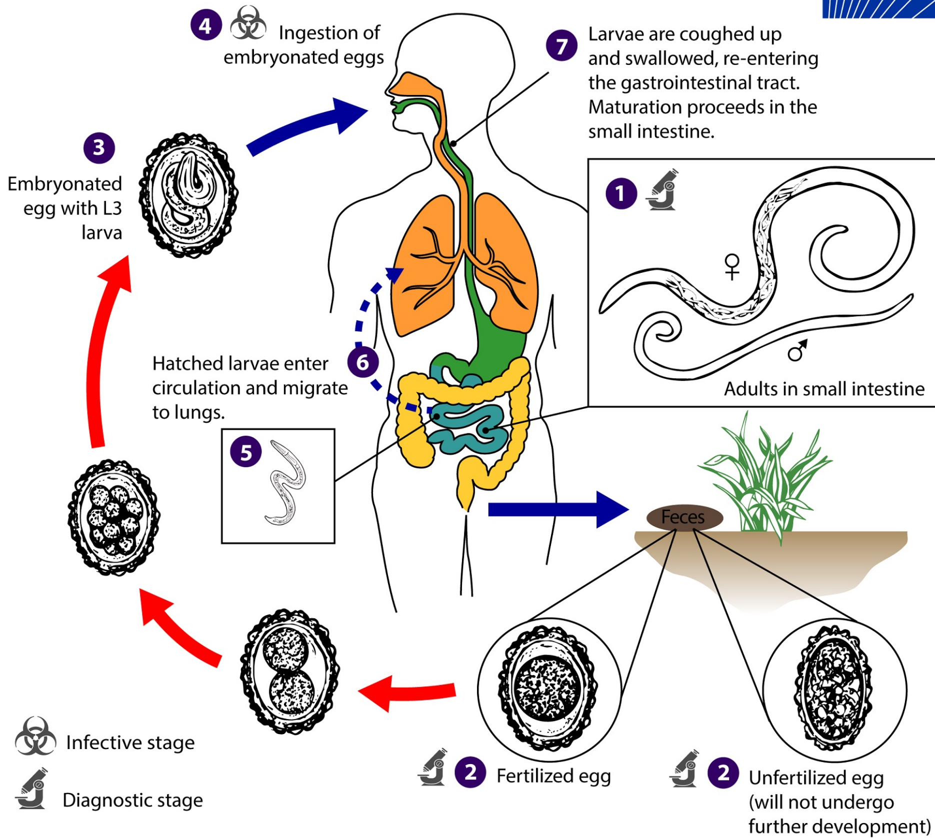
A more common cause of zoonotic helminth infections is caused by a round worm of cats and dogs, *Toxocara canis* and *T. cati* [48]. Human exposure to these parasites can vary greatly with seroprevalence of 6 – 87% reported [49] [50] [51] [52] [53] [54]. Zoonotic infection with *Toxocara* typically takes place via ingestion of eggs by direct

contact with dogs or cats or via contaminated soil. Parks and other recreational spots in nature can be potential sites of exposure to the eggs.

Based on the limited literature from Europe, infections with *Ascaris* and *Toxocara* might be more prevalent than previously expected.

1.4 Life cycle of *Ascaris* and *Toxocara* and immune response towards them

A. lumbricoides and *A.suum* are round worms, or nematodes, with humans and pigs as their definitive hosts, respectively, and may well be the same species [55]. Both are capable of infecting and carrying out their full life cycle in humans (see Figure 2; image courtesy of DpDx, Centers for Disease Control and Prevention (CDC)). The adult worms usually reside in the small intestine and produce large numbers of eggs that pass into the environment with faeces. These eggs can survive in a variety of harsh conditions [56], but require embryonation that takes place in the soil, in order to be infective to the next host. Infection occurs via ingestion of eggs. After hatching in the host intestine L3 larvae penetrate the intestinal wall and migrate via the portal system to the liver. After some migration and growth, they advance to the lungs, penetrate the alveolar spaces, where they further maturate and move to the pharynx where they get coughed up and swallowed, finishing their life cycle [57] (also see Figure 2). The entire cycle takes approximately 67–73 days in humans [58]. Although the infection is usually asymptomatic when infected with a low number of eggs, clinical manifestations may include vague abdominal pains, unexplained fever, anaemia, malaise, and upper respiratory tract infections. Intestinal obstruction and infections are among the severe complications that can occur [59] [60].



Adult worms ❶ live in the lumen of the small intestine. A female may produce approximately 200,000 eggs per day, which are passed with the feces ❷ . Unfertilized eggs may be ingested but are not infective. Larvae develop to infectivity within fertile eggs after 18 days to several weeks ❸ , depending on the environmental conditions (optimum: moist, warm, shaded soil). After infective eggs are swallowed ❹ , the larvae hatch ❺ , invade the intestinal mucosa, and are carried via the portal, then systemic circulation to the lungs ❻ . The larvae mature further in the lungs (10 to 14 days), penetrate the alveolar walls, ascend the bronchial tree to the throat, and are swallowed ❼ . Upon reaching the small intestine, they develop into adult worms. Between 2 and 3 months are required from ingestion of the infective eggs to oviposition by the adult female. Adult worms can live 1 to 2 years.

***Figure 2*. Life cycle of *A. lumbricoides* in humans.**

Toxocara has a similar life cycle in dogs and cats as *Ascaris* does in humans, but they cannot finish their life cycle in accidental hosts, such as humans[48]. Human toxocariasis manifests in four forms: visceral larvae migrans (a systemic disease caused by larval migration through major organs), ocular larvae migrans (a disease limited to the eyes and optic nerves), covert/common toxocariasis (a syndrome comprising chronic dyspnoea and weakness, skin rash, pruritus and abdominal pain, often with eosinophilia) [61] and neurotoxocariasis (a relatively rare entity yet associated with severe sequelae: including meningitis, encephalitis, myelitis, cerebellar vasculitis, cerebral/cerebellar lesions and behavioural abnormalities) [62].

In addition to the direct tissue damage caused by the migrating larvae, helminths can regulate the host's immune response to survive. This can be achieved through shedding of their outer layer during moulting, through production and release of excretory-secretory (ES) products or extracellular vesicles (EVs) that often contain immunomodulatory proteins that protect them against host immunity [63]. Murine models have identified detailed pathways which demonstrate how helminth infections illicit immune responses. The first line of defence against helminths is the innate immune system, which is ready to elicit an attack even without prior contact with the pathogen. The invasion of the parasites through the intestinal wall causes tissue damage and release of alarmins including IL-33 [64]. Additionally, mucosal Tuft cells can sense the presence of helminths via chemosensory receptors, leading to the release of IL-25 which activates intestinal type 2 innate lymphoid cells (ILC-2s) [65]. This is followed by IL-5 and IL-13 production [66] which leads to goblet cell hyperplasia and increased secretion of mucin - a critical step for helminth expulsion. Th2 cytokines such as IL-4, IL-5 and IL-13 are induced soon after helminth infection. Although the expulsion and clearance of helminths usually requires pathogen-specific Th2-mediated immunity, early induction of Th2 cytokines during the innate immune phase is important for host protection from helminth invasion [66] [67], leading together to the "weep and sweep" response [68], this increases the permeability of the intestinal mucosa as well as the motility of the intestinal smooth muscles. This is supported by pig models, where *A. suum* infection profoundly modulates the immunological milieu in the intestinal mucosa, downregulating transcriptional pathways related to immune function.

Specifically modulating proinflammatory, Th1, and antigen processing pathways [69]. Studies in mice with cell specific disruption of Type2 immunity have also shown that immune signalling on smooth muscle cells ([70] [71] [72] and epithelial cells is critical for early resolution of infection [73].

Ascaris and other important helminth pathogens pass through the respiratory system during their natural life cycle in humans. Thus the lungs are an important site of host immunity against helminths [74]. Direct damage/stress on, for example, host pulmonary epithelium can lead to release of alarmin cytokines (e.g. IL-25, Thymic stromal lymphopoietin (TSLP), IL-33) which promote induction of effective type 2 immune response. Combined antigen and stress driven challenges, therefore, promote type 2 interleukin secretin of IL-4, IL-5, IL-9 and IL-13, as well as associated chemokines (e.g. leukotriene, eotaxin etc) that shape the subsequent host immune response.

The classic helminth innate response is characterized by raised M2 macrophages, basophils, eosinophils, mast cells and type 2 innate lymphoid cells (ILC2) in the lungs [63]. Aspects of this host response may contribute to long-term lung pathology following larval migration through the lung. For example, *N. brasiliensis* infection results in deterioration in lung function, destruction of alveoli and emphysema like changes [75]. One of the mechanisms related to such changes is an IL-25 promoted IL-13 release from ILC2s that can drive collagen deposition in the lungs, as demonstrated in *Schistosoma mansoni* egg-induced pulmonary fibrosis in mice [76] . This bears analogy with evidence from a small human cohort where increased pulmonary expression of IL-25 and up-regulated ILC2s from bronchoalveolar lavage (BAL) was associated with the presence of pulmonary fibrosis [77].

Secondary or chronic pulmonary helminth infection in the lungs also promotes expansion of both Th2 and Th17 cells, eosinophilia, basophilia and B cell class switching to type 2-associated antibody isotypes (IgE, IgG1, IgG4). The antibody response has been demonstrated to potentially enhance anti-larval responses following *Ascaris* infection. Here specific antibodies against the parasite promote macrophage adherence to larvae and upregulate the production of chemokines that attract

eosinophils and neutrophils to the site of infection which together attach to the larva and kill them [78] [79]. Basophils enhance Th2 polarization through the release of IL-4, eosinophils release transforming growth factor beta (TGF-β) and IL-13. This cascade culminates in the activation of profibrotic macrophages, smooth muscle contraction, and mucus production [80].

Differences in the response to ascariasis between those suffering acute (one-off or seasonal exposure) or chronic infection (continuous transmission throughout the year) [81] might be explained by multiple exposures eliciting a polarized systemic Th2 response. This would lead to an increased number of circulating inflammatory cells, production of higher levels of systemic cytokines (IL-4, IL-5, IL-6, IL-10, IL-17A, tumour necrosis factor alpha (TNF-α)), and a more intense pulmonary inflammation [82].

1.5 Association between helminth infections, asthma, and allergies

According to the "old friends" hypothesis, modern living conditions and the absence of sufficient heterogenous immunostimulation can lead to defective maturation of different parts of the immune system. It has been proposed that in the absence of optimal levels of immunoregulation, the individual may develop a Th1 or a Th2 mediated inflammatory disorder depending on his/her own particular Th1/Th2 bias, immunological history, and genetic background [83]. Helminth infections and the corresponding host immune responses are products of a prolonged dynamic co-evolution between the host and the parasite. For parasites, it is advantageous to trick the host into developing an ineffective immune response to find a suitable niche for maturation and propagation, and to do so without killing or unduly harming the host. Conversely, the host has to ideally generate an effective immune response to expel the parasite and minimize its harmful effects, while not sacrificing its ability to effectively respond to other pathogens [68]. Helminth infections can influence lung health and allergies by either direct damage caused by the migration of larvae and the subsequent innate immune response, or by the release of immunomodulators either during larval migration or by adult worms in the gut lumen. Both can alter the normal lung function

and modify responses to subsequent pathogen or allergen exposure [80]. Löffler's syndrome, the direct damage from migrating larvae and the subsequent eosinophilic pneumonitis, has been reported and is mostly related to zoonotic exposure to *A. suum* [47, 84, 85] [86].

The relationship between *Ascaris* infection, allergies and asthma is complex, and conflicting results have showed that infection can both increase and decrease the risk of allergies and asthma. Active vs previous infection, worm burden and atopic state also seem to play an important role [87]. Severe ascariasis has been shown to be protective against asthma symptoms and atopic sensitization, [88] [89] and treatment of infection in endemic regions can increase risk of atopic reactivity [90] [91]. Studies in highly endemic indigenous populations from Bolivia show that helminth infection can also have sex-specific effects on the dampening of immune responses and these differences in immune responses are more pronounced in case of *Ascaris* infections [28] as compared to other parasites. At the same time, it has been shown that mild infection, as well as *Ascaris* specific IgE sensitization, can be a risk factor for allergy and asthma in children [92] [93]. A recent meta-analyses including 80 studies and almost 100 000 participants concluded that *A.lumbricoides* infections were associated with an increased risk of bronchial hyperreactivity in children and an increased risk of atopic sensitization among adults. However, the overall strength of the underlying evidence was rated as low to moderate [94]. Most of these studies have been conducted in endemic regions and amongst children. The knowledge of the potential of *Ascaris* infection altering risk of allergy and asthma in non-endemic populations is very limited.

1.6 Detection of helminth infections

Conventional microscopy-based methods such as direct Kato-Katz smear (which is the WHO suggested gold standard [95]) or mounts after stool centrifugation/flotation-based concentration techniques have been the mainstay of diagnosis, especially in resource-poor countries where these infections are common. The diagnostic reliability of molecular methods such as PCR depends on the efficiency of DNA extraction and sample preservation before sample testing. Stool samples alos contain components that

affect the sensitivity of PCR. Addtionally, preservation of samples in formalin can also inhibit PCR efficacy. Furthermore, quantitative PCR (qPCR) methods have showed lower reproducibility of test results compared to Kato-Katz [96]. Assessments on treatment efficacy comparing traditional microscopic to newly emerging molecular approaches are scarce and hampered partly by the lack of an established molecular diagnostic gold standard [96]. Therefore, multiple samples per participant is suggested to be analysed to achieve a reliable diagnosis of STH infection. However, the assessment of environmental samples from water treatment plants has demonstrated qPCR and digital PCR (dPCR) methods to be extremely sensitive and they are able to detect very low amounts of eggs [97]. Together these insights suggest that PCR based diagnostics are valuable but need to be carried out under conditions that control for these potential confounders.

In research settings, the collection and proper storage of multiple samples is not always easily achievable. Exposure to *Ascaris* can also be underestimated when measured with copromicroscopy [98]. Interestingly, coproantigen detection tests have been developed and used in veterinary practice for the detection of STH, but these have not been evaluated for use in humans [95]. However, a recent study using the ABA-1 coproantigen, detected *Ascaris* infection with a sensitivity of 91.5% and a specificity of 95.3% in stool samples [99].

Serological tests offer the opportunity to detect antibodies against specific helminths in human serum. IgG4 response against *A. suum* haemoglobin (AsHb) has been shown to reflect recent exposure to *Ascaris* in humans [98, 100]. IgG4 levels have been shown to normalize 6 months after treatment with anti-helminthics [101], which is supported by a Brazilian study where anti-*Ascaris* IgG4 was not detected in children treated within a large public deworming scheme. Raised anti-*Ascaris* IgE levels have been associated with current *Ascaris* infection, with low worm burdens, and natural immunity against *Ascaris* [102] [103, 104]. Although, in Bangladeshi children, raised IgG1, IgG4, and IgE levels to *Ascaris,* were higher in heavily infected children [105]. However, the detection of IgG4 antibodies against *Ascaris* proteins might be elevated due to cross-reactivity with other allergens. The use of *Ascaris*-specific antigens can therefore

increase the sensitivity and specificity of serodiagnosis. One such protein is ABA-1, which has no cross-reactive counterpart in mites suggesting its usefulness as a more specific marker of *Ascaris* infection [106].

1.7 Potential cross-reactivity with allergens

One possible mechanism contributing to how helminth infections can lead to allergic manifestations is the potential of helminth allergens to drive a cross-reactive IgE immune response towards other allergens. It has been suggested that many environmental allergens have helminth counterparts capable of this [107]. Many *Ascaris*-specific antigens have been discovered in the search for a potential vaccine candidate [108]. Three of these have also been linked with cross-reactivity with other allergens. Tropomyosin is a pan-allergen, and a high grade of homology has been shown among tropomyosins from different species such as crustaceans, mites, other helminths (e.g. *Anisakis simplex[109]*) and insects, which supports the hypothesis of cross-reactivity among tropomyosins from divergent species. Tropomyosin and enolase are the two major allergens that *Ascaris spp* share with house dust mites, with around 70% homology in their structure [110] [111]. Another cross-reactive allergen is *Ascaris* glutathione transferase (GSTA), which is also present in cockroaches, house dust mites, moulds (e.g. *Alternaria alternata*) and blood flukes (*Schistosoma spp.)* [112].

It is nearly impossible to determine where and when people get sensitized to certain allergens. However, animal models have shown that immunization of naïve rabbits and mice with *A. lumbricoides* antigens induces the production of antibodies cross-reactive to house dust mite [113, 114], and suggest that sensitization to HDM and development of IgE-mediated allergic diseases is faster in individuals with a previous history of *Ascaris* infection [114]. Controversially, it has also been show in a murine model that HDM-induced allergic inflammation followed by *Ascaris* infection drives an eosinophil-rich pulmonary type 2 immune response (Th2 cells, M2 macrophages, ILC2s, raised IL-33, IL-4, IL-13, and mucus production) that directly hinders larval development and reduces markedly the parasite burden in the lungs, but not in the liver [115]. In a similar mouse model, sensitization with HDM-extract drove marked IgE and

IgG1 responses that were cross-reactive with *Ascaris* larval antigens. Moreover, HDM-induced IgE antibodies were found to be functional as they mediated immediate hypersensitivity responses in skin testing with helminth-derived recombinant tropomyosin. However, they showed that allergen-driven cell-mediated larval killing is not antibody-dependent [110]. Whether HDM sensitization drives elevated helminth-specific IgE or IgG responses or whether helminth infections can drive cross-reactive HDM sensitization in humans is yet to be explored.

1.8 Epigenome-wide association studies (EWAS)

Genome-wide association studies (GWAS) linking different genes to certain traits or diseases have provided valuable insights into the pathogenesis of many illnesses. However, the genetic variance from GWAS has explained only a very small proportion of familial clustering in diseases like asthma [116], although the family history of asthma has shown to be one of the most important risk factors for developing the disease [117, 118].

Epigenetics is traditionally defined as the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. There are four main classes of epigenetic changes —DNA methylation (and demethylation), histone modification, chromatin remodelling and non-coding RNAs such as microRNAs — each of which can influence gene expression and may be influenced by the environment, diet, diseases, and ageing [119]. The epigenetic marker that has been most studied is DNA methylation, the presence of methyl groups on a cytosine nucleotide in a CpG dinucleotide [120]. Methylation is achieved by the addition of a methyl group to the 5 position of a cytosine ring mediated by DNA methyltransferases (DNMTs) [121]. CpG methylation is crucial for gene repression and expression [122]. It is potentially reversible depending on the exposure. However, many methylation patterns are long-lasting [123] [124] [125]. Furthermore, viral, bacterial and parasitic infections can also cause long-lasting DNA methylation [126] [127] [128] [129], some of which have been linked to altered host immunity [130]. Moreover, preconception and in utero exposures have been linked with allergic diseases also in their future

offspring [131]. DNA methylation signatures associated with concurrent disease and with the development of asthma have been identified, but their significance is not easily interpretable. As epigenetic mechanisms link gene regulation to environmental exposures and developmental trajectories, their contribution to asthma and allergy pathogenesis is under active investigation [132].

1.9 Infections across generations

It is well-established that maternal infection plays a role in determining the health of offspring and their risk of allergies [133]. This has been shown with maternal influenza A [134] as well as *Schistosoma mansoni* [135] infection. Maternal infection may lead to expanded regulatory T-cell populations in the offspring, which can impair their responsiveness to allergenic challenge [136].

Additionally, maternal transfer of antigens to offspring can lead to tolerance towards allergic airway inflammation in mice. This has been shown also in preconception exposures [137] [138]. Preconception infections altering the health of offspring are supported by a study using Norwegian registry data investigating the timing of parental tuberculosis infection in relation to the offspring's birth year and the association with offspring asthma. It revealed an increased risk of asthma in the offspring of parents who had a diagnosis of tuberculosis in childhood or in a period before conception of the offspring, as compared to those whose parents had tuberculosis after the birth of the offspring [139]. The authors speculate that tuberculosis-induced epigenetic reprograming might alter parental immunity, which could alter the offspring type 2 immunity characteristics [139]. Mice models support these findings showing that preconception helminth infection can alter offspring's immunity and microbiome, [140] and that maternal immune profile can be transferred via breastmilk to the offspring [141]. These studies suggest that maternal infection well before conception might alter their children's risk to allergic diseases. Another mechanism for the transfer of parental immunity to the offspring is via the male line. Germline epigenetic changes in the preconception period have been illustrated by *Toxoplasma gondii* infection in male

mice, in which the infection caused small RNA profile changes in their sperm and led to subsequent altered behaviour in the offspring [142].

Given this body of literature, it is plausible that parental infections during as well as before conception, may contribute to immunologic changes which could result in allergic outcomes in the next generation.

2. Aims of thesis

2.1 Main objective

The overall aim of this thesis was to investigate how helminth exposure in Northern Europe is associated with allergies and lung health in a non-endemic setting.

2.2 Specific objectives

1) To describe the prevalence of *Toxocara spp.* and *Ascaris spp*. seropositivity and associations with allergic diseases and sensitization, in two generations of younger and older adults in Bergen, Norway (Paper I)

2) To explore the association between *Ascaris* exposure and lung function in three Northern European countries, and to address potential sex differences in such associations; further, to investigate whether *Ascaris* exposure is associated with differentially methylated DNA in whole blood (Paper II).

3) To evaluate the binding of *Ascaris*-specific serum IgG4 and IgE to *Ascaris* larvae, and to assess whether house dust mite sensitization drives cross-reactive antibodies towards *Ascaris* (Paper III).

3. Material and methods

Table 1. Overview of materials and methods used in papers I-III

	Paper I	**Paper II**	**Paper III**
Aims	Measure *Ascaris* and *Toxocara* seropositivity and their associations with allergic outcomes	Explore association of *Ascaris* exposure with lung function, and with differentially methylated DNA, in men and women	Evaluate binding of IgE and IgG4 to *Ascaris* L3 larvae. Assess cross-reactivity with HDM.
Design	Cross-sectional data from 2 generations	Cross-sectional data from 3 study centres	Cross-sectional
Data source	ECRHS III and RHINESSA	RHINESSA	RHINESSA
Study population	171 parents from ECRHS III and their 264 offspring from RHINESSA Bergen	671 participants from RHINESSA Aarhus (Denmark), Bergen (Norway) and Tartu (Estonia)	24 men from RHINESSA Bergen, age matched in 4 groups
Exposures	IgG4 antibodies against *Ascaris* and *Toxocara*	Anti-*Ascaris* IgG	HDM sensitization and *Ascaris* IgG4 seropositivity
Outcomes	SPT reactivity, allergic symptoms	Ever asthma, FVC, FEV_1, FEV_1/FVC, DNA methylation.	Serum IgG4 binding to *Ascaris* larvae
Covariates	Sex, age, BMI and clustering within families	Age, sex , smoking	Age matched 4 groups compared
Statistical methods	Logistic regression	Logistic and linear regression	Kruskal-Wallis test

Laboratory assays	End-point titration ELISAs	Methylation: Illumina EPIC; ImmunoCAP; NovaTec ELISA	Fluorescence imaging, ELISAs

3.1 Data sources

This thesis is based on the data from the European Community Respiratory Health Survey (ECRHS) III and Respiratory Health in Northern Europe, Spain and Australia (RHINESSA) studies (see Figure 3).

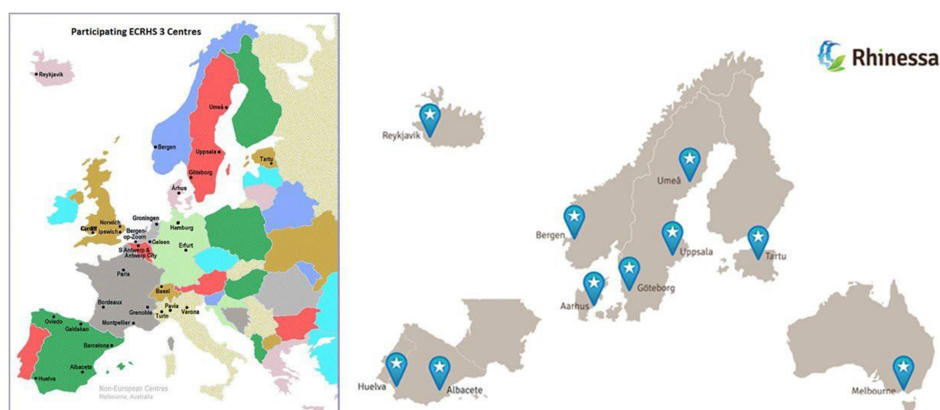


Figure 3. **Illustrative overview of all the ECRHS III and RHINESSA study centres.**

3.2 Study populations and design

ECRHS was planned to answer specific questions about the distribution of asthma and health care given for asthma in the European Community. It was the first study to assess the prevalence of asthma and allergic disease in young adults in many countries using a standardized protocol [143]. The study began in 1990 collecting data from a randomly sampled general population from 25 mainly European countries, with about 26,000 participants in the first clinical stage. In 14 countries these participants were invited to a follow up approximately 10 and 20 years later, with ECRHS III taking place between 2010-2013 (see Figure 4).

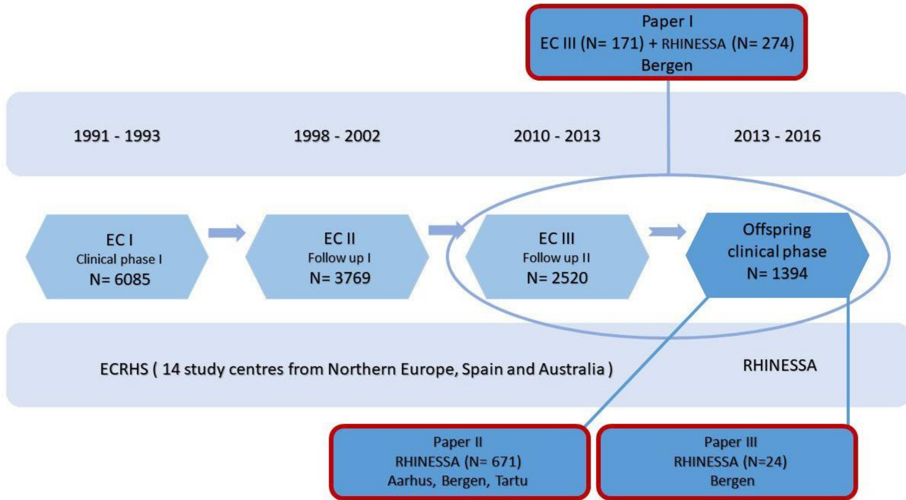


***Figure 4*. Flow chart of the ECRHS and RHINESSA clinical phase participants and overview of study populations in each paper.**

RHINESSA participants are the offspring of ECRHS. It is a project that studies asthma, allergies, and lung health across generations and throughout a person's lifespan in seven countries in ten study centres. The aim of the study is to understand how lifestyle and environmental factors influence one's own health and the health and potential illnesses of their children and future generations. During the ECRHS and RHINESSA clinical phase, participants were invited to the study centres where they answered extensive interviewer lead questionnaires, spirometry and SPTs were performed, and biological material including blood, was collected.

In Paper I, 171 parents from the ECRHS, born from 1945 to 1972 were include, who had at least one offspring that participated in the RHINESSA study. A total of 264 offspring born from 1969 to 2003 were included. The study population was selected based on the availability of serum samples and p skin prick test (SPT) results.

In Paper II, we included 671 participants from the RHINESSA study from Aarhus, Bergen and Tartu centres, with spirometry and available serum samples. Of these, 551 participants had full blood samples available for analyses of DNA methylation.

In Paper III, samples from 24 men who participated in the RHINESSA Bergen study were selected based on their house dust mite sensitization and *Ascaris* seropositivity. 4 groups were created and matched by age. Only six participants were both HDM sensitized and *Ascaris* seropositive, they made up group A (HDM+;ASC+). The other groups also consisted of six participants with Group B not sensitized towards HDM but *Ascaris* seropositive (HDM-;ASC+), Group C sensitized towards HDM but not *Ascaris* seropositive (HDM+;ASC-) and Group D not sensitized towards HDM and *Ascaris* seronegative (HDM-;ASC-). Two participants from each group were selected for fluorescence imaging of IgG4 binding to larvae.

3.3 Questionnaires

Analyses in Papers I and II were based on the interview-based questionnaires from ECRHS III (Paper I) and RHINESSA (Paper I and II). The questionnaires included questions on respiratory symptoms, allergies, smoking, pet keeping and education among others (APPENDIX A and B)

3.4 Collection of study material

3.4.1 Collection of serum samples

Blood samples were collected and sera separated in SST Vacutainer glasses, centrifuged within 30-60 min after collection (at 3400 RPM for 10 minutes at room temperature). The samples were stored at −80°C. Some of the samples were transported on dry ice for further analyses.

3.4.2 Collection of *Ascaris* larvae and embryonation of eggs

A. lumbricoides adult worms were harvested from an infected patient from Red Cross Children's Hospital in Cape Town, South Africa. The worms were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 µg/mL Pen-strep (Thermo Fisher Scientific, Massachusetts, USA), 10 µg/mL L-glutamine (Merck, Darmstadt, Germany), 10 µg/mL Gentamycin (Thermo Fisher Scientific) and 10 µg/mL Glucose (w/v). Embryos were harvested according to Kang et al [144]. The eggs were embryonated on a shaker at 30 °C for 4 weeks in 0.1% formaldehyde (in water) in a glass bottle, covered with perforated parafilm for better oxygenation. This resulted in about 70% of embryonated eggs. The eggs containing larvae were washed three times with sterile saline. After the last wash, the eggs were incubated with 5% NaOCl for three minutes with mild shaking. This leads to damage of the hard chitinous layer. Then, the eggs were washed another five times with sterile saline at 37°C and were transferred to a prewarmed sterile flask containing 20ml of HBSS. Autoclaved glass beads with 4-6mm diameter were added and shaken mildly to induce mechanical breakage of the eggs. Larvae were separated from the eggs using a modified Baermann column with tissue paper and kept in a KW-2 medium in a humidified cell culture incubator at 37°C until cytospun. The protocol was adapted from FH Falcone 1996 PhD thesis [145].

3.5 Exposure measurements

In Paper I, II and III, the exposure was based on antibody levels against *Ascaris*. In Paper III, HDM sensitization was considered also as an exposure for having elevated antibodies towards *Ascaris*.

3.5.1 IgG4, IgG and IgE antibody measurement by ELISA and ImmunoCAP

For Papers I and II, we used change-point analysis for interpretation of the ELISA results.

This method does not require the presence of known positive or negative sera (blind analysis). Change-point analysis is aimed at identifying points in a dilution series where the statistical properties change. In particular, such analysis can be used to detect abrupt steps in the mean level of a such a dilution series. In the case of ELISA, if absorbance values of a micro-titer plate are ordered in ascending order, negative samples are supposed to be the lower ones in the series while positive ones (if they exist) would be the higher. However, values are not supposed to increase regularly if positive samples exist in the series. Indeed, as positive controls are supposed to be “different” from negative ones, a step, even small, should appear in the series, separating the negative from the positive values. Therefore, change-point algorithms might be used to detect such a change and locate the value where in the series this change occured. The detected value is therefore a kind of specific cut-off proxy that discriminates between positive and negative samples [146].

In Paper I, detection of IgG4 antibodies against *T. canis* and *A. lumbricoides* was achieved using an indirect ELISA. 96-well Nunc Immunosorb ELISA plates (Thermo Scientific) were coated with 10 µg/mL of soluble helminth antigen diluted in carbonate buffer. The serum from participants was diluted 1:20, 1:100, 1:500 and 1:2500 in Phosphate-buffered saline (PBS). Bound antibodies were detected using alkaline phosphatase-conjugated mouse anti-human IgG4 antibodies (Sigma-Aldrich). ELISA plates were read at 405 nm to determine optical density (OD). To reduce cross-reactivity in assessment of *Toxocara*, sera were pre-incubated on *A. lumbricoides* antigen-coated plates and then transferred to *Toxocara* antigen-coated plates. Total IgE and specific IgEs were measured according to standardized laboratory methods at Haukeland University Hospital in Bergen, Norway. IgE positivity was defined by IgE ≥ 0.35 kU/L to at least 1 of 4 allergens tested (cat, timothy grass, birch and house dust mite). In Paper II, levels of *Ascaris*-specific IgG antibodies were measured by ImmunoCAP (Thermo Fisher Scientific, Waltham, Mass). In order to account for potential cross-reactivity between other helminths, 446 Bergen centre samples were tested using

NovaLisa IgG ELISA (NovaTec Immunodiagnostica, Dietzenbach, Germany) for the presence of IgG antibodies against *Ascaris lumbricoides, Echinococcus spp, Taenia solium, Toxocara canis*, and *Trichinella spiralis* according to the manufacturer's instructions. A result of >11 NovaTec units (NTU) was considered positive. A result of 9 to 11 NTU was considered a gray zone and <9 NTU negative.

In Paper III, similar indirect ELISA was used as in Paper I, but the plates were coated with 5 µg/mL of either *A. lumbricoides* adult worm somatic, adult excretory-secretory (ES), larval somatic or adult extracellular vesicles (EVs) antigen diluted in carbonate buffer. The serum from participants was diluted in six dilutions: 1:3, 1:9, 1:27, 1:81, 1:243 and 1:729 in PBS. Bound antibodies were detected using alkaline phosphatase-conjugated mouse anti-human IgG4 antibodies (Merck) at a dilution of 1:4000 in PBS-T in 5% BSA. p-Nitrophenyl Phosphate (PNPP) was used for visualization (incubated for 30 minutes at 37°C) and the plates were read (ClarioStar Plus, BMG, Labtech, Germany) at 405 nm to determine the optical density (OD). Anti-HDM IgG4 was measured using the same protocol with a mixture of *D. pteronyssinus* and *D. farinae extract* (ALK-Abello) with the concentration of the coating antigen at 5 µg/mL. The same applies for detection of anti-*Ascaris* IgE using a secondary mouse anti-human IgE antibody (Sigma-Aldrich) at a dilution of 1:4000 in PBS-T in 5% BSA. Log transformed arbitrary values of the OD were used for figures and statistical analyses.

3.5.2 Preparation of *Ascaris* antigens

For A. lumbricoides adult and larval somatic antigen, tissue or larvae suspended into RIPA lysis buffer (Sigma-Aldrich) at room temperature for 30 minutes. It was homogenized using an electric homogenizer followed by centrifugation at 2000g for 30 minutes at 4 °C in RIPA buffer (Merck, Darmstadt, Germany) and then centrifuged at 2000g for 30 minutes at 4°C. Supernatants were then used for subsequent analysis. A. lumbricoides excretory-secretory (E/S) proteins were obtained by maintaining live adult A. lumbricoides in Dulbecco modified essential medium with 10 µg/mL Pen-strep (ThermoFisher Scientific, Waltham, Mass), 10 µg/mL L-glutamine (Merck), 10 µg/mL gentamicin (ThermoFisher Scientific), and 10 µg/mL glucose (wt/vol). Live adult A. lumbricoides were maintained at 37°C. Media were harvested every three days. E/S

proteins were concentrated by using an Amicon ultraconcentrator (Merck) and resuspended in 5 mL of PBS. The BCA protein estimation kit (Pierce, Rockford) determined protein of the supernatant. The lysates were then normalized to a total protein concentration of 500 µg/mL in 100 µL aliquots frozen at -80 degrees until further use [15]. Adult extracellular vesicles (EVs) isolation involved polyethylene glycol (PEG) precipitation of EVs from the E/S proteins, which was then further purified using size exclusion chromatography. Briefly, thawed E/S protein solution was filter sterilized using 0.22 µm filters and sequential differential centrifugation steps at 500g and 2000g at 4°C, each for 30 min. The supernatant collected was then added in equal volume to a 2x stock solution containing 16% PEG 5000 (Merck) and 1M NaCl [16]. This combined solution was gently mixed by inversion and left overnight at 4°C. To pellet the EVs, the E/S protein-PEG solution was spun at 3128g at 4°C for 1 h. The resultant pellet was resuspended in 1 mL of sterilized PBS. The 1 mL samples were then loaded on to a gel filtration column (Sepharose CL-B4) for the final purification step. 20 sequential fractions of 0.5 mL each were collected and using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific), A280 readings were obtained for each fraction collected, to quantify the level of protein present.

3.6 Spirometry

NDD EasyOne spirometer was used following American Thoracic Society/European Respiratory Society criteria [147]. Reversibility was assessed 15 minutes after inhalation of 200µg of salbutamol. The spirometer was calibrated each morning prior to the arrival of study participants. Participants were asked to perform up to eight spirometry maneuvers. At least three acceptable maneuvers were required to achieve reproducibility, where the two highest values for FVC and FEV_1 should not vary >200 mL from each other. The highest FVC and FEV_1 of each participant were used in the analyses.

3.7 Skin prick tests (SPTs)

SPTs were performed using 12 aeroallergens (ALK-Abelló, Hørsholm, Denmark): timothy grass, ragweed, *Dermatophagoides pteronyssinus, D. farinea,* cat, dog, birch,

Blatella germanica, olive*, Alternaria spp., Cladosporium spp.* and *Parietaria spp.;* 0.9% saline and 10 mg/mL histamine solution were used for negative and positive controls. Reactions to the allergens were read after 15 minutes. Reactivity was considered positive if the mean wheal size was 3 mm greater than the negative control. "Any positive HDM SPT" was defined as positive SPT reactivity toward *D. pteronyssinus* and/or *D. farinae*. SPTs were carried out by trained nurses following a standardized protocol, similar in all study centers.

3.8 Outcomes and covariates

Allergies

In Paper I, allergic outcomes were based on SPT reactivity, on specific IgE sensitization towards cat, timothy grass, house dust mite, and questionnaire data that involved questions on nasal symptoms and eczema. Hay fever was based on the following question: "Do you have any nasal allergies, including hay fever?". (For exact wording of the questions please see Appendix B) .

Asthma and asthma symptoms

The asthma outcomes used in Paper I and II were based on self-reported questions. "Ever asthma" was defined as answering yes to both of the following questions: "Have you ever had asthma?" and "Was this confirmed by a doctor?". Having three or more of the following symptoms 12 months prior to the study were defined as positive for asthma symptoms: wheeze, nocturnal chest tightness, nocturnal breathlessness, nocturnal cough, any asthma attack, or use of asthma medication.

Lung function

In Paper II, lung function was assessed by spirometry. FVC, FEV_1 and FEV_1/FVC were used as outcomes. To account for lung function in a wide age range, we calculated the percentage of predicted values for FVC and FEV_1 according to the Global Lung Initiative reference values [22] with the online calculator.

DNA methylation: EWAS analyses

Methylation data were profiled from peripheral blood using HumanMethylationEPIC chip (Illumina, San Diego, Calif), which captures >850,000 CpG sites. Methylation input data (IDAT files) were processed using the R/Bioconductor package minfi [148]. The batch effect was adjusted using COMBAT from SVA [149]. Normalization was carried out using BMIQ [150]. Probes with a detection P value above 0.01 in 1 or more samples, probes with a bead count of <3 in at least 5% of samples, non-CG probes, cross-reactive probes, and probes on the X or Y chromosome were excluded from the analysis, resulting in a total of 747,053 probes used for downstream analysis. To identify differentially methylated CpGs, a robust multiple regression model was applied using limma [151] on methylation beta value stratified by sex, adjusting for age, personal smoking status, and estimated cell type proportions (B, CD4T, CD8T, natural killer, monocytes, and granulocytes) [152]. Multiple test correction was applied using the Benjamini-Hochberg false discovery rate at a significance level of 0.05. Manhattan plots were generated using the R package qqman [153]. Differential methylated regions were predicted using DMRcate [154]. Functional enrichment was identified using Enrichr in R [155]. Transcription factor enrichment was carried using eFORGE [156].

Fluorescence microscopy for imaging of antibody binding to *Ascaris* larvae

L3 larvae from embryonated eggs were spun down (Thermo Scientific Cytospin 4 Centrifuge) onto positively charged glass slides at 800 x/min for five minutes, fixed with 10% formalin, and incubated at 4°C overnight with 25µl of undiluted serum from the four groups previously mentioned under section 3.2. After incubation with serum, the slides were washed with PBS three times and incubated for one hour at room temperature, with 100 µl of Hoechst 33342 (Invitrogen, USA) (diluted 1:1000 in 1xPBS). After the incubation and washes, the slides were incubated with 50 µl of Alexa-Fluor 647-conjugated anti-human IgG4 (SouthernBiotech, USA) diluted 1:250 in 1% BSA-PBS for one hour at room temperature. For visualizing IgE binding, Alexa-Fluor 647-conjugated anti-human IgE (SouthernBiotech, USA) was used following the above described steps. The slides were fixed with moviol (Merck) and anti-fade (Thermo Fisher Scientific) (diluted 1/50). All slides were imaged on Delta Vision Elite deconvolution microscope system (GE Healthcare, Pennsylvania, USA) using

softWoRx 7.0.0 software with 60X oil immersion objective on FITC, CY5 and DAPI fluorescence channels. All intact larvae on each slide were visualized for IgG4 and IgE binding, with duplicate slides per participant, and two participants per group.

Covariates

A covariate is a variable that is not the main factor or variable in a study but needs to be considered because it can potentially influence the outcome variable. A confounder is a covariate that is both associated with the exposure and outcome, but is not one the causal pathway between the exposure and outcome [157]. In Paper I, logistic regression models assessing the association between helminth seropositivity with allergic outcomes, age and sex were considered as confounders, as both have been shown to be associated with helminth infections, as well as allergies and specific IgE levels. Additionally, in the offspring generation, BMI was positively associated with helminth seropositivity and was included in the models as it is also associated with allergic outcomes.

In Paper II, all analyses were stratified by sex. Logistic regression models were adjusted for height and age, and clustered by study centre. In addition to these, HDM sensitization was considered as a potential confounder based on the literature that HDM sensitization can drive cross-reactive antibody response toward *Ascaris,* and HDM sensitization itself is a risk factor for asthma [158].
In Paper III, four age matched groups based on their HDM sensitization and *Ascaris* IgG4 seropositivity were created. No other covariates were considered.

3.9 Statistical analyses

All analyses in Paper I and II were performed using STATA versions 15.0-17.0 (Stata Statistical Software, Statacorp, College station, TX: StataCorp LLC). Additionally, ELISA data was analysed using GraphPad Prism 7 (GraphPad Software, San Diego, USA) in Paper I and GraphPad Prism 9 in Paper III.

Descriptive statistics for the study population were reported as mean and range or standard deviation for continuous variables, and count and percentages for categorical variables.

In Paper I, logistic regression was applied to assess associations between pet keeping, place of upbringing, age and sex as well as other potential variables that might be associated with helminth exposure. Similar models were applied to study associations between *Toxocara* and *Ascaris* seropositivity, and allergic sensitization, rhinitis, hay fever and asthma. Models were performed separately or combined for the 2 generations (ECRHS parent and RHINESSA offspring generations). In all regression models with the combined study populations and when analysing the offspring generation, we corrected for clustering within families (parent-offspring and/or siblings) by applying a cluster for family-id.

In Paper II, logistic regression was used to model the association between *Ascaris* seropositivity and asthma status, wheeze, and ≥3 asthma symptoms (dichotomous) 12 months prior to the study. FVC and FEV_1 were included as continuous outcomes. All models were stratified by sex, and adjusted for age, height (measured before spirometry), smoking (interview data), and clustered within study centre. Linear regression was used to model the association between anti-*Ascaris* IgG concentration and % predicted FVC and FEV_1 values.

For non-parametric data, the Kruskal-Wallis test was used to assess difference between the categories. Group D (HDM-;ASC-) was used as the reference category. Differences were considered statistically significant when $p < 0.05$.

3.10 Ethical considerations

Ethical permissions were obtained for each study centre from regional ethics committees. All study participants provided written informed consent prior to participation. Approval numbers and the names of ethics committees can be found below:

Denmark: Ethical Scientific Committee for Mid Region Jylland, approval number 1-10-72-301-15. Norway: Bergen Regional Committee of Medical and Health Research Ethics (REK VEST) approval number 2012/1077. Estonia: Tartu Research Ethics Committee of the University of Tartu (UT REC) approval number 233/ T-7. Appropriate measures have been exercised in order to facilitate safe data storage and protection. SAFE (secure access to research data and e-infrastructure), developed at the IT-department at the University of Bergen, was used for secure processing of sensitive personal data. SAFE is based on "Norwegian Code of conduct for information security in the health and care sector" (Normen) and ensures confidentiality, integrity, and availability are preserved when processing sensitive personal date.

4. Summary of main results

4.1 Zoonotic helminth exposure and risk of allergic diseases: A study of two generations in Norway

We investigated the seroprevalence of tow helminths, *Ascaris spp.* and *Toxocara spp.* in two generations in population-based cohorts from Bergen, Norway and the association of seroprevalence with allergic outcomes. We used logistic regression models to study the association between seropositivity, skin prick test reactivity, and allergic symptoms based on questionnaire data.

We found an unexpectedly high seroprevalence among these cohorts with 29% in the parent (ECRHS: born 1945-1972) and 17.5% in the offspring generation (RHINESSA: born 1969-2003). We showed that the seroprevalence is decreasing based on the birth cohort among the offspring.

In the offspring seroprevalence towards *Toxocara* was associated with pet keeping before age 15 (OR = 6.15; 95% CI = 1.37-27.5) and with rising BMI (1.16[1.06-1.25] per kg/m2). Among parents, *Ascaris* seropositivity was positively associated with childhood cat keeping and growing up on a farm with livestock. *Toxocara* seropositivity was associated with increased odds of ever wheezing, having hay fever, eczema and cat sIgE sensitization among the offspring generation, but not among the parent generation.

We concluded that zoonotic helminth exposure in Norway was less frequent in offspring than parents, *Toxocara* spp. seropositivity was associated with increased risk of allergic manifestations in the offspring generation, but not among parents. We suggested that changes in response to helminth exposure may provide insights into the increase in allergy incidence in affluent countries.

4.2 *Ascaris* exposure and its association with lung function, asthma, and DNA methylation in Northern Europe

We aimed to further explore whether the Bergen offspring cohort in Paper I had any exposure to other helminths. We used a commercially available ELISA kits to detect IgG antibodies against 5 helminths and found that *Ascaris* is the most prevalent exposure. Further, we wanted to explore how *Ascaris* exposure is associated with lung function, asthma, and asthma symptoms in three different study centres of the RHINESSA cohort, and explore whether *Ascaris* exposure is associated with differentially methylated CpG sites.

We found that *Ascaris* seropositivity was associated with lower FEV_1 (-247 mL; 95% CI, -460, -34), higher odds for having asthma (adjusted odds ratio, 5.84; 95% CI, 1.67, 20.37), and asthma symptoms 12 months prior to the study among men but not women. This was consistent across study centres and also after further adjustment for house dust mite sensitivity. At a genome-wide level, *Ascaris* exposure was associated with 23 differentially methylated sites in men and three in women. We identified hypermethylation of the MYBPC1 gene, which can regulate airway muscle contraction. We also identified genes linked to asthma pathogenesis such as CRHR1 and GRK1, as well as a differentially methylated region in the PRSS22 gene linked to nematode infection.

We concluded that *Ascaris* exposure was associated with substantially lower lung function and increased asthma among men. Seropositive participants had sex-specific differences in DNA methylation compared to the unexposed, thus, suggesting that exposure may lead to sex-specific epigenetic changes associated with lung pathology.

4.3 IgE or IG4 response against *Ascaris* in Northern Europe is independent of house dust mite sensitization.

We aimed to further validate our findings using four different antigens to measure *Ascaris*-specific IgG4 antibodies in Bergen RHINESSA men. We were investigating whether these measured antibodies actually bind to *Ascaris lumbricoides* L3 larvae

using fluorescence imaging. Further, we wanted to explore whether recent findings from murine models also apply to humans: whether HDM sensitization elicits elevated *Ascaris*-specific IgE and IgG antibody responses.
We showed that our findings are consistent when using different antigens and that people with elevated *Ascaris*-specific IgG4 levels also show IgG4 binding using fluorescent microscopy. However, none of the samples demonstrated any IgE binding, also in HDM IgE sensitized groups. Furthermore, we demonstrated that *Ascaris* exposure leads to elevated IgG4 levels that cross-react with HDM antigens. HDM sensitization itself, however, does not lead to cross-reactive IgE or IgG4 response (demonstrated by no elevated IgG4 or IgE in group C: HDM+ASC-).

We concluded that HDM sensitization by itself does not lead to cross-reactive IgE or IgG4 response towards *Ascaris*. HDM and *Ascaris*-specific IgG4 levels seem to reflect *Ascaris* exposure rather than HDM sensitization.

5. Discussion

In this chapter the strengths and weaknesses concerning the study methods, and results from each paper will be discussed.

5.1 Methodological considerations

5.1.1 Study design

Although the ECRHS and RHINESSA studies are both prospective cohort studies, we have used the available serum samples collected at one time point for the current thesis and the analyses are limited to cross-sectional data. The study design, therefore, limits our possibility to infer any causal relationship between exposure to *Ascaris spp.*, asthma and allergic outcomes, but we can describe associations of helminth exposure to allergies and lung health. Moreover, measuring IgG4 antibodies against *Ascaris* has been shown to be sensitive for determining infection. These antibodies usually rise 4-5 weeks after the infection and, therefore, the presence of these IgG4 antibodies places the measured exposure before the measured outcome.

In Paper I, we use measurements from two generations (ECRHS and RHINESSA) collected at two different time points (ECRHS 2013 and RHINESSA 2015). Assessing parental exposure that might have happened before offspring birth and outcomes in the offspring is therefore limited. We cannot conclude whether the measured IgG4 response reflects current or past infection in the parent's generation. However, the existence of long-lived memory/plasma B cell populations as a source of this IgG4 could be plausible.

In Paper II, the inclusion of three different study populations from different geographical sites and using several ELISA methods to measure exposure strengthens our findings.

In Paper III, the number of participants was selected based on previous HDM sensitization and IgG4 antibody measurements against *Ascaris* using the crude worm antigen. The number of participants in each group was limited to six as only six participants were both sensitized to HDM and were anti-*Ascaris* IgG4 seropositive. We limited the research to men as the association with lower lung function and *Ascaris* seropositivity in Paper II was not seen in women.

5.1.2 Study population

The ECRHS cohort was originally recruited from a random sampling of a general population living in respective study sites, the overall response rate was 78% [3]. Only ECRHS participants from the third follow up in Bergen were included in Paper I. All offspring of ECRHS participants were identified through national registries. The participation rate in the RHINESSA clinical study was quite low, 35% among the eligible offspring. However, the demographic characteristics of the participants did not differ substantially from the general population [159]. The low participation rate in some of the RHINESSA centres could partially be explained by the sites being university towns. During the first recruitment of ECRHS adults aged 20 – 44 years were included. After a long follow-up period, a large proportion of these people would have moved away from the study centres. Their offspring may also have relocated to other

cities, making it less likely for them to attend the clinical visits. Overall, our results should be generalizable to adult Europeans and other urban lifestyle populations, although the study population was quite homogenous, and white Caucasians made up the majority of the cohort.

5.1.3 Measuring exposure to helminths

Unfortunately, we did not have any available faecal samples to confirm the diagnosis of ascariasis which is considered the gold standard of diagnosis. However, the absence of eggs in the faecal samples would not exclude recent infection or light infection. The collection, storage, and analyses of faecal samples from three consecutive sampling rounds to increase the sensitivity and specificity of diagnosis, would also be laborious and expensive. With that in mind, serodiagnosis is a feasible method to apply in such a research setting.

5.1.4 Cross-reactivity with other allergens

Cockroach proteins could be potential cross-reactive allergens with *Ascaris*. Cockroach allergen was included in the SPT panel, but only 3% were sensitized, too few to test for associations with *Ascaris* seropositivity. HDM sensitization is commonly assumed to be a a potential source of cross-sensitization due to the close structural homology between HDM and *Ascaris* proteins, such as tropomyosin and enolase [110]. Another potential source of cross-reactive allergens is exposure to *Anisakis simplex*, a round worm present in fish in the Atlantic Ocean. However, we excluded *Anisakis* from our studies, as the IgE seroprevalence has been shown to be non-existent among Norwegian blood donors from the same region [160]. Furthermore, the participants also answered a food frequency questionnaire, where no association was found between anti-*Ascaris* IgG or IgG4 levels and frequency of fish consumption. As other nematodes are a potential source of cross-reactivity, we pre-incubated the sera in Paper I on A. *lumbricoides*-coated plates and then transferred the sera to *T. canis*-coated plates in order to reduce cross-reactivity.

A strength in our studies is also the use of different antigens for measuring *Ascaris*

exposure (Paper III) and measuring IgG antibodies towards five other helminths (Paper II), which also showed *Ascaris* to be the most prevalent. One potential source of cross-reactivity that we did not consider at the time of conducting the studies was potential sensitization to alpha-1,3 galactose (alpha-gal), which is present in *Ascaris* as well as ticks [161], and has been of great interest in relation to red meat allergy all over the world, including Europe [162] [163] [164]. However, when assessing the food frequency questionnaire among RHINESSA participants, *Ascaris*-specific IgG/IgG4 levels were not related to recent consumption of any kind of meat.

5.1.5 Lung function and asthma

Spirometry was conducted by trained study personnel according to ATS/ERS guidelines in all study centres following the same protocol. The EasyOne spirometer, which was used in all study centres, was calibrated every morning prior to the arrival of study participants. Asthma status was based on self-reported questionnaire data, which might lead us to overestimate the prevalence of asthma. However, the high prevalence of asthma among the study centres is in accordance with other studies [4]. Moreover, we did also include asthma symptoms and showed that *Ascaris* seropositivity was associated with increased odds of having more than 2 asthma symptoms during the last 12 months prior to the study among men. Spirometry was not performed in participants positive for any of the following criteria: last trimester of pregnancy; thoracic or abdominal surgery within last three months; myocardial infarction within last 3 months; hospitalization due to cardiac problems within the past month; detached retina or any eye surgery the past 3 months; heart rate >120 bpm; current tuberculosis treatment. A strength of this thesis is the extensive data on respiratory symptoms and spirometry data, as well as *Ascaris* serology, for population-based cohorts. Further, inclusion of three study centres from different countries contributes to higher credibility and generalizability of the results.

5.1.6 Measuring allergic outcomes

ALK-Abello allergen extracts are widely used in clinical and research settings, and the results from Paper I can be easily comparable by other researchers investigating sensitization with SPTs. SPT positivity was determined as a mean wheal size larger than 3mm as compared to the negative control. The sensitivity of the test also depends on the pressure applied, the mean diameter of positive histamine wheals among the participants was 4.3 mm. The participants were asked about their regular usage of antihistamines and were advised not to use any anti-allergic medicines prior to coming to the study centre. We did not perform duplicate tests because the original protocol for the ECRHS, with which the RHINESSA protocol was harmonized, did not include duplicates. Moreover, it has been shown that performing SPT twice with the same allergen batch does not enhance the validity of the test. Hay fever was self-reported from questionnaires. However, self-reporting of hay fever has been shown to be quite sensitive [165].

5.1.7 Fluorescence imaging of antibody binding

Live *Ascaris* L3 larvae were hatched from eggs and cytospun to positively charged slides (Thermo Fisher Scientific: Superfrost Plus). The supernatant with the larvae also contained some debris (eggshells, broken worms) which interfered with clear visualization of IgG4 binding as the eggs themselves were auto-fluorescent at a similar wave length (far red) as the secondary antibody that was used to visualize the IgG4 binding. For future reference, only supernatants that have minimal debris should be used. A weakness in our methods is that we did not have a positive IgE control, where we could show IgE binding. The potential of IgG epitopes to bind to the larvae blocking IgE binding is also of concern. Another weakness in our methods is the assessment of IgG4 and IgE binding qualitatively: binding was assessed as whether it was present or not, and we did not quantify the binding. However, when binding was present, it was clearly distinguishable from slides that had no binding.

5.1.8 EWAS

A limitation of the epigenetic analysis is that it was carried out on DNA extracted from whole blood as other relevant tissues (e.g. sorted cells, Bronchoalveolar lavage (BAL) samples) were not available. Although, the epigenetic analysis was adjusted for blood cell type composition, it is possible that differences in cell subtype composition between *Ascaris*-exposed and -unexposed individuals may partly account for the observed associations. A strength of the EWAS analyses is the relatively large sample size, including participants from 3 study centres and the use of the Illumina EPIC assay which measures the methylation of 850 000 CpG sites. However, we cannot address whether the identified DNA methylation changes lie on the causal pathway between *Ascaris* exposure and lung function [166].

5.2 Main findings and previous literature

5.2.1 Prevalence of asthma and allergy

In Paper I, our reported prevalence of SPT positivity and asthma is in accordance with previous studies. However, we did not see any increase in self-reported asthma prevalence between two generations [4]. Wheezing was less frequent among the offspring generation than in the parents (14% and 25% accordingly), which is most likely attributed to a lower percentage of smokers among the offspring generation. In Paper II the reported 20% prevalence of ever asthma in Denmark in our cohort is much higher than previously reported [5]. The participation rate for all the RHINESSA centres was quite low, only 35%. This could lead to a selection bias. However, it is unlikely that selection bias could cause association between exposure to *Ascaris* and lung function or asthma. It has been previously reported that offspring who participated in RHINESSA more often had a parent with asthma [159]. This could induce a response bias among offspring who have a history of asthma in the family, leading to higher prevalence of self-reported asthma. However, the prevalence of asthma symptoms 12 months prior to the study was around 6% in all centres and the association with asthma symptoms and *Ascaris* exposure was consistent throughout the study centres. The

reported prevalence in Bergen and Tartu were in agreement with previous studies [4, 7].

5.2.2 Helminth exposure and potential cross-reactivity with other allergens

To the best of our knowledge, Paper I and Paper II are the first studies to address the prevalence of exposure to helminths in Norway. The source of this exposure remains unknown as the natural life cycle of STH is believed to be broken in countries like Norway, Estonia and Denmark. In Denmark, a potential source of exposure to humans could be from pig farms as ascariasis is considered a zoonosis [47], and is still a problem long after first reports about it [167]. For Estonia and Norway this has not been shown. One potential source of exposure could also be travelling. More than 20% of tourists can be affected by traveller's diarrhoea, showing that a large proportion of them ingest *Escherichia coli* or some other pathogen [168]. Ascariasis is mostly asymptomatic, so there is no indication of how many people could be infected while travelling. Many studies have shown a high level of *Ascaris* contamination of fruit and vegetables in markets etc. (e.g. in Ethiopia, Nigeria, Pakistan) [169] [170] [171]. Exposure from imported fruit or vegetables is unlikely, as there is a study showing that imported fruits in Norway are not contaminated with *Ascaris* [172]. However, there are Polish studies showing >90% of sludge samples in Poland having infective *Ascaris* eggs [37] (this sludge may be used as a fertilizer) and also soil from farms have been shown to have a high rate of *Ascaris* and *Toxocara* contamination [38] [173]. Moreover, endemic ascariasis due to contamination of imported vegetables has previously been reported in the Nordics [174]. Therefore, imported fruit/vegetables/gardening soil should not be excluded as a potential source of infection to helminths in Norther European countries.

The main source of cross-reactivity is believed to be from house dust mites. In Paper I, no association was observed for *A. lumbricoides* and *T. canis* IgG4, and allergic sensitization towards HDM. However, as we did not have data on specific IgE towards *Ascaris* or *Toxocara* in Paper I, we could not exclude the possibility that exposure to HDM or cockroach allergens may have influenced *A. lumbricoides* IgG4 seropositivity. Adjusting our analyses for HDM sensitization in Paper II did not alter the results for

either lung function or asthma. Furthermore, in Paper III we addressed this issue and showed that HDM sensitization is not the source of the measured IgG4. We showed that participants sensitized to HDM, but who were *Ascaris* IgG4 seronegative (Group C) did not have elevated IgG4 response towards any of the *Ascaris* antigens nor to HDM extract. In addition, no larval binding of IgG4 could be visualized in group C, which supports our findings that the measured IgG4 response is due to *Ascaris* and not HDM exposure.

The use of somatic *T.canis* antigen in Paper I as opposed to excretory-secretory (ES) antigen may have reduced the specificity and sensitivity of our ELISA. The association with *Toxocara* exposure in Paper I could have been attributed to *Ascaris* exposure, as when using a more specific *Toxocara* ES antigen in the NovaTec ELISA kits (Paper II), *Toxocara* seroprevalence was only 0.7%. At the same time *Ascaris* had the highest seroprevalence.

The association between farm upbringing and pet keeping in childhood with helminth seropositivity was not surprising, as one of the potential sources of infection is contact with animals [44]. In the parent generation in Paper I, *Ascaris* seropositivity was associated with being brought up on a farm with livestock. Given the nature of the exposure and the known association between *Ascaris* spp. and farm animals, especially pigs, it would have been valuable to know which type of livestock the participants had been in contact with. Unfortunately, this information was not captured in the questionnaires and interviews. Childhood contact with animals and seropositivity measured years later suggest the existence of long-lived memory/plasma B cell populations as a source of this IgG4, as current pet keeping was not associated with elevated IgG4 levels.

We cannot rule out that cross-reactivity with some unknown exposure or allergen is the source of the elevated antibody response in these cohorts but the consistent results with using different ELISA methods and IgG4 antibody binding to *Ascaris* larvae all point to genuine *Ascaris* exposure.

5.2.3 Lung function, asthma, allergies and association with *Ascaris* exposure

Our study populations are thoroughly characterized, but the relatively low number of study participants could limit our ability to detect all associations with lung function and allergies as related to *Ascaris* exposure.

An unexpected finding from our study was the large differences in allergic risk association between the parent and offspring generations in Paper I. This might imply that especially early-life exposure to helminths is a risk factor for the development of allergic disease, as seropositivity was associated with childhood animal exposure and that the risk resulting from this exposure may not be lifelong. Moreover, changes in microbial diversity in the environment [175] might potentially be of importance for response to helminth infections. Bacterial infections can alter the response to helminths [176], and helminth infections themselves can lead to worse outcomes in bacterial infections, as shown with *Mycobacterium tuberculosis* infection [177]. Although, the difference between the generations might also reflect a time trend rather than a biological age pattern.

An intriguing finding in Paper I was the association of parental *Toxocara* spp. seropositivity with offspring allergic outcomes, namely paternal exposure increased risk of allergy in daughters and maternal exposure increased risk in sons. Adjustment for parental allergies did not alter the findings, suggesting that heritability in allergy or reverse causation did not explain the findings [178]. Others have shown that maternal helminth infections can influence offspring susceptibility to allergy [179], which is also supported by the previously mentioned murine models.

To the best of our knowledge, our study was the first to investigate associations between serum *Ascaris* IgG antibody levels and lung function assessed by spirometry in humans. Men who were seropositive to *Ascaris* had lower FEV_1, unaltered FVC, and more asthma symptoms. A dose–response pattern was found: FEV_1 decreased with increased concentration of anti-*Ascaris* IgG. In women, no association was found with lung function, but asthma symptoms were significantly less common in seropositive women. However, higher *Ascaris* infection rates have been reported in women [180] [181], which is in accordance with our findings. The sex differences in the associations of

Ascaris seropositivity with lung function and asthma are striking. Infection intensity and burden can be influenced by sex-related behavioural and environmental factors that contribute to risk of exposure to infectious eggs. Differences in sex hormone levels could hypothetically influence the pathogenic outcomes from *Ascaris* exposure. Oestradiol, the main female sex hormone, is known to be important for many tissue repair processes, notably inflammation and granulation [182] [183], and tissue damage is an essential element in the pathology caused by *Ascaris*. Murine models have also shown that female mice can have a delayed Th2 response to helminth infections compared to males [184]. Such regulatory mechanisms could possibly lead to a higher worm burden in female subjects and higher *Ascaris* IgG antibody levels, as also found in our studies. Sustained Th2 responses in male subjects, on the other hand, could enhance pathology such as pulmonary inflammation. Interestingly, we discovered hypomethylation in dmCpGs in NAV3 (a gene involved in immunoregulatory processes through IL-2) in women, suggestive of sex differences in immunoregulation of helminth responses. In the whole cohort, we found differentially methylated CpG sites among the seropositive subject that have previously been associated with nematode infestation, which supports our findings. The apparent lower likelihood of asthma risk in seropositive women is still surprising, and we speculate that altered immunoregulation may play a role [166].

Our findings regarding increased asthma symptoms among seropositive men are supported by animal models [185]. However, most previous research in humans linking *Ascaris* infection and asthma is based on children's cohorts [92, 93, 186]. A recent meta-analysis concluded that *A. lumbricoides* infections were associated with an increased risk of bronchial hyperreactivity in children and an increased risk of atopy among helminth infected adults [94]. However, the evidence is still conflicting and not sufficient to assess the effect of *Ascaris* infection on induction or exacerbation of asthma [187].

It is possible that the negative association between *Ascaris* exposure and FEV_1 is an example of reverse causation and reflects a dampened response to *Ascaris* leading to higher worm burden and more pronounced lung damage in men. Higher IgG4 values could be a sign of this inability to clear the infection, as they can function as blocking

antibodies. In case of repeated or prolonged presence of an antigen, class-switching of B cells to IgG4 occurs. The latter outcompetes any other IgG isotypes by higher affinity to the antigen and can stop the inflammatory response of the IgG1/FcγR activation by high-affinity interaction with the only inhibitory IgG receptor, FcγRIIb. Furthermore, elevated IgG4 is also associated with immune escape in helminthic infections [188, 189].

It is possible but unlikely that some unmeasured confounding, except from cross-reactivity with other allergens, would influence the association of lung function and elevated antibody response towards *Ascaris*.

5.2.4 Fluorescence imaging of antibody binding

The average slide contained about 25 intact larvae. We did also visualize some debris on the slide (egg shells, broken larvae, parts of sheaths). Antibody binding to *Ascaris* larvae for IgG4 and IgE was assessed in the far-red spectrum on fluorescence microscope. As the eggs also emitted red autofluorescence, they added some difficulties in the visualization. The measured IgG4 response in groups A and B (seropositive for IgG4 against *Ascaris*) showed clear IgG4 binding whereas no binding was seen in group C (only HDM sensitized) or D ("naïve"). This supports our ELISA data that HDM sensitization itself does not drive a cross-reactive IgG4 response towards *Ascaris*, and shows that there is no unspecific binding of IgG4 to the larvae. We did not see any IgE binding, which also shows that there is no specific or unspecific IgE binding to the larvae. However, we cannot exclude that due to higher amounts of IgG in the serum, the potential unspecific binding of IgG might block the IgE binding to the larvae.

6. Conclusions

The overall objective of this thesis was to investigate how helminth exposure affects lung health and allergies in a non-endemic region. The objective was addressed through three papers. Paper I focused on the association of helminth exposure and allergies across generations. With findings from Paper I indicating a relationship between exposure and allergic outcomes in a younger generation, we focused on assessing

exposure to five helminths in this generation, and narrowed our research down to the most prevalent helminth – *Ascaris*. In Paper II, we assessed *Ascaris* exposure and its relation to lung function and epigenetic changes associated with the exposure in three Northern European study centres. In Paper III, we validated our measurement of exposure by demonstrating antibody binding to larvae grown in vitro. Additionally, we showed that HDM sensitization is not the source of the measured antibodies against *Ascaris*.

Based on Paper I, we concluded that in an affluent young adult Northern European population, helminth exposure is still relatively common, and is a risk factor for allergic outcomes. Although helminth exposure was associated with childhood exposure to farm animals and pets, this did not explain the associations between helminth seropositivity and allergies. When investigating an older parental generation, helminth seropositivity was associated with allergic outcomes in their offspring, but not in themselves. Our findings suggest that zoonotic helminth exposure may modify the risk of allergic disease.

Based on Paper II, our findings suggest that men and women respond differently to *Ascaris* infections, and that *Ascaris* exposure may possibly have long-term consequences for lung health. Our findings show that detection of higher serum IgG antibody levels against *Ascaris* is associated with substantially lower lung function and more asthma among Northern European men. Moreover, the effect magnitude for lung function was larger than that of current smoking. In women, no association was found with lung function, but asthma symptoms were significantly less common in seropositive women. Regarding EWAS analyses of *Ascaris* exposure, a wide range of sex-specific DNA methylation markers associated with *Ascaris* exposure were identified in genes linked to traits such as asthma, lung function, and immunoregulation. These findings support the notion that *Ascaris* exposure may have severe consequences for lung function, and that DNA methylation changes due to *Ascaris* exposure may be involved in the pathogenesis.

In Paper III, we find that the IgG4 antibodies measured in human serum against *Ascaris* bind to cultured *Ascaris* larvae. Moreover, it seems that raised IgG4 levels towards

HDM antigens reflect *Ascaris* exposure rather than HDM sensitization. Contrary to murine models, it seems that HDM sensitization in humans, does not lead to cross-reactive IgE or IgG4 response towards *Ascaris*. Both these findings support the notion that our IgG4 antibody measurements specifically reflect *Ascaris* exposure. Overall, our findings identify a need to investigate the role of helminths on long-term lung health globally, including in high- and middle-income countries, as well as in low-income countries, where helminth exposure is highly prevalent.

7. Future perspectives

This thesis has added valuable knowledge on the potential of helminth infections to influence the risk of allergies and lung health in Northern Europe. We found that *Ascaris* exposure was associated with increased risk of allergies and substantially lower lung function in men. Both the relatively high prevalence of helminth exposure ,and the strong associations with allergies and low lung function were surprising in these populations, and needs further research. The sex differences in the risk-associations are compelling, and new studies are emerging that have stratified their analyses by sex that support our findings. Our epigenetic analyses also support true sex differences in response to helminths, still, the underlying mechanisms are poorly understood. We believe that this thesis will further fuel the research into the relationship of helminths, lung health and allergies, also in countries in which helminths are believed to be non-endemic.

The origin of exposure to *Ascaris* remains unknown in the Northern European cohorts we have studied. Identification of the potential sources is of great interest for public health, and could specifically be relevant regarding policies e.g. on regulations of imported fruit, vegetables, fertilizers etc. Given the opportunity, we plan to analyse samples from water treatment plants in Norway to see whether we can detect any *Ascaris* eggs via PCR methods, that have a lower limit of detection of 1 egg per 500ml. This would support our findings, that *Ascaris* is a neglected public health threat also in

Europe. Furthermore, we plan to assess soil samples from parks and commercially available gardening soil as potential source of helminth infections.

Based on the compelling results from Paper I, and from animal models, that suggest preconception parental helminth infections could influence offspring immune response, we plan to investigate whether preconception helminth exposure in humans is associated with offspring allergic and respiratory outcomes. This could be addressed using maternal serum from the ECRHS I study samples in Tartu collected in 1992, before the conception of the offspring, and offspring data for outcomes from the RHINESSA study, collected in 2015. This is the only study centre with such material available.

Overall, this thesis presents intriguing results suggesting that helminth exposure may possibly still be a major public health concern in affluent Northern European countries. We speculate that further insights into the role of helminth exposure for immune maturation across generations might open new perspectives in research on asthma and allergies. However, our studies cannot prove causal associations, our understanding of helminth exposure in our study setting is limited, and our results should further be replicated in high-endemic areas. In this regard, I find that this thesis can serve as a catalyst for future research and a reminder to public health authorities as well as scientists not to forget helminth infections as a potentially overlooked health hazard.

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9. Papers I – III

I

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WILEY

ORIGINAL ARTICLE

Clinical Allergy

Zoonotic helminth exposure and risk of allergic diseases: A study of two generations in Norway

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Summary

Background: Animal and human studies indicate that definitive host helminth infections may confer protection from allergies. However, zoonotic helminths, such as *Toxocara* species (spp.), have been associated with increased allergies.

Objective: We describe the prevalence of *Toxocara* spp. and *Ascaris* spp. seropositivity and associations with allergic diseases and sensitization, in 2 generations in Bergen, Norway.

Methods: Serum levels of total IgG4, anti-*Toxocara* spp. IgG4 and *Ascaris* spp. IgG4 were established by ELISA in 2 cohorts: parents born 1945-1972 (n = 171) and their offspring born 1969-2003 (n = 264). Allergic outcomes and covariates were recorded through interviews and clinical examinations including serum IgEs and skin prick tests.

Results: Anti-*Ascaris* spp. IgG4 was detected in 29.2% of parents and 10.3% of offspring, and anti-*Toxocara* spp. IgG4 in 17.5% and 8.0% of parents and offspring, respectively. Among offspring, anti-*Toxocara* spp. IgG4 was associated with pet keeping before age 15 (OR = 6.15; 95% CI = 1.37-27.5) and increasing BMI (1.16 [1.06-1.25] per kg/m^2). *Toxocara* spp. seropositivity was associated with wheeze (2.97[1.45- 7.76]), hayfever (4.03[1.63-9.95]), eczema (2.89[1.08-7.76]) and cat sensitization (5.65[1.92-16.6]) among offspring, but was not associated with allergic outcomes among parents. Adjustment for childhood or current pet keeping did not alter associations with allergies. Parental *Toxocara* spp. seropositivity was associated with increased offspring allergies following a sex-specific pattern.

Conclusions & Clinical Relevance: Zoonotic helminth exposure in Norway was less frequent in offspring than parents; however, *Toxocara* spp. seropositivity was associated with increased risk of allergic manifestations in the offspring generation, but not among parents. Changes in response to helminth exposure may provide insights into the increase in allergy incidence in affluent countries.

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KEYWORDS
allergy, Ascaris, Asthma, ECRHS, helminths, IgG4, Norway, RHINESSA, Toxocara, zoonosis

1 | INTRODUCTION

Exposure to micro-organisms may alter subsequent risk of non-infectious diseases, such as allergy. For example, protection against allergy as a result of being brought up on a farm[1] has been explained by the innate immune response being trained by the local microbial environment.[2] Conversely, pathogenic lower respiratory tract viral infections in early life, such as those caused by respiratory syncytial virus (RSV) infections, lead to a remodelling of the pulmonary immune system with potential elevated risk of subsequent allergic airway disease.[3]

It is hypothesized that exposure to helminths might be an important contributor to the risk of allergic disease. Helminth infections result in the host eliciting a type 2/Th2 immune response. This is characterized by helminth interaction with epithelial cells causing epithelial release of alarmin cytokines (eg, IL-25/IL-33) which drive innate lymphoid type 2 (ILC2) secretion of IL-4, IL-5 and IL-13. These cytokines support the induction of characteristic eosinophilia and M2 macrophage polarization along with CD4 T cell polarization to a Th2 phenotype and B cell secretion of IgE, a response similar to that which drives allergic pathology.[4] However, pre-clinical studies have demonstrated that helminth infections limit induction of type 2 allergic pathology by secreting substances that directly influence host immunity by, for example, raising regulatory immune response components (eg, regulatory T and B cells: Treg and Breg)[5,6] or limiting epithelial cell alarmin secretion.[7] Clinical studies in areas endemic for helminth infections support these findings.[8-10] The presence of helminth infection may be required for this protection as antihelminth therapy can lead to increased prevalence of allergic disease in helminth endemic regions.[11] These and other related studies have led to the suggestion that increasing and high rates of allergy in the developed world might be, at least in part, due to the loss of effective immune control by host-adapted parasitic helminth infections.[12]

However, helminth exposure may not always be protective against allergy. In high-income countries, the major human parasitic helminths associated with allergy protection have been controlled by strong public health provision breaking the cycle of infection.[13] However, zoonotic exposure is not uncommon through exposure to parasites of livestock and companion animals.[14-16] Indeed, a number of relatively recent studies have identified *Ascaris suum* infection and associated pathology (such as Loffler syndrome) to occur (albeit at low levels) in northern European and North American pig farming areas.[17-20] A more common cause of zoonotic helminth infections comes from *Toxocara canis and T. cati* which naturally infect dogs and cats.[21] According to recent studies, levels of human exposure to these parasites can vary from 6% to 87% depending on age and local environmental factors.[21-28] Zoonotic infection by *Toxocara* spp. is typically via ingestion of eggs by direct contact with dogs or cats or via fecally contaminated soil. Human infection by *Toxocara* spp. is, in most cases, asymptomatic.[21,22,29] However, a proportion of infections can provoke pathologies with rheumatic, neurologic and asthmatic symptoms.[23,30,31] Zoonotic *Toxocara* spp. exposure is also associated with increased risk of asthma and atopy.[23,25,29,32,33]

In this study, we aimed to address how common seroprevalence to *Ascaris* spp. and *Toxocara* spp. was in a Norwegian intergenerational cohort. Seropositivity to both parasites has been reported in northern Europe, but not in Scandinavia. Here, we established exposure by detecting the prevalence of circulating immunoglobulin G4 (IgG4) against both *Ascaris spp.* and *Toxocara* spp. by enzyme-linked immunosorbent assay (ELISA) in a Norwegian two-generation cohort. We then establish what associations existed between seropositivity to these parasites and allergic sensitization and diseases.

2 | METHODS

2.1 | Study population

This study is based on information and samples from Norwegian participants in 2 linked studies, the European Community Respiratory Health Survey (ECRHS; www.ecrhs.org)[34] and the Respiratory Health In Northern Europe, Spain and Australia study (RHINESSA; www.rhinessa.net).

The parent population comprised of 171 ECRHS participants born 1945-1972 from the study centre in Bergen (originally recruited from the general population aged 20-44 years in 1992-1994) that were followed up in 2010-2013. Participants underwent an interviewer-led questionnaire, lung function measurements and skin prick tests to aeroallergens, and provided blood samples for measurements

of total and aeroallergen-specific immunoglobulin Es (IgE) and serum parasite-specific IgG4s.

The offspring population included 264 adult and adolescent offspring (≥10 years of age, born 1969-2003) of the Bergen ECRHS participants. They were examined in 2014-2015 as part of the RHINESSA study, with questionnaires, clinical examination, skin prick tests and measurements of serum IgEs and IgG4s, following protocols comparable to those applied to the parents.

2.2 | Ethical approval

Approval was obtained from the Regional Committee for Medical and Health Research Ethics in Western Norway (approval numbers #2010/759 and #2012/1077). All participants provided informed written consent.

2.3 | Allergic sensitization and diseases

Allergic sensitization was determined by skin prick tests (SPT) to 12 allergens (ALK-Abello): timothy grass, ragweed, *Dermatophagoides pteronyssinus, D. farinea*, cat, dog, birch, *Blatella germanica*, olive, *Alternaria* spp., *Cladosporium* spp. *and Parietaria* spp., and 0.9% saline and 10 mg/mL histamine solution were used for negative and positive controls. Reactions to the allergens were read after 15 minutes. Reactivity was considered positive if the mean weal size was 3 mm greater than the negative control. Blood samples were collected and sera separated in SST Vacutainer glasses, centrifuged within 30-60 minutes after collection (at 2081 9 g for 10 minutes, room temperature). The samples were stored at −20°C. Total IgE and specific IgE were performed according to standardized laboratory methods in Haukeland University Hospital in Bergen, Norway. IgE positivity was defined by IgE ≥ 0.35 kU/L to at least 1 of 4 allergens tested (cat, timothy grass and house dust mite).

Allergic diseases were assessed through standardized interviews, including questions on doctor's diagnosed asthma, symptoms of wheezing, hayfever (seasonal rhinitis), rhinitis (all year round) and eczema (see www.ecrhs.org and www.rhinessa.net for wording of questions).

2.4 | Preparation of helminth antigen

Toxocara canis worms were kindly provided by Professor Philip Cooper, Ecuador. *Ascaris lumbricoides* worms were obtained from Professor Mike Levin, Red Cross Children's Hospital, Cape Town. Whole worms were washed in distilled water with penicillin, streptomycin and fungizone to reduce contamination, and then washed 4 times with distilled water. Worms were then homogenized in filter-sterilized phosphate-buffered saline (PBS). The homogenate was centrifuged at 15 292 9 g for 20 minutess and the soluble fraction collected and filtered through a 0.20-Lm filter. Protein concentration of soluble worm antigen preparations was established using a bicinchoninic acid protein assay by Thermo Scientific (Rockford, IL, USA).

2.5 | Detection of total IgG4-, *Toxocara canis*- and *Ascaris lumbricoides*-specific antibodies by ELISA

Analysis of IgG4 towards *T. canis* and *A. lumbricoides* was performed for sera from both ECRHS3 and RHINESSA Bergen participants, whereas total IgG4 was quantified in sera from the RHINESSA participants only. Total IgG4 concentration was detected by ELISA using 96-well Nunc Immunosorb ELISA plates (Thermo Scientific) coated with 20 Lg/mL of mouse monoclonal antibody against human IgG4 heavy chain in PBS. Participant plasma was diluted 1:20, 1:100, 1:500 and 1:2500 in PBS containing 1% bovine serum albumin (PBS-BSA). Serum antibody was detected using alkaline phosphatase-conjugated mouse anti-human IgG4 antibodies from Sigma-Aldrich (St. Louis, MI, USA). ELISA plates were read at 405 nm to determine optical density.

Detection of IgG4 antibodies against *T. canis and A. lumbricoides* was achieved using an indirect ELISA. Ninety-six-well Nunc Immunosorb ELISA plates (Thermo Scientific) were coated with 10 Lg/mL of soluble helminth antigen diluted in carbonate buffer. The serum from participants was diluted 1:20, 1:100, 1:500 and 1:2500 in PBS-BSA. Bound antibodies were detected using alkaline phosphatase-conjugated mouse anti-human IgG4 antibodies (Sigma-Aldrich). ELISA plates were read at 405 nm to determine optical density (OD). Relative plasma recognition of soluble worm antigen was calculated from optical density vs sample dilution curve.[35]

Antihelminth immunoglobulin responses can be cross-reactive between helminth species.[36,37] Anti-*A.lumbricoides* IgG4 (anti-*Ascaris* IgG4) was used as a general marker of exposure to parasitic nematodes. To reduce cross-reactivity in assessment of *Toxocara,* sera were pre-incubated on *A. lumbricoides* antigen-coated plates and then transferred to *Toxocara* antigen-coated plates.

2.6 | Covariates

Data relating to age, sex, education level, smoking status, parental history of allergic disease, place of upbringing (farm with livestock, farm without livestock, village in rural area, small town, suburb of city, inner city) and pet ownership (cats and dogs in childhood and current pet keeping) were retrieved from interviews performed during the clinical examinations of the ECRHS and RHINESSA participants, the same day as the blood samples were taken. Smoking (in adults) was categorized into never smokers, previous smokers and current smokers. The study subject's level of education was categorized as primary school, secondary/technical education and college/university, whereas the adolescents were categorized as being students/still in school.

2.7 | Statistical analyses

Descriptive statistics for the study population were reported as mean and range or standard deviation for continuous variables and count and percentages for categorical variables.

Logistic regression was applied to assess associations between pet keeping, place of upbringing, age and sex as well as other

potential variables that might be associated with helminth exposure (anti-*Toxocara* and anti-*Ascaris* IgG4 positivity). Similar models were applied to study associations between *Toxocara* seropositivity and allergic sensitization (specific IgE and SPT towards inhalant allergens), total IgE, rhinitis, hayfever and asthma. Models were performed separately or combined for the 2 generations (ECRHS parent and RHINESSA offspring generations). In all regression models with the combined study populations and when analyzing the offspring generation, we corrected for clustering within families (parent-offspring and/or siblings) by applying a cluster for family-id. To discriminate between helminth infections that can translate into high specific IgE which does not necessarily reflect SPT reactivity, we performed sensitivity analyses with separate models for *Toxocara* and *Ascaris* IgG4 sero positivity and associations with SPT and specific IgE towards any inhalant, cat, HDM and timothy (grass) allergens.

STATA (StataCorp, College Station, TX, USA), version IC 14.0, was used in all analyses.

3 | RESULTS

3.1 | Characteristics of study populations

The median age was 26 years in the offspring generation and 53 years in the parent generation (Table 1). The education level in this population was high, with more than 50% of study participants reporting University or college degrees. The parent generation had more often kept cats in childhood, but there was no statistical difference in dog keeping between the 2 generations (Table 1). Current pet keeping was similar between the 2 cohorts. Of the parent generation, 15% had grown up on a farm with livestock, compared to only 0.8% of the offspring generation. The 2 generations had similar lifetime prevalence of rhinitis and hayfever, but the offspring generation had a higher prevalence of positive allergy test (positive SPT or IgE towards at least 1 inhalant allergens) than the parent generation (44.5% vs 31.1%, respectively, $P = .006$) (Table 1).

3.2 | Detection of IgG4 to helminth antigens

Overall, 11.7% had detectable levels of anti-*Toxocara* spp. IgG4, with a higher prevalence among the parents (17.5%) than among the offspring (8.0%), $P = .002$ (Table 1). Overall, 17.9% of the study population had detectable levels of anti-*Ascaris* spp. IgG4; 29.2% in the parent generation and 10.3% in the offspring generation. Among the participants with elevated levels of *Toxocara* spp. IgG4, 88% also had elevated levels of anti-*Ascaris* spp. IgG4, suggesting sera cross-reactivity and/or simultaneous exposure to other parasitic nematodes. The seroprevalence of *Toxocara* and *Ascaris* IgG4 was decreasing in more recent cohorts (Figures 1 and 2) with a statistically significant trend for *Toxocara* in offspring (Figure 1) and decreasing trend for *Ascaris* for parent and offspring combined ($P = .07$, Figure 2). Among the offspring, we also detected total IgG4 in 77% and 86% of the subjects with either anti-*Ascaris* spp. or anti-*Toxocara* spp. IgG4, respectively. Total IgG4 was associated with cat and dog keeping in childhood.

3.3 | Factors associated with exposure to helminths

Seropositivity to *Toxocara* spp. and *Ascaris* spp. increased with age and with BMI among the offspring, but did not differ significantly by gender (Table 2A and B). *Toxocara* spp. seropositivity was associated with pet keeping before the age of 15 among the offspring (OR = 6.15 [1.37-27.54], $P = .02$), but was not associated with pet keeping among the parents (Table 2A). For *Ascaris* spp. seropositivity, associations were seen for cat keeping during both early and late childhood in the parent generation (Table 2B). Current pet ownership was not associated with raised anti-*Toxocara* spp. IgG4 or anti-*Ascaris* spp. IgG4. The risk of *Ascaris* seropositivity was increased among parents who had grown up on a farm with livestock (OR = 3.38 [1.31-8.69]) or in a small town (OR = 2.36 [1.06-5.27], $P = .04$ for trend) as compared to those growing up in the city or in the suburbs (Table 2B). There was no association between *Toxocara* spp. and *Ascaris* spp. seropositivity and total IgE (data not given).

3.4 | Exposure to helminths as associated with risk of allergic sensitization and diseases

In the offspring generation, anti-*Toxocara* IgG4 was associated with 3 to 4 times increased risk of reported wheeze, hayfever and eczema (Table 3A). Anti-*Toxocara* IgG4 was further associated with increased risk of positive SPT/IgE towards cat allergens (OR = 5.65 [1.92-16.6]). In the parent generation, anti-*Toxocara* IgG4 was not significantly associated with any allergic outcome; the associations were generally in a negative direction (Table 3A). For anti-*Ascaris* IgG4, the patterns were generally similar, with anti-*Ascaris* IgG4 being associated with increased allergic outcomes in the offspring generation but not in the parent generation (Table 3B). In the model assessing cat allergy, we tested for interaction between *Toxocara* seropositivity and childhood cat keeping. No significant interaction was found ($P = .34$). The associations between allergies and *Toxocara* seropositivity were not altered when adjusted for childhood or current pet keeping. The results of the sensitivity analyses for *Toxocara* spp. and *Ascaris* spp. with separate models for specific IgE and SPT results differed from the models using a combined measure for specific IgE and SPT. Positive results were seen for offspring *Toxocara* spp. positivity and both SPT and IgE towards timothy (aOR = 2.91 [95% CI: 1.11, 7.63] and 3.36 [1.24, 8.44], respectively) (Table S1). *Ascaris* spp. positivity was borderline significant for SPT towards cat (aOR = 2.67 [0.99, 7.24], $P = .05$), but non-significant for *Ascaris* spp. and cat-specific IgE (Table S2).

Although parents' *Toxocara* seropositivity was not associated with their own allergic manifestations, parents' *Toxocara* seropositivity appeared to be associated with allergic manifestations in their offspring (Table 4). Gender-specific patterns, indicating associations between paternal exposure and their daughters' outcomes (significant for asthma, eczema and timothy grass) and maternal exposure and their sons' outcomes (significant for any specific IgE positivity and sensitization to cat allergens), were also found (Table 4).

TABLE 1 Characteristics of study populations (in percent unless otherwise specified)

	All (n = 435)	Parents (n = 171)	Offspring (n = 264)	*P*-value
Gender (% men)	53.9	52.6	54.8	.7
Age in years (median (range))	35 (10-63)	53 (39-63)	26 (10-45)	
BMI (kg/m^2) median (range)		25.6 (18.8-42.0)	24.6 (15.1-40.1)	
Education level				
Student/adolescent	6.3		10.8	
Primary	4.3	8.2	1.6	
Secondary	38.0	43.5	34.2	
University or college	51.4	48.2	53.7	.003[a]
Cat ownership				
Early childhood	22.8	30.6	17.7	.003
Late childhood	43.0	46.8	40.6	.2
Current	21.6	21.3	21.7	.9
Dog ownership				
Early childhood	11.8	8.9	13.7	.2
Late childhood	28.6	27.6	29.2	.7
Current	18.2	18.3	18.0	.9
Place of upbringing				
City or suburb	57.8	46.2	65.8	
Small town	16.6	28.1	8.6	
Village in rural area	15.4	3.5	23.8	
Farm without livestock	3.6	7.0	1.2	
Farm with livestock	6.8	15.2	0.8	<.001
Smoking status				
Never	55.3	40.6	66.8	
Previous smoker	28.9	38.2	21.7	
Current smoker	15.8	21.2	11.5	<.001*
Parental asthma	14.7	7.7	19.8	
Parental asthma not known	8.2	15.4	3.0	.004
Wheeze, ever	18.3	24.7	13.9	.005
Hayfever, ever	24.2	25.4	23.4	.6
Rhinitis, ever	49.8	51.2	48.9	.6
Asthma, ever	14.2	14.1	14.2	.97
Eczema, ever	43.7	49.4	40.0	.06
Any positive allergy test (specific IgE or SPT)[b]	39.3	31.1	44.5	.006
IgE positivity[c]	32.7	19.5	41.2	<.001
Total IgE in kU/L (mean (SD))	71.6 (193.9)	69.9 (240.9)	72.9 (156.7)	.6
Total IgG4 (% positive)[d]			11.4	
Ascaris IgG4 (% positive)	17.9	29.2	10.3	<.001
Toxocara IgG4 (% positive)	11.7	17.5	8.0	.002

[a]Does not include students.
[b]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards at least 1 of 4 allergens (cat, timothy grass, birch and house dust mite).
[c]Positive IgE ≥ 0.35 kU/L to at least 1 of 4 allergens tested (cat, timothy grass, birch and house dust mite).
[d]Only measured for offspring. Information missing for sex and age (n = 3), BMI (n = 3 for offspring, n = 7 for parents), education level (n = 19), father/mother asthma (n = 34) place of upbringing (n = 20), cat ownership (early childhood: n = 41, late childhood: n = 35, current: n = 22), dog ownership (early childhood: n = 44, late childhood: n = 36, current: n = 22), wheeze (n = 20), hayfever, rhinitis, asthma, positive allergy test (n = 5), eczema (n = 7), total and specific IgE (n = 4), *Ascaris* (n = 11),* smoking status only available for adults. Parents=ECRHS. Offspring=RHINESSA.

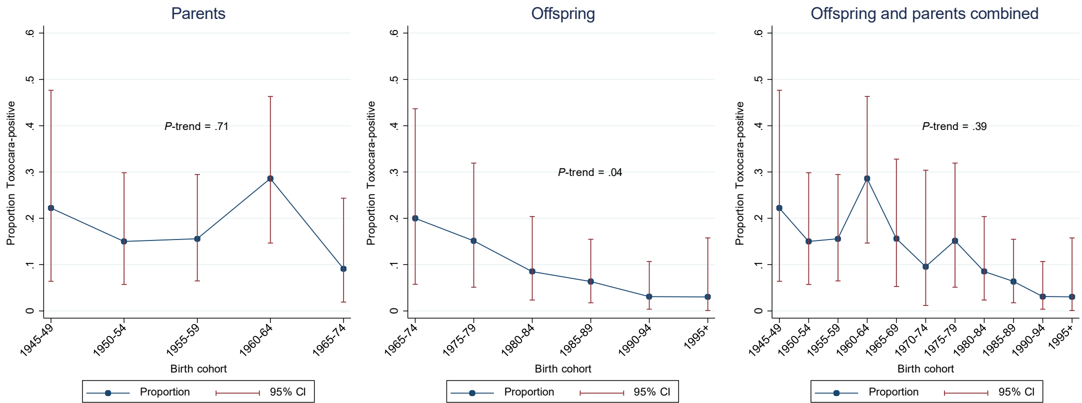

FIGURE 1 Anti-*Toxocara* seropositivity according to birth cohort in the parent generation, the offspring generation and in both combined

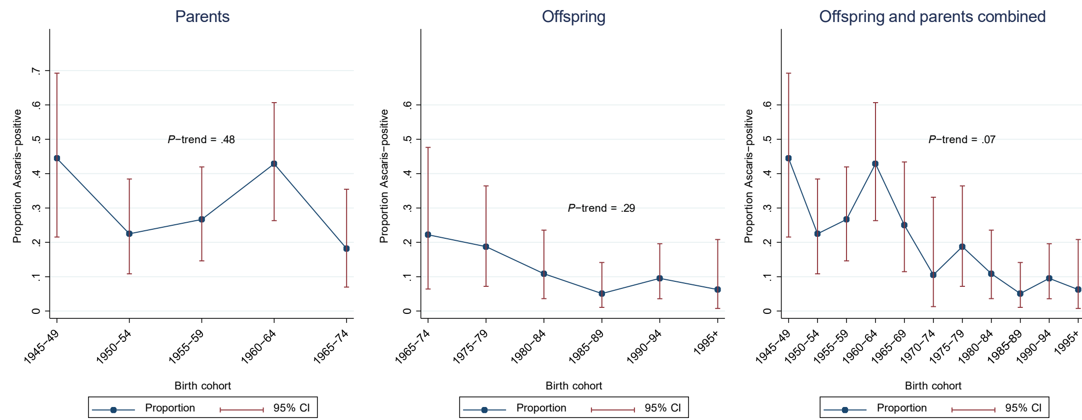

FIGURE 2 Anti-*Ascaris* seropositivity according to birth cohort in the parent generation, the offspring generation and in both combined

4 | DISCUSSION

In this study, we present evidence that exposure to helminths exists in a Norwegian population and that this parasitic exposure may have important health implications. To the best of our knowledge, this is the first study to address prevalence of zoonotic exposure to helminths in Norway. We detected antibodies against *Toxocara* spp. and *Ascaris* spp. in 12% and 18% of the study participants, respectively. We found a higher prevalence of exposure in participants born between 1945 and 1972 (parents) compared to participants born between 1969 and 2003 (offspring).

Helminth exposure was associated with childhood cat keeping in both the parents and offspring generations, place of upbringing in parents, and increasing age and increasing BMI in offspring. Exposure to *Toxocara* spp. was an important risk factor for allergic disease. In the offspring generation, *Toxocara* spp. seropositivity was associated with a 3-4 times increased risk of several allergic outcomes. However, in the parent generation, *Toxocara* spp. seropositivity was not associated with parental allergic outcomes, but did associate with allergic outcomes in their offspring following a complex sex-specific pattern. Neither childhood nor current pet keeping explained the associations of *Toxocara* spp. with allergies.

The 12% prevalence of anti-*Toxocara* IgG in our study corresponds with reports from other European countries and the United States, with 8%-11% reported for Dutch children,[32,33] 8% for Dutch adults,[14] 14% in a US population-based study (NHANES),[28] but lower than among Spanish adults (29%).[29] We found that *Toxocara* spp. seropositivity was associated with allergic diseases and sensitization among the offspring. This is in agreement with findings reported from other regions; for example, Mughini-Gras et al[14] reported an association of anti-*Toxocara* IgGs with hayfever in the Netherlands. Higher prevalence of exposure to *Toxocara* spp. has also been reported in atopic populations in low to middle income countries including Malaysia (21% among asthmatics and 9% in non-

TABLE 2 Odds ratio (OR) and 95% CI for (A) anti-*Toxocara* spp., (B) anti-*Ascaris* spp. IgG4 positivity and associations with gender, age, BMI, cat and dog keeping and place of upbringing

	Parents (n = 171)		Offspring (n = 264)	
	OR (95% CI)	*P*-value	OR[a] (95% CI)	*P*-value
(A)				
Gender (ref men)	0.69 (0.31, 1.55)	.4	0.63 (0.26, 1.51)	.3
Age	1.06 (0.99, 1.14)	.7	1.10 (1.03, 1.17)	.009
BMI (per kg/m2)	0.98 (0.88, 1.09)	.7	1.16 (1.06, 1.25)	<.001
Cat keeping				
Early childhood	1.42 (0.60, 3.39)	.3	1.94 (0.61, 6.19)	.2
Late childhood	1.53 (0.66, 3.52)	.3	6.25 (1.94, 20.12)	.002
Current	0.51 (0.17, 1.58)	.3	0.70 (0.22, 2.22)	.5
Dog ownership				
Early childhood	1.35 (0.35, 5.22)	.9	1.50 (0.39, 5.76)	.5
Late childhood	0.90 (0.35, 2.32)	.8	1.63 (0.57, 4.68)	.4
Current	0.44 (0.13, 1.56)	.2	0.86 (0.29, 2.62)	.8
Any pets from birth to age 15	1.79 (0.70, 4.53)	.2	6.15 (1.37, 27.54)	.02
Place of upbringing				
City or suburb (ref)		.06		.6
Small town	1.05 (0.38, 2.94)		1.18 (0.24, 5.89)	
Village in rural area	3.09 (0.50, 18.9)		0.83 (0.24, 2.94)	
Farm without livestock	1.24 (0.24, 6.41)		-	
Farm with livestock	2.75 (0.96, 7.84)		-	
(B)				
Gender (ref men)	0.83 (0.43, 1.60)	.6	0.55 (0.25, 1.21)	.2
Age	1.07 (1.01, 1.13)	.5	1.06 (1.00, 1.12)	.08
BMI (per kg/m2)	0.99 (0.91, 1.09)	.9	1.17 (1.08, 1.26)	<.001
Cat keeping				
Early childhood	2.32 (1.11, 4.84)	.04	1.21 (0.40, 3.65)	.6
Late childhood	2.02 (0.99, 4.15)	.06	1.76 (0.69, 4.44)	.2
Current	0.52 (0.21, 1.29)	.2	0.72 (0.25, 2.07)	.5
Dog ownership				
Early childhood	1.54 (0.48, 4.89)	.8	0.79 (0.16, 3.76)	.8
Late childhood	0.77 (0.34, 1.74)	.5	1.62 (0.63, 4.15)	.3
Current	0.41 (0.15, 1.14)	.09	1.91 (0.80, 4.58)	.1
Any pets from birth to age 15	2.75 (1.21, 6.25)	.02	1.94 (0.70, 5.35)	.2
Place of upbringing				
City or suburb (ref)		.04		.4
Small town	2.36 (1.06, 5.27)		0.82 (0.17, 4.05)	
Village in rural area	1.97 (0.33, 11.72)		0.72 (0.23, 2.29)	
Farm without livestock	0.78 (0.16, 3.96)		-	
Farm with livestock	3.38 (1.31, 8.69)		-	

[a]Corrected for clustering within families. Parents=ECRHS; Offspring=RHINESSA. Statistically significant odds ratios highlighted in bold.

asthmatics),[30] Turkey (13% of asthmatics and 2% of non-asthmatics)[31] and Sri Lanka (29% of asthmatics and 10% in non-asthmatics).[23] Why zoonotic *Toxocara* spp. infections do not confer the protection associated with natural helminth infections is not known, but may possibly be due to *Toxocara* spp.-secreted immune regulatory products being active against dog and cat immune responses and not against human.[38] Therefore, the *Toxocara* spp. induced type 2 immune response may lack effective regulation in a human host, thereby promoting allergic pathology and potentially sensitization to other allergens.[39]

TABLE 3 Adjusted odds ratio (aOR) and 95% CI for (A) anti-*Toxocara* spp. IgG4 (B) anti-*Ascaris* spp. IgG4 positivity and associations with respiratory symptoms and allergic sensitization

	Parents (n = 171)		Offspring (n = 264)	
	aOR[a] (95% CI)	*P*-value	aOR[a] (95% CI)	*P*-value
(A)				
Wheeze, ever	0.77 (0.28, 2.08)	.6	2.97 (1.45, 7.76)	.03
Asthma, ever	0.86 (0.27, 2.77)	.8	1.24 (0.29, 5.19)	.7
Hay fever, ever	0.97 (0.38, 2.49)	1.0	4.03 (1.63, 9.95)	.003
Rhinitis, ever	0.61 (0.27, 1.37)	.2	3.06 (0.97, 9.72)	.06
Eczema, ever	1.02 (0.45, 2.32)	1.0	2.89 (1.08, 7.76)	.04
Any positive allergy test[b]	0.56 (0.21, 1.48)	.2	1.22 (0.52, 2.88)	.6
Any IgE-positive[c]	0.76 (0.26, 2.19)	.6	1.84 (0.78, 4.38)	.2
Cat SPT/IgE-positive[d]	0.72 (0.15, 3.46)	.7	5.65 (1.92, 16.6)	.002
HDM SPT/IgE-positive[e]	0.46 (0.10, 2.13)	.3	1.41 (0.52, 3.81)	.5
Timothy grass SPT/IgE-positive[f]	1.00 (0.31, 3.24)	1.0	2.12 (0.85, 5.33)	.1
(B)				
Wheeze, ever	0.72 (0.31, 1.64)	.4	1.81 (0.70, 4.68)	.2
Asthma, ever	0.94 (0.36, 2.46)	.9	0.47 (0.10, 2.25)	.3
Hay fever, ever	1.60 (0.75, 3.41)	.2	3.50 (1.42, 8.63)	.007
Rhinitis, ever	0.87 (0.45, 1.71)	.7	2.11 (0.82, 5.40)	.1
Eczema, ever	0.83 (0.42, 1.65)	.6	2.06 (0.85, 5.98)	.1
Any positive allergy test[b]	0.80 (0.38, 1.70)	.6	1.16 (0.50, 2.69)	.7
Any IgE-positive[c]	0.45 (0.17, 1.18)	.1	1.66 (0.71, 3.86)	.2
Cat SPT/IgE-positive[d]	0.62 (0.16, 2.33)	.5	3.31 (1.19, 9.17)	.02
HDM SPT/IgE-positive[e]	0.75 (0.26, 2.19)	.6	1.29 (0.48, 3.49)	.6
Timothy grass SPT/IgE-positive[f]	1.32 (0.52, 3.39)	.6	2.66 (1.10, 6.47)	.03

[a]Adjusted for: gender, age, BMI and corrected for clustering within families.
[b]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards at least 1 of 4 allergens (cat, timothy grass, birch and house dust mite).
[c]Positive IgE ≥ 0.35 kU/L to at least 1 of 4 allergens tested (cat, timothy grass, birch and house dust mite).
[d]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards cat allergen.
[e]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards house dust mite (*D. pteronyssinus* and/or *farinae*).
[f]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards timothy grass. Parents=ECRHS. Offspring=RHINESSA. Statistically significant odds ratios highlighted in bold

An unexpected finding from our study was the large differences in allergic risk association between the parent and offspring generations. No effects from *Toxocara* spp. seropositivity were indicated in the parent generation. However, *Toxocara* spp. seropositivity was strongly associated with increased risk of allergic outcomes in the offspring generation. This may imply that early-life exposure to *Toxocara* is a risk factor for development of allergic disease and that the risk resulting from this exposure may not be lifelong. Changes in microbial diversity in the environment might potentially be of importance for response to helminths, as it has been demonstrated that gut microbiota can alter the response to *Toxocara* in mice.[40]

The age effect in our study, with *Toxocara* seroprevalence increasing with age in the offspring generation, might reflect a time trend rather than a biological age patterns, and decreasing seroprevalence of *Toxocara* in more recent birth cohorts is presented in Figure 1. Similar trends were observed for *Ascaris*. Comparison of offspring and parents in the present study indicated that both *Toxocara* and *Ascaris* exposure were lower in offspring. Rural areas tend to exhibit higher prevalence of human exposure to helminths (35%-42%) than semi-rural (15%-20%) or urban (2%-5%) areas.[41] Although urban living is the most fitting description of the populations in the present study, urban areas, such as parks and town squares, can contain high numbers of *Toxocara* eggs and may represent a substantial risk factor for infection by *Toxocara* spp. Indeed, some studies have reported higher levels of *Toxocara* seroprevalence among subjects living in urban compared to rural areas.[27,29]

An intriguing finding in our study was the association of parental *Toxocara* spp. seropositivity with offspring allergic outcomes, namely paternal exposure increased risk of allergy in daughters and maternal exposure increased risk in sons. Adjustment for pet keeping did not alter the associations; thus, shared environment did not appear to explain the findings. In addition, adjustment for parental allergies did not alter the findings, suggesting that heritability in allergy or reverse causation did not explain the findings. Others have demonstrated maternal helminth exposure to influence offspring susceptibility to allergy,[42] and there are mice models demonstrating that maternal

TABLE 4 Adjusted odds ratio (aOR) and 95% CI for parental anti-*Toxocara* spp. IgG4 positivity and associations with respiratory symptoms and allergic sensitization in offspring

	All offspring (n = 264) aOR[a] (95% CI)	Paternal line		Maternal line	
		Sons (n = 71) aOR[b] (95% CI)	Daughters (n = 69) aOR[b] (95% CI)	Sons (n = 69) aOR[b] (95% CI)	Daughters (n = 48) aOR[b] (95% CI)
Wheeze, ever	1.99 (0.54, 7.34)	0.95 (0.11, 8.04)	0.66 (0.08, 5.49)	6.93 (0.50, 95.2)	-
Asthma, ever	2.85 (1.16, 7.00)	0.57 (0.06, 5.14)	5.43 (1.29, 22.9)	4.96 (0.96, 25.6)	-
Hay fever, ever	1.66 (0.77, 3.61)	0.69 (0.17, 2.71)	2.26 (0.68, 7.44)	3.91 (0.98, 15.67)	0.91 (0.10, 8.62)
Rhinitis, ever	1.47 (0.68, 3.19)	0.62 (0.16, 2.39)	1.78 (0.52, 6.01)	1.95 (0.54, 7.06)	0.72 (0.08, 6.21)
Eczema, ever	1.94 (0.93, 4.04)	1.75 (0.51, 6.03)	3.89 (1.18, 12.8)	0.68 (0.12, 3.89)	2.76 (0.28, 27.5)
Any positive allergy test[c]	1.92 (0.90, 4.09)	1.41 (0.35, 5.65)	2.21 (0.74, 6.64)	4.42 (0.96, 20.29)	0.86 (0.16, 4.62)
Any IgE-positive[d]	1.13 (0.50, 2.56)	0.99 (0.25, 3.95)	1.26 (0.36, 4.36)	6.74 (1.52, 29.9)	0.44 (0.07, 2.99)
Cat SPT/IgE-positive[e]	1.50 (0.53, 4.20)	1.24 (0.25, 6.10)	1.58 (0.29, 8.64)	5.40 (1.22, 23.8)	0.77 (0.10, 5.77)
HDM SPT/IgE-positive[f]	1.31 (0.51, 3.40)	2.21 (0.52, 9.38)	1.09 (0.26, 4.46)	1.56 (0.43, 5.65)	0.54 (0.07, 4.37)
Timothy grass SPT/IgE-positive[g]	2.27 (1.12, 4.60)	1.32 (0.37, 4.76)	3.82 (1.21, 12.0)	2.12 (0.52, 8.62)	1.75 (0.31, 9.81)

[a]Adjusted for: offspring age, parent age, offspring gender, parent gender, offspring *Toxocara* seropositivity, offspring education, offspring BMI, parental asthma/allergy, and corrected for clustering within families.
[b]Adjusted for: offspring age, parent age.
[c]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards at least 1 of 4 allergens (cat, timothy grass, birch and house dust mite), also associated with parental allergy.
[d]Positive IgE ≥ 0.35 kU/L to at least 1 of 4 allergens tested (cat, timothy grass, birch and house dust mite).
[e]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards cat allergen.
[f]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards house dust mite (*D. pteronyssinus* and/or *farinae*).
[g]Positive IgE ≥ 0.35 kU/L and/or skin prick test positivity towards timothy grass. Parents=ECRHS. Offspring=RHINESSA. Gender missing for 3 offspring. Statistically significant odds ratios highlighted in bold.

helminth infection influences immunological characteristics in pups.[43] With regard to differential risk outcome by gender, research focused on other exposures than helminths has found that parental pre-conception exposure might influence disease risk in offspring differently through the maternal and paternal lines.[44,45] The risk of allergy has been reported not only to be affected by the maternal or paternal line, but also to depend on the sex of the child.[44,46,47] In our study, parental anti-*Toxocara* IgG4 was measured years after the offspring were born. The half-life of IgG4 in general is approximately 21 days,[48] but *Toxocara* larvae may stay capsulated in tissues for many years in accidental hosts.[49] Our findings may suggest either ongoing antigenic exposure or the existence of long-lived memory/plasma B cell populations as a source of this IgG4. The mechanisms that may underlie the parental effect on offspring are beyond the scope of our current study. The effect may be due to any range of potential parental influences on the offspring including (but not limited to) post-translational modification,[50] shared environment,[51] microchimerism[52] or passive transfer of immune markers or antibodies.[53,54]

In the present study, we also addressed *Ascaris* seroprevalence in the cohort and found higher rates of seropositivity in the parent (29%) than in the offspring generation (10%). *Ascaris* spp. exposure could be acquired via a number of sources, most plausibly via exposure to agricultural or wildlife sources of *Ascaris* spp. (especially *A. suum*), which are recognized zoonoses in northern Europe.[17,20] That *Ascaris* spp. IgG4 was also more commonly detected in individuals associated with exposure to livestock farm supports this hypothesis. Less likely may be the maintenance of *A. lumbricodes* infection cycle in the study area. The data may also reflect non-specific ELISA reaction due to antigen cross-reactivity. Helminth proteins targeted by IgE have been shown to be cross-reactive with other allergens which include proteins from house dust mite and cockroach,[55] and cross-reactivity between *Ascaris lumbricoides* and *Dermatophagoides farinae* IgG has been demonstrated in rabbits.[56] However, as documented cases of toxocariasis are rarely described in Norway, we would not expect our study participants to have specific IgE towards *Toxocara* spp. We therefore focused on assessing IgG4 towards *Toxocara* spp., which is most commonly used for serodiagnosis of toxocariasis.[57] Although we did not have data on specific IgE towards *Ascaris* or *Toxocara* in the present study, we cannot exclude the possibility that exposure to HDM or cockroach allergens may have influenced *A. lumbricoides* IgG4 seropositivity as observed in the present study. However, we did not observe any association between *Ascaris* or *Toxocara* IgG4 and allergic sensitization (SPT or specific IgE) towards HDM, which is the most commonly described cross-reactive allergen with helminths. Anti-*Ascaris* antibodies are also recognized as being cross-reactive to other helminth antigens[58]; in particular, species within the group of ascaridoid nematodes, such as *A. lumbricoides*, *A. suum* and *T. canis*, show antigenic relationship.[36] Thus, detection of anti-*Ascaris* IgG4 may reflect exposure to other helminths or allergens. Similarly, our use of somatic *Toxocara canis* antigen as opposed to excretory-secretory *Toxocara canis* antigen may have reduced the specificity and sensitivity of our ELISA. However, somatic *Toxocara* spp. antigen ELISA is

acceptable for detecting exposure to *Toxocara* spp.[57] Moreover, we based our definition of exposure on elevated levels of *Toxocara* spp. IgG4 which lends greater specificity[59] to our findings and therefore greater confidence that our measurements genuinely reflect exposure to the parasite. Furthermore, sera were pre-incubated on *A. lumbricoides*-coated plates and then transferred to *T. canis*-coated plates. By applying this method, we limited the possibility that *Toxocara* spp. positivity is a marker for *Ascaris* spp. exposure. There is little data on cross-reactivity between *Toxocara* and environmental allergens. In our study, no association was observed for *T. canis* IgG4 and allergic sensitization towards HDM. In our study, the RHINESSA offspring were tested for SPT towards cockroach allergen. However, only 3% were sensitized, too few to test for associations with *Ascaris* and *Toxocara* IgG4 positivity.

Our study populations are thoroughly characterized, but the relatively low number of study participants has limited our ability to detect associations. This might be 1 reason that indicated protective associations between helminth exposure and allergic outcomes in the parent population did not reach statistical significance, despite the fact that this population had higher prevalence of *Ascaris* and *Toxocara* seropositivity. We reported association with *Ascaris* spp. seropositivity and growing up on farms with livestock. Given the nature of the exposure and the known association between *Ascaris* spp. and farm animals, especially pigs, it would have been valuable to know which type of livestock the participants had been in contact with. Unfortunately, this information was not captured in the interview or questionnaires. The study design limits our possibility to infer any causal relationship between exposure to *Ascaris* spp. and *Toxocara* spp. However, the associations reported in the present study raise some intriguing possibilities of zoonotic helminth exposures as a potential modifier for allergic disease development which warrants more research.

In conclusion, this study shows that in an affluent northern European population, helminth exposure is still relatively common and that *Toxocara* seropositivity appeared to be a strong risk factor for allergic outcomes in the younger generation. *Toxocara* was associated with childhood cat keeping, but this did not explain the associations between *Toxocara* and allergies. The risk associations differed between the parent and offspring generations, and parental *Toxocara* seropositivity was associated with allergic outcomes in their offspring but not in themselves. These intriguing findings might shed light on the increase in allergies during the last decades. Our findings suggest that zoonotic helminth exposure may modify the risk of allergic disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTION

NOJ, CS, WGCH and RJB contributed to conception and design of the study and drafted the manuscript. NOJ, CS, AN, SPS, FGR, WGCH and RJB were involved in acquisition of data. NOJ, CS, JI, WGCH and RJB were involved in statistical analysis. All authors contributed to interpretation of data for the work, revised the manuscript critically for important intellectual content and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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II

Ascaris exposure and its association with lung function, asthma, and DNA methylation in Northern Europe

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Background: *Ascaris* infections, with a worldwide prevalence above 10%, can cause respiratory pathology. However, long-term effects on lung function in humans are largely unknown.
Objective: We investigated the associations of *Ascaris* exposure with lung function, asthma, and DNA methylation.
Methods: Serum *Ascaris* IgG antibodies were measured in 671 adults aged 18 to 47 years (46% women) from Aarhus, Bergen, and Tartu RHINESSA study centers. Seropositivity was defined as IgG above the 90th percentile. Linear and logistic regressions were used to analyze *Ascaris* seropositivity as associated with lung function and asthma, adjusted for age, height, and smoking and clustered by center. DNA methylation in blood was profiled by a commercial methylation assay.
Results: *Ascaris* seropositivity was associated with lower FEV_1 (−247 mL; 95% CI, −460, −34) and higher odds for asthma (adjusted odds ratio, 5.84; 95% CI, 1.67, 20.37) among men but not women, also after further adjusting for house dust mite sensitivity, consistent across study centers. At a genome-wide level, *Ascaris* exposure was associated with 23 differentially methylated sites in men and 3 in women. We identified hypermethylation of the *MYBPC1* gene, which can regulate airway muscle contraction. We also identified genes linked to asthma pathogenesis such as *CRHR1* and *GRK1,* as well as a differentially methylated region in the *PRSS22* gene linked to nematode infection.
Conclusion: *Ascaris* exposure was associated with substantially lower lung function and increased asthma risk among men. Seropositive participants had sex-specific differences in DNA methylation compared to the unexposed, thus suggesting that exposure may lead to sex-specific epigenetic changes associated with lung pathology. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: Ascaris, *helminth, lung function, asthma, EWAS, DNA methylation, RHINESSA*

Ascaris lumbricoides infection is estimated to affect at least 800 million people worldwide.[1] Seropositivity to *Ascaris* is high in low-income countries, but it is believed to have decreased over time in affluent countries as a result of improved sanitary conditions.[2] Reports of *Ascaris* seroprevalence and infection rates are scarce in Europe. However, studies suggest unexpectedly high seroprevalence, with 42%[3] reported in the Netherlands, 29% reported in our analysis of a Norwegian cohort (parents of the current study population),[4] and 13% reported in a general population in Estonia.[5] There are no standardized assays for assessing *Ascaris* exposure; mostly in-house end-point titration ELISAs are used. Stool sampling is the reference standard for determination of current *Ascaris* infections but is unsuitable for assessing previous exposure to *Ascaris*. Evidence for infective *Ascaris* life cycles in Europe have been presented in a study of sewage treatment plants, where 95% of sewage sludge contained viable *Ascaris* eggs.[6] Thus, the potential risk for acquiring helminth infections in affluent countries could be higher than previously recognized.

Respiratory pathology caused by *Ascaris* infections is well known. The natural life cycle of *Ascaris* includes migration

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Abbreviations used
- aOR: Adjusted odds ratio
- CpG: 59-C-phosphate-G-39
- dmCpG: Differentially methylated CpG
- DMR: Differentially methylated region
- EWAS: Epigenome-wide association study
- HDM: House dust mite
- NTU: NovaTec units
- RHINESSA: Respiratory Health in Northern Europe, Spain, and Australia
- SPT: Skin prick test

through the lungs,[7] which has been demonstrated to cause eosinophilic pneumonitis and related alterations on computed tomography.[8-11] *Ascaris* infection is also associated with increased risk of asthma symptoms,[12,13] especially among children in endemic regions.[12,14,15] Importantly, detection of anti–*A lumbricoides* IgE antibodies, rather than current *A lumbricoides* infection, is associated with wheezing in atopic children.[14] Elevated IgE levels toward *Ascaris* have been associated with current infection,[13] and higher levels are in particular observed in children with chronic infections.[16] One study suggests that increased levels of *Ascaris*-specific IgE reflects protection from infection rather than exposure.[17] Measuring *Ascaris*-specific IgE as marker for ever exposure can be therefore questionable. On the other hand, *Ascaris*-specific IgG may overestimate the prevalence of infection as a result of the persistence of antibodies long after patients undergo deworming therapy.[18] Thus, IgG is assumed to be more suitable for assessing previous exposure.

The potential for long-term effects on lung function have been highlighted in murine studies. Recurrent infection with *Ascaris suum* increases lung cytokine responses, promoting severe impairment of respiratory function and a polarized systemic T_H2/T_H17 immune response.[19] Furthermore, infection with the murine parasitic nematode *Nippostrongylus brasiliensis,* which, like *Ascaris* in humans, transits the lungs of mice, has demonstrated long-term effects on lung cellular and physiologic characteristics.[20] The infection has also been associated with development of fibrosis and emphysema-like changes.[21] Infection with gut-restricted helminths can also result in immunologic and structural changes in the lung.[22] Sex differences in parasite infections are described in animal models,[23] with a different response to infection between male and female mice.[24] In humans, infection rates have been reported to be higher among women,[25,26] and one of our previous studies revealed sex-specific patterns in the associations of parental helminth exposure with the allergic outcomes of their offspring.[4] This body of work suggests that parasite infection could be an important predictor of long-term respiratory health in humans and that there might be substantial sex differences.

Further, viral, bacterial, and parasitic infections in humans have been shown to cause long-lasting changes in DNA methylation,[27-30] some of which have been linked to altered host immunity.[31] In addition to the potential direct damage and subsequent changes in the airways, epigenetic changes due to infection might contribute to the pathogenesis of allergic diseases related to helminth infections.

The role of exposure to *Ascaris* in determining lung function in humans has not yet been addressed, but *Ascaris* infection has previously been associated with asthma severity.[13] The aim of our study was to explore the association between *Ascaris* exposure and lung function, the potential sex differences in such associations, and whether *Ascaris* exposure was associated with differentially methylated DNA.

METHODS

Study population

The study population included 671 adults from Aarhus (Denmark) (n 5 53), Bergen (Norway) (n 5 474), and Tartu (Estonia) (n 5 144), investigated in 2014-15 as part of the RHINESSA study (Respiratory Health In Northern Europe, Spain and Australia study; www.rhinessa.net). The RHINESSA clinical study included all adult offspring of the participants of the population-based European Community Respiratory Health Survey study in 10 study centers. Helminth serology was performed in Danish, Estonian, and Norwegian study centers. The present analysis included RHINESSA study participants from these study centers with available lung function and serum samples (86% of the total population). Protocols for questionnaires and clinical examination were standardized across study centers.

Approval was obtained from the local ethics committees for each center. On the basis of cross-sectional data, the main objective of the study was to investigate the association of *Ascaris* exposure with lung function and genome-wide DNA methylation profiles in blood.

Lung function, asthma symptoms, and sensitization

Lung function was measured using a standard spirometry method with a NDD EasyOne spirometer following American Thoracic Society/European Respiratory Society criteria.[32] Interview data were used to define ever having asthma, wheeze, and >_3 asthma symptoms (wheeze, nocturnal chest tightness, nocturnal breathlessness, nocturnal cough, any asthma attack, or use of asthma medication) during the last 12 months before the study. Further details are provided in the Online Repository available at www.jacionline.org, and questionnaire forms are available at www.rhinessa.net. In order to account for lung function in a wide age range, we calculated the percentage of predicted values for FVC and FEV_1 using the Global Lung Initiative reference values[33] using the online calculator. Bronchodilator reversibility was defined according to 2019 Global Initiative for Asthma guidelines: increase in FEV_1 of >12% and >200 mL from baseline 15 minutes after inhalation of 200 mg salbutamol. Allergic sensitization was determined by skin prick test (SPT) to 12 allergens (ALK-Abell' o, Hørsholm, Denmark) including *Dermatophagoides pteronyssinus and Dermatophagoides farinae;* 0.9% saline and 10 mg/mL histamine solution were used for negative and positive controls. The house dust mite (HDM) allergens used were Der p 1 (9.8 mg/100,000 Standardized Quality Units [SQ-U]) 19.6 mg/mL in 10 HEP Soluprick) and Der p 2 (0.7 mg/100,000 SQ-U 1.4 mg/mL in Soluprick). Reactions to the allergens were read after 15 minutes. Reactivity was considered positive if the mean wheal size was 3 mm greater than the negative control. The mean diameter of positive histamine wheals was 4.3 mm. ''Any positive HDM SPT'' was defined as positive SPT reactivity toward *D pteronyssinus* and/or *D farinae*. SPTs were carried out by trained nurses following a standardized protocol, similar in all study centers.

Ascaris IgG antibodies

Ascaris IgG antibodies levels were measured by ImmunoCAP (Thermo Fisher Scientific, Waltham, Mass). Additionally, in a subsample of the Bergen cohort, *Ascaris* IgG_4 antibodies were measured using an indirect end-point titration (in 4 dilutions) ELISA (n 5 265) with *Ascaris lumbricoides* crude worm antigen preincubated with *Toxocara* somatic antigen to reduce cross-reactivity.[4] On the basis of previous findings from Europe as well as from our previous study,[4] we assume IgG seropositivity toward *Ascaris* to be around

10%, and therefore seropositivity was defined as values above the 90th percentile (cutoff, 4.53 mg/L).

Epigenome-wide association studies

Methylation data were profiled from peripheral blood using HumanMethylationEPIC (Illumina, San Diego, Calif). Methylation input data (IDAT files) (n 5 551) were processed using the R/Bioconductor package minfi.[34] The batch effect was adjusted using COMBAT from SVA.[35] Normalization was carried out using BMIQ.[36] Probes with a detection *P* value above .01 in 1 or more samples, probes with a bead count of <3 in at least 5% of samples, non-CG probes, cross-reactive probes, and probes on the X or Y chromosome were excluded from the analysis, resulting in a total of 747,053 probes used for downstream analysis. To identify differentially methylated 59-C-phosphate-G-39 (CpG), a robust multiple regression model was applied using limma[37] on methylation beta value stratified by sex, adjusting for age, personal smoking status, and estimated cell type proportions (B, CD4T, CD8T, natural killer, monocytes, and granulocytes).[38] Multiple test correction was applied using the Benjamini-Hochberg false discovery rate at a significance level of .05. Manhattan plots were generated using the R package qqman.[39] Differential methylated regions were predicted using DMRcate.[40] Functional enrichment was identified using Enrichr in R.[41] Transcription factor enrichment was carried using eFORGE.[42]

Potential cross-reactivity with other helminths

In order to account for potential cross-reactivity with helminths, 446 Bergen center samples were tested using NovaLisa IgG ELISA (NovaTec Immunodiagnostica, Dietzenbach, Germany) for the presence of IgG antibodies against *Ascaris lumbricoides, Echinococcus* spp, *Taenia solium, Toxocara canis,* and *Trichinella spiralis* according to the manufacturer's instructions. A result of >11 NovaTec units (NTU) was considered positive. A result of 9 to 11 NTU was considered a gray zone and <9 NTU negative.

Sensitivity analyses

For sensitivity analyses, we applied a separate 90th percentile cutoff for men and women that was additionally adjusted for pet ownership, occupation, body mass index, and education. To account for differences in lung function in different age groups, a percentage of predicted spirometry values was used. In a subsample of *Ascaris* IgG measurements obtained via 3 different methods, we selected the samples that were negative according to both NovaTec ELISA and in-house ELISA, then used the mean concentration of these negative samples 63.3 SD[43] as an alternative cutoff for the ImmunoCAP results. In a subsample of 98 participants with the highest IgG values, we also measured *Ascaris*-specific IgE serum concentrations (ImmunoCAP) and its relation to lung function and asthma.

Statistical analyses

Descriptive statistics for the study population are reported as means and SDs for normally distributed data and otherwise as median and interquartile range.

Logistic regression was used to model the association between *Ascaris* seropositivity and asthma status, wheeze, and >_3 asthma symptoms (dichotomous) 12 months before the study. FVC and FEV_1 were included as continuous outcomes. Linear regression was used to model the association between anti-*Ascaris* IgG concentration and FVC and FEV_1 values.

All models were adjusted for age, sex, height (measured before spirometry), and smoking (interview data) and clustered within study center. For sensitivity analyses, we applied a separate 90th percentile cutoff for men and women, adjusted for pet ownership, occupation, body mass index, and education. To account for differences in lung function in different age groups, the percentage of predicted spirometry values were used. Stata 16.0 (StataCorp, College Station, Tex) was used for regression analyses.

RESULTS

Population characteristics

The study participants were aged 18 to 47 years (median, 28 years) (Table I). A total of 14.9% were current smokers (Bergen 12%, Aarhus and Tartu 22%). Ever asthma was higher among Aarhus participants (21%) compared to Bergen and Tartu (15% and 7%, respectively), while 3 or more asthma symptoms during the last 12 months showed a similar prevalence in all study centers (6%). Sensitization to *D pteronyssinus* and/or *D farinae* by SPTs was lower in Tartu (11%) compared to Aarhus and Bergen (21% and 24%, respectively).

Ascaris seropositivity was present in 6.9% of participants from Tartu and in 11% of participants from Aarhus and Bergen (Table I). Women were more often seropositive than men (15.5% vs 5.5%, respectively). We compared the characteristics of *Ascaris*-seropositive and -seronegative participants and found that women were more often seropositive (Table II).

In a Norwegian subpopulation (n 5 446), 5 helminths were measured using a NovaLisa ELISA with *Ascaris* seroprevalence of 5.6% (additionally 9.1% including gray zone) *Echinococcus* 0.7% (13.6% including gray zone), *Taenia* 2.5% (14.7% including gray zone), *Toxocara* 0.7% (11.6% including gray zone), and *Trichinella* 1.3% (10.9% including gray zone) (see Fig E1, *A,* in the Online Repository available at www.jacionline.org). When stratified by sex, women accounted for most of the seropositive samples (Fig E1, *B*).

Ascaris IgG antibody levels and HDM SPT sensitivity

There was a statistically significant difference in *Ascaris* IgG antibody level between HDM SPT-positive and -negative study participants (Wilcoxon Mann-Whitney *U* test, *P* 5.04) with a median difference of 0.33 mg/L higher IgG antibody levels among the HDM SPT positive results (see Fig E2 in the Online Repository available at www.jacionline.org). Overall, 129 participants (19%) were sensitized to any HDM, and 68 (10%) were, according to our definition, *Ascaris* IgG positive, with 14 (2%) positive toward both (see Fig E3 in the Online Repository).

Ascaris IgG antibody serum level, lung function, and asthma symptoms

Among men, *Ascaris* IgG seropositivity (according to ImmunoCAP results) was associated with lower FEV_1 (2247 mL; 95% CI, 2460, 234) (Table III) with an effect size larger than that of current smoking (2151 mL; 95% CI, 2501, 199). There was a clear dose–response pattern, with subsequently decreasing FEV_1 with increasing *Ascaris* IgG antibody levels (see Fig E4 in the Online Repository available at www.jacionline.org). In a linear regression model, a 1 mg/L rise in *Ascaris* IgG concentration was associated with 40 mL lower FEV_1 among men (95% CI, 260, 221). Seropositive men had 5% lower predicted FEV_1 and 4% lower FEV_1/FVC ratio compared to seronegative men; this pattern was not seen for women (see Fig E5, *A* and *B*, in the Online Repository). These associations remained after adjusting for allergic sensitization to HDM (Table III) and when using an in-house ELISA method for detection anti-*Ascaris* IgG_4 antibodies (see Fig E6 in the Online Repository). No association was seen for FVC among men. For women, no clear pattern was seen for FEV_1 or FVC (Fig E4), apart from a slightly lower FVC for

TABLE I. Characteristics of study populations

Characteristic	Total (n 5 671)	Aarhus (n 5 53)	Bergen (n 5 474)	Tartu (n 5 144)
Female (%)	46.2	50.9	46	45.1
Age (years), mean (range; SD)	28 (18-47; 6.6)	28 (19-47; 7.7)	28 (18-47; 10)	29 (18-42; 5.6)
Height (cm), median (range; IQR)	175 (146-200; 14)	176 (159-191; 15)	175 (146-200; 13)	177 (149-197; 16)
FVC (mL), median (range; IQR)	4.77 (1.52-8.28; 1.61)	4.64 (3.13-6.61; 1.07)	4.75 (1.52-8.28; 1.59)	4.86 (2.84-7.62; 1.72)
FEV_1 (mL), median (range; IQR)	3.86 (1.18-6.79; 1.2)	3.79 (2.36-5.25; 0.99)	3.82 (1.18-6.79; 1.23)	3.94 (2.25-5.86; 1.22)
FEV_1/FVC ratio, median (range; IQR)	0.82 (0.49-1.26; 0.08)	0.81 (0.68-0.93; 0.07)	0.82 (0.58-0.98; 0.07)	0.83 (0.49-1.26; 0.08)
Smoking				
Never smokers (%)	63.4	66.7	63.7	61
Ex-smokers (at least 1 year) (%)	21.7	11.1	24.1	17.1
Current smoking (%)	14.9	22.2	12.2	22
Asthma, ever (%)	13.9	20.5	15.3	6.5
Wheeze, ever (%)	13.1	20	12.8	11.8
Asthma symptoms, last 12 months (%)	6.2	6.7	6.1	6.4
D pteronyssinus–specific IgE positive (%)	24.3	35.9	25.1	17.5
Any HDM SPT positivity (%)*	20.9	20.8	23.8	11.1
Atopic (any SPT or sIgE positive) (%)	45.1	44.2	49.3	31.5
Anti-*Ascaris* IgG (mg/L) median (range; IQR)	2.21 (0.03-23.3; 1.62)	2.31 (0.73-6.6; 1.78)	2.18 (0.03-23.3; 1.73)	2.24 (0.69-6.66; 1.32)
Ascaris seropositive at 90th percentile (cutoff, 4.53 mg/L) (%)	10	11.3	11	6.9
Ascaris seropositive (cutoff at mean 6 3.3 SD of negative samples at 6.1 mg/L) (%)	3.9	3.8	4.4	2.1
Anti-*Ascaris* IgG levels (%)				
<2 mg/L	42.2	35.9	43.9	39.2
2-4 mg/L	43.4	47.2	41.1	49.0
4-6 mg/L	10.3	13.2	10.3	9.2
6-8 mg/L	2.1	3.8	1.7	2.6
>8 mg/L	2.1	0	3.0	0

*SPT positive to *Dermatophagoides pteronyssinus* and/or *Dermatophagoides farinae*.

TABLE II. Characteristics of study population stratified by *Ascaris* seropositivity

Variable	*Ascaris* seropositive (≥90th percentile, 4.53 mg/L)	
	Seronegative	Seropositive
Sex*		
Female (%)	84.5	15.5
Male (%)	94.5	5.5
Age (years), mean (range; SD)	28 (18-47; 6.6)	29 (18-43; 6.9)
Height (cm), median (range; IQR)	176 (146-200; 20)	170 (152-195; 12)
Smoking (%)		
Never smokers	64.2	56.1
Ex-smokers (at least 1 year)	21.3	25.8
Current smoking	14.5	18.2
Asthma, ever (%)	13.4	18.2
Wheeze, ever (%)	12.7	16.7
Asthma symptoms, last 12 months (%)	6.2	6.1
Mother's education (%)		
Primary	9.5	18.2
Secondary	38.4	34.9
University or college	52.1	47
Father's education (%)		
Primary	8.6	10.8
Secondary	38.6	41.5
University or college	52.8	47.7

*Difference between groups $P < .05$.

Ascaris IgG antibody level of 6-8 mg/L compared to *Ascaris* IgG level <2 mg/L.

Among men, *Ascaris* seropositivity was associated with increased odds of ever having asthma (adjusted odds ratio [aOR], 5.84; 95% CI, 1.67, 20.37), ever wheezing (aOR, 3.78; 95% CI, 1.85, 7.74), and having ≥3 asthma symptoms during the last 12 months (aOR, 3.59; 95% CI, 2.01, 6.47) compared to seronegative men (Table IV). Among women, *Ascaris* seropositivity was associated with decreased odds of ever having asthma (aOR, 0.42; 95% CI, 0.18, 0.96), ever wheezing (aOR, 0.63; 95% CI, 0.16, 2.44), and ≥3 asthma symptoms during the last 12 months before the study (aOR, 0.24; 95% CI, 0.15, 0.40). These associations for men and women did not change when adjusting for HDM sensitivity (Table IV) and were consistent for men across study centers (Fig 1) and with different methods for detection of antibodies against *Ascaris* among the Bergen cohort (Fig E5; Fig E6). For women, the number of *Ascaris*-seropositive participants was too low in Aarhus (n 5 2) for us to perform a meta-analysis by study center. There was no statistically significant difference in FEV_1 reversibility between the seropositive and seronegative participants (3% vs 3.5%, respectively).

Sensitivity analyses

The results did not change when we used the 90th percentile cutoff separately for men and women. When adjusting for pet ownership, occupation, body mass index, smoking, and parental or own education in the models, the effect size remained significant. Analyses of postbronchodilator lung function measures (n 5 352) gave comparable results as for prebronchodilator measures (association with postbronchodilator FEV_1 191 [95% CI,2434, 43] mL). For men, *Ascaris* seropositivity was associated with 6% lower predicted FEV_1 (95% CI, 11.8, 20.2) (Table III). The associations with percentages predicting FVC in men or with percentages predicting FEV_1 or FVC in

TABLE III. Associations of *Ascaris* seropositivity with lung function

Characteristic	Male sex (n 5 361)		Female sex (n 5 310)	
	AD (95% CI)	AD-HDM (95% CI)	AD (95% CI)	AD-HDM (95% CI)
FVC (mL)	297 (2535, 340)	287 (2501, 324)	242 (2174, 89)	242 (2179, 95)
FEV_1 (mL)	2247 (2460, 234)	2232 (2408, 256)	229 (280, 21)	230 (276, 16)
FEV_1/FVC ratio	23.2% (29.3, 2.9)	23% (29.3, 3.3)	10.1% (22.6, 3.5)	10.5% (22.4, 3.3)
FVC % predicted*	21.9% (213.2, 9.4)	21.7% (211.2, 8.6)	20.3% (22.9, 2.3)	20.3% (23.1, 2.4)
FEV_1 % predicted*	26% (211.8, 20.2)	25.7% (210.5, 20.8)	21.7% (22.9, 0.4)	20.5% (22.1, 1.1)

Shown are the adjusted differences (AD) as well as the AD with adjustment for HDM sensitivity (AD-HDM). AD are adjusted for height and age, clustered by study center; in addition to these, AD-HDM are adjusted for any HDM SPT positivity.
*Not adjusted for age or height.

TABLE IV. Adjusted odds ratio (95% CI), stratified by sex, for *Ascaris* seropositivity as associated with respiratory symptoms in a logistic regression model

Characteristic	Male sex (n 5 361)		Female sex (n 5 310)	
	AD	AD-HDM	AD	AD-HDM
Asthma, ever	5.84 (1.67, 20.37)	5.44 (1.71, 17.34)	0.42 (0.18, 0.96)	0.38 (0.23, 0.65)
Wheeze, ever	3.78 (1.85, 7.74)	3.4 (1.99, 6.81)	0.63 (0.16, 2.44)	0.61 (0.18, 2.07)
Combined asthma symptoms*	3.59 (2.01, 6.47)	3.8 (1.96, 7.34)	0.24 (0.15, 0.40)	0.24 (0.12, 0.47)

*More than 2 asthma symptoms (wheeze, tightness of breath, nocturnal breathlessness, nocturnal cough, asthma attack, use of asthma medication) during the last 12 months.

women were not significant. Serum concentrations of *Ascaris*-specific IgG and IgE were not correlated. Elevated *Ascaris*-specific IgE levels were not associated with increased odds of having asthma, asthma symptoms, or lower spirometry measurements (among the 98 participants with IgE measurements). Seven subjects had a serum IgE concentration of >0.35 kU/L. Only 2 participants had an IgE response in radioallergosorbent test (RAST) class 3 or higher, and these participants also had a highly elevated IgG response (7.5 and 9.3 mg/L), which could be indicative of current or chronic infection.

DNA methylation as related to *Ascaris* exposure

We identified 5 differentially methylated CpGs (dmCpGs) associated with *Ascaris* exposure that achieved genome-wide significance (at adjusted *P* value of .05 and inflation of 1.17) when analyzing men and women together. Two of these dmCpGs were mapped to known genes: *MYBPC2* (myosin-binding protein C) and *NAV3* (neuron navigator 3). Three dmCpGs were mapped to intergenic regions. CpG cg20041612 showed association at adjusted *P* 5 .056 (borderline) mapped to *EGFR* (epidermal growth factor receptor). The dmCpG cg04671734 (adjusted *P* 5 .046) was significantly enriched in proximity to the binding site of transcription factor DEAF1 (deformed epidermal autoregulatory factor 1 homolog) at a *q* value of 0.003.

By analysis of sex-stratified epigenome-wide association study (EWAS) results, 23 dmCpGs were identified as genome-wide significantly associated with *Ascaris* exposure in men that were mapped to 19 known genes (see Table E1 in this article's Online Repository at www.jacionline.org; Fig 2, *A*). Three dmCpGs (close to *RADIL, NAV3,* and *ACSL5*) were significantly associated in women (false discovery rate, <0.05) (Fig 2, *B*). *RADIL* and *NAV3* were hypomethylated while *ACSL5* was hypermethylated in the *Ascaris*-exposed group. The effect size and direction of association between exposed and unexposed groups for the top dmCpGs are shown in Fig 3. The regression coefficient of *NAV3* (20.06) shows the strongest association in female subjects, while *CRHR1* (0.097) and cg04671734 (20.079) show the strongest association in male subjects. Sex differences in relation to *Ascaris* exposure are illustrated in Fig 4.

Ascaris exposure and its association with differentially methylated regions

Five differentially methylated regions (DMRs) significantly associated with *Ascaris* seropositivity were identified in male subjects and 4 DMRs in female subjects. One DMR was identified in subjects of both sexes in the locus of *PRSS22* (brain-specific serine protease 4) (17 CpGs within 2016 bp) at Stouffer 5 0.01; this was hypomethylated in the *Ascaris*-exposed group.

EWAS Atlas enrichment showing genes linked to lung function and asthma pathogenesis

To gain pathophysiologic insight into these CpGs, the significant genes were compared against the EWAS Atlas repository (https://ngdc.cncb.ac.cn/ewas/index). The methylation status of the genes *GOT1, TPD52L2, RAPGEF4, GRK1, DLEU7, CRHR1, DCAF17, SUMF1, PROCR, CLEC16A,* and *MYBPC1* was found to be linked to asthma pathogenesis[44] (see Table E2 in this article's Online Repository at www.jacionline.org).

Functional enrichment

To gain further biological insight, the significant genes (19 from men and 3 from women) were used for functional enrichment with Enrichr. In men, the top enriched biological processes include fatty acid homeostasis (GO:0055089) (*GOT1, GPAM*), amide transport (GO:0042886) (*SLC38A7, CRHR1, RAPGEF4*), peptide hormone secretion (GO:0030072) (*CRHR1, RAPGEF4*), and lipid homeostasis (GO:0055088; *GOT1, GPAM*). In women, the top enriched biological pathway was triglyceride biosynthesis (GO:0019432; *ACSL5, AGPAT3*) (Table E2).

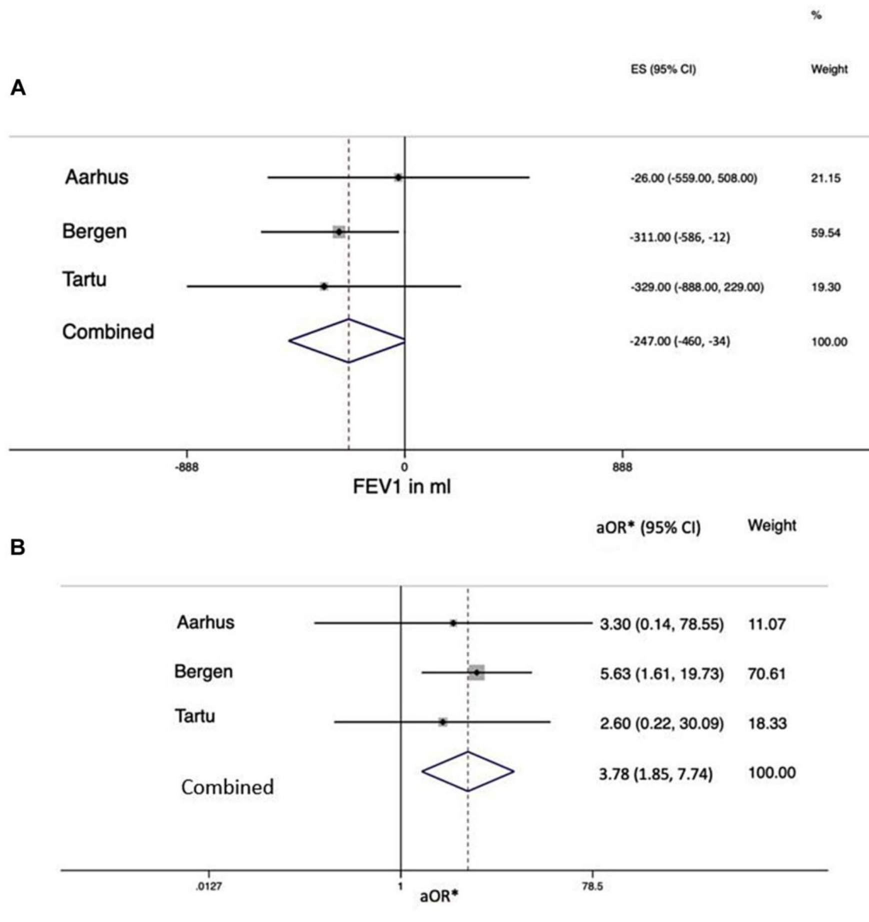

FIG 1. Meta-analysis of study centers specific associations among men of *Ascaris* seropositivity (>_90th percentile) with (A) FEV_1 (adjusted difference in mL) and (B) wheeze (aOR).

DISCUSSION

Seropositivity to *Ascaris lumbricoides* consistently showed an association with substantially lower lung function in young adult men from a population-based study in Northern Europe. Men who were seropositive to *Ascaris* had lower FEV_1, unaltered FVC, and more asthma symptoms. A dose–response pattern was found: FEV_1 decreased with increased concentration of anti-*Ascaris* IgG. In women, no association was found with lung function, but asthma symptoms were significantly less common in seropositive women. Genome-wide analyses uncovered DNA methylation characteristics associated with *Ascaris* seropositivity, including differentially methylated sites related to lung pathology, such as regulation of airway muscle contraction and asthma pathogenesis, and to immune regulation and specifically to nematode infection. The associations of *Ascaris* seropositivity with differentially methylated DNA sites were different in men and women, supporting a sex-specific role of *Ascaris* exposure. The findings from epidemiologic analyses, supported by DNA methylation analyses, suggest that the lower lung function and higher asthma risk observed in *Ascaris*-exposed individuals may be mediated by infection-driven epigenetic changes.

Identification of a hypomethylated CpG close to DEAF1 binding site and the differential methylation in *Ascaris*-exposed individuals of genes associated with helminth infection, namely *NAV3* and *EGFR,*[45] suggests that *Ascaris*-associated changes have the potential to alter the function of genes involved in type 2 immunity and therefore lung function. Hypermethylation of deoxyhypusine hydroxylase linked to immune regulation,[46] chronic lung disease progression,[47] being a putative antiparasitic drug target,[48] and respiratory muscle contraction modulating *MYBPC1* (myosin-binding protein C, slow-type)[49] also support epigenetic change influencing lung function.

To our knowledge, our study is the first to investigate associations between serum *Ascaris* IgG antibody levels and lung function in humans. Our findings with respect to increased asthma (among men) associated with *Ascaris* is in accordance

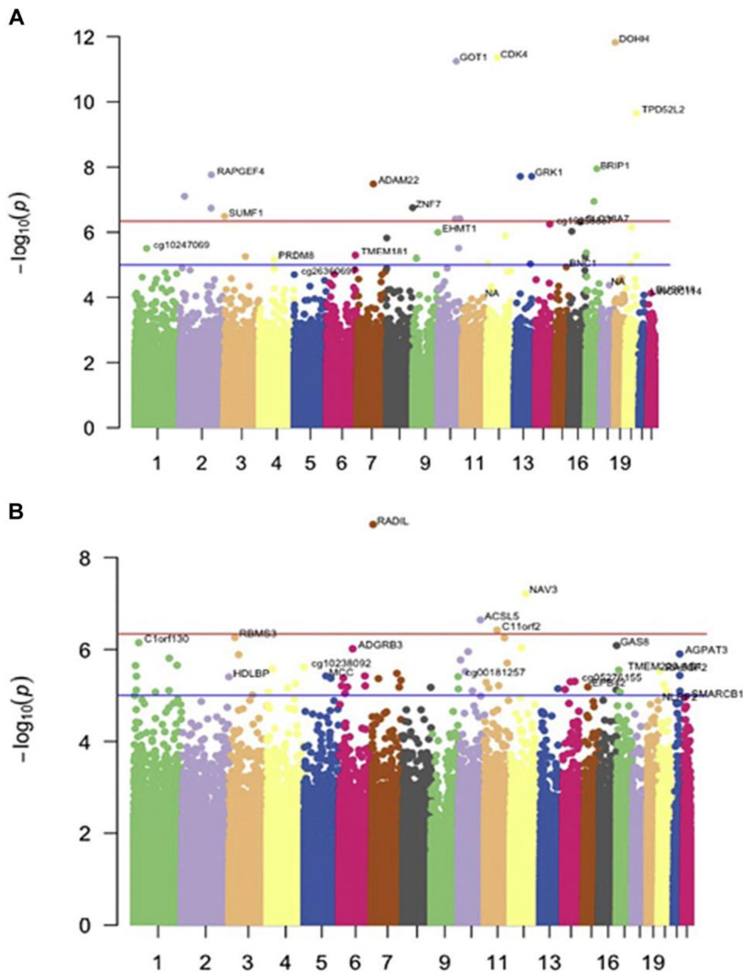

FIG 2. Manhattan plot for *Ascaris* seropositivity EWAS showing autosomal chromosomes in male (A) and female (B) subjects. The *vertical axis* (log_{10} transformed) indicates observed *P* values; *horizontal axis,* chromosomal positions with the points indicating individual CpGs. The *red line* indicates the multiple testing correction threshold (false discovery rate <0.05); *blue,* the suggestive line.

with other publications; however, most previous research has been based on pediatric cohorts.[50-54] The scale of lung damage evidenced by computed tomographic scan in *Ascaris*-infected persons strongly supports the likelihood that reduced long-term lung function would not be unexpected after infection. Our finding of impaired lung function in seropositive young men from Northern Europe therefore breaks new ground in presenting *Ascaris* infection as potentially important cause of long-term reduction in lung function.

The sex differences in the associations of *Ascaris* seropositivity with lung function and asthma are striking and have not been described before. The intensity of *A lumbricoides* can be influenced by sex-related behavioral and environmental factors that contribute to risk of exposure to infectious inoculum.[23] Higher infection rates have previously been reported in women,[25,26] which is in accordance with our results. Higher seroprevalence rates among women were seen for all measured helminth antibodies. Differences in sex hormone levels could hypothetically influence the pathogenic outcomes from *Ascaris* exposure. Estradiol, the main female sex hormone, is known to be important for many tissue repair processes, notably inflammation and regranulation,[55,56] and tissue damage is an essential element in the pathology caused by *Ascaris.* Moreover, murine models have shown that female mice can have a delayed T_H2 response to the murine nematode *Trichuris muris* compared to male mice.[57] On the one hand, such mechanisms could possibly lead to a higher worm burden in female subjects and therefore higher *Ascaris* IgG antibody levels. Sustained T_H2 responses in male subjects, on the other hand, could enhance pathology such as pulmonary inflammation. Interestingly, we discovered hypomethylation in dmCpGs in *NAV3* (a

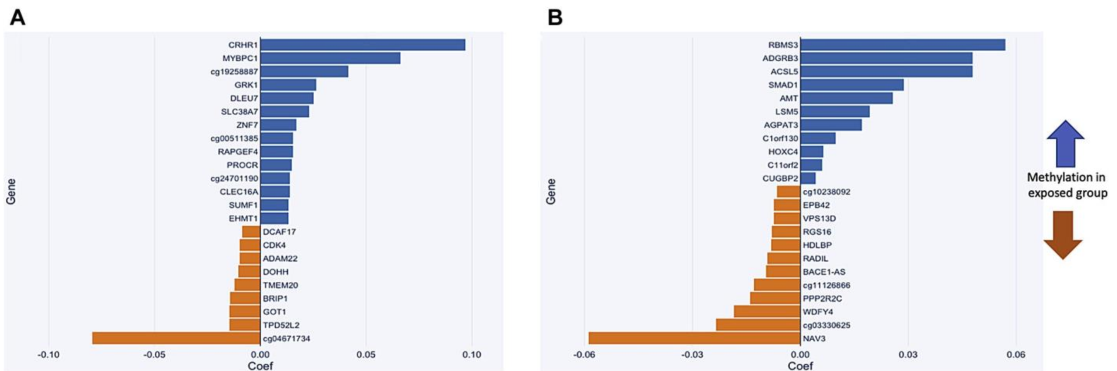

FIG 3. The top dmCpGs showing a different methylation pattern between exposed and unexposed groups in male (A) and female (B) subjects. *Positive regression coefficient shows that the mean methylation is higher in the exposed group, whereas negative regression coefficient shows lower mean methylation.

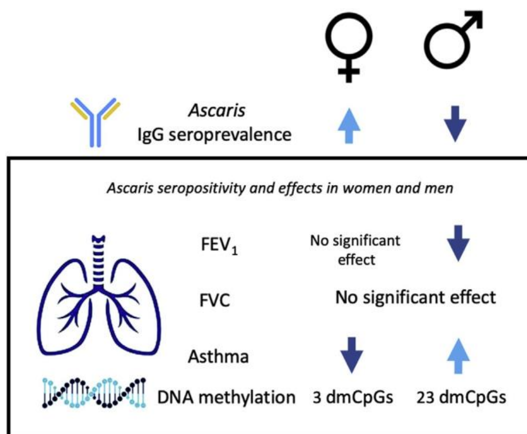

FIG 4. Sex differences in relation to *Ascaris* exposure.

gene involved in immunoregulatory processes through IL-2) in women, suggestive of sex differences in immunoregulation of helminth responses. The apparent *lower* likelihood of asthma risk in seropositive women is still surprising, and we speculate that altered immunoregulation may play a role.

Our findings suggest that men and women might respond differently to *Ascaris* infections and that these responses could have long-term outcomes. The EWAS analyses also showed that lower lung function in men may be explained by changes in the function of signaling pathways related to asthma pathogenesis and muscle contraction. The likelihood of a long-term effect is supported by findings from others that demonstrate long-term changes in DNA methylation after helminth infection.[31]

A strength of the present study is the extensive data on respiratory symptoms and spirometry data, as well as *Ascaris* serology, for population-based cohorts. Further, the inclusion of 3 study centers from different countries contributes to higher credibility and generalizability of the results. Interestingly, the Estonian study center had the lowest seroprevalence compared to Norway and Denmark.

Defining *Ascaris* exposure is a challenge because of potential cross-reactivity with other helminths and allergens. For Bergen samples, we used an additional method in which serum was preincubated on plates covered with *Toxocara* somatic antigen before transferring to *Ascaris* plates; this should reduce cross-reactivity. Moreover, using the same methodology, anti-*Ascaris* IgG_4 seropositive serum has been shown to react with live larvae.[58] Both strategies strengthen our findings and suggest that we are truly measuring antibodies toward *Ascaris.* Exposure to *Anisakis simplex* due to fish consumption could be a potential cause of cross-reactivity; however, a study of Norwegian blood donors showed a prevalence of almost 0 for IgE sensitization toward *Anisakis.*[59] HDM sensitization is commonly assumed to be a confounder as a result of possible cross-reactivity and the close structural homology between HDM and *Ascaris* proteins, such as tropomyosin, enolase, and enoyl-CoA hydratase.[60] However, adjusting our analyses for HDM sensitivity did not alter the results for either lung function or asthma. Thus, we believe that anti-*Ascaris* IgG as measured in our study reflects exposure to *Ascaris* rather than any other helminth or cross-reactivity with other allergens. For defining HDM sensitivity, we used a single SPT procedure, harmonized with the European Community Respiratory Health Survey protocol. We do note that a study by Thomsen et al[61] found that performing SPT twice with the same allergen batch did not enhance the validity of the test. Still, we do not know whether *Ascaris* IgG antibody seropositivity reflects past infection, current infection, or parasite exposure without disease manifestation. The reference standard for diagnosing ongoing infection is real-time quantitative PCR or microscopy of eggs in stool. Past infection, however, cannot be assessed that way.

EWAS analyses found that *Ascaris* seropositivity was associated with DMR *PRSS22*. This region has been associated with nematode infection, and this finding strengthens the interpretation that the participants who are *Ascaris* seropositive have actually been exposed to the helminth. A limitation of the epigenetic analysis is that it was carried out on DNA extracted from whole blood while other relevant tissues were not available. Further, although the epigenetic analysis was adjusted for blood cell type composition, it is possible that differences in cell subtype composition

between *Ascaris*-exposed and -unexposed individuals may partly account for the observed associations. Finally, our study cannot address whether the identified DNA methylation changes lie on the causal pathway between *Ascaris* exposure and lung function.

In conclusion, our findings show that detection of higher serum IgG antibody levels against *Ascaris* is associated with substantially lower lung function and more asthma among Northern European men. The effect magnitude for lung function was larger than that of current smoking. In women, no association was found with lung function, but asthma symptoms were significantly less common in seropositive women. A wide range of sex-specific DNA methylation markers associated with *Ascaris* exposure were identified in genes linked to asthma pathogenesis, lung function, and immunoregulation. These findings support the notion that DNA methylation changes due to helminth exposure may contribute to the pathogenesis underlying reduced lung health. Further, our findings identify a need to investigate the role of helminths on long-term lung health globally, including in high- and middle-income countries, as well as in low-income countries where helminth exposure is highly prevalent.

We thank all the study participants, clinical field workers, and laboratory personnel involved.

Clinical implications: *Ascaris* exposure may be an important public health challenge in terms of respiratory function. Our results highlight a need for further research on long-term effects of helminth exposure on host lung health.

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III

Appendix A

ECRHS III MAIN QUESTIONNAIRE

Centre number
Personal number
Sample
Date

You were last seen as part of this survey in _______ (month) ___________ (year)

I AM GOING TO ASK YOU SOME QUESTIONS. AT FIRST THESE WILL BE MOSTLY ABOUT YOUR BREATHING. WHEREVER POSSIBLE, I WOULD LIKE YOU TO ANSWER 'YES' OR 'NO'.

1. Have you had wheezing or whistling in your chest at any time in the last ***12 months***? — NO YES

   IF 'NO' GO TO QUESTION 2, IF 'YES':

   1.1 Have you been at all breathless when the wheezing noise was present? — NO YES

   1.2. Have you had this wheezing or whistling when you did ***not*** have a cold? — NO YES

   1.3 How old were you when you first had wheezing or whistling in your chest? — YEARS
   (If started 'as a baby' enter '01')

   1.4 How frequently have you had wheezing or whistling in the last 12 months? — TICK ONE BOX ONLY
   - everyday — 1
   - at least once a week, but not everyday — 2
   - occasionally — 3

2. Have you woken up with a feeling of tightness in your chest at any time in the last ***12 months***? — NO YES

3. Have you had an attack of shortness of breath that came on during the day when you were at rest at any time in the last ***12 months***? — NO YES

   IF 'NO' GO TO QUESTION 4, IF 'YES':

   3.1 How old were you when you first had an attack of shortness of breath that came on during the day when you were at rest? — YE/

4. Have you had an attack of shortness of breath that came on ***following*** strenuous activity at any time in the last ***12 months***? — NO

5. Have you been woken by an attack of shortness of breath at any time in the last ***12 months?*** — NO

6. Have you been woken by an attack of coughing at any time *in the last* ***12 months***? — NO

7. How often have you experienced bouts or spasms of coughing in the last 12 months? — TICK ONE BOX ONLY
   - less than once a month — 1
   - every month, but less than every week — 2
   - every week, but not every day — 3
   - every day — 4

NO YE

ECRHS III MAIN QUESTIONNAIRE

8. Do you ***usually*** cough first thing in the morning in the winter? ☐ ☐
[IF DOUBTFUL, USE QUESTION 9.1 TO CONFIRM]

9. Do you ***usually*** cough during the day, or at night, in the winter? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 10, IF 'YES':

9.1 Do you cough like this on most days for as much as three months each year? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 10, IF 'YES':

9.2 How many years have you had this problem (coughing on most days for as much as three months each year? YEARS ☐☐

10. Do you ***usually*** bring up any phlegm from your chest first thing in the morning in the winter? NO ☐ YES ☐
[IF DOUBTFUL, USE QUESTION 11.1 TO CONFIRM]

11. Do you ***usually*** bring up any phlegm from your chest during the day, or at night, in the winter? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 12, IF 'YES':

11.1 Do you bring up phlegm like this on most days for as much as three months each year? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 12, IF 'YES':

11.2 How many years have you had this problem (of bringing up phlegm from your chest on most days for as much as three months each year)? YEARS ☐☐

IF 'NO' TO QUESTIONS <u>3-11</u> GO DIRECT TO QUESTION 13;
IF 'YES' TO ANY OF QUESTIONS <u>3-11</u> PLEASE COMPLETE QUESTION 12

12. In the last ***12 months***, have you had any episodes/times when your symptoms (cough, phlegm, shortness of breath) were a lot worse than usual? NO ☐ YES ☐

IF 'NO' TO QUESTION 12 GO TO QUESTION 13; IF 'YES'

In the last ***12 months***:

12.1 How many times have these episodes occurred? TIMES ☐☐

12.2 How many times have these episodes forced you to consult your doctor? TIMES ☐☐

12.3 How many times was your therapy changed after these episodes? TIMES ☐☐

12.4 How many times have you visited a hospital casualty department or emergency room or have you spent a night in hospital after these episodes? TIMES ☐☐

13. Do you ever have trouble with your breathing? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 14, IF 'YES':

13.1 Do you have this trouble (TICK ONE BOX ONLY)
a) continuously so that your breathing is never quite right? 1 ☐
b) repeatedly, but it always gets completely better? 2 ☐
c) only rarely? 3 ☐

14. Are you disabled from walking by a condition ***other than*** heart or lung disease? NO ☐ YES ☐

IF 'YES' STATE CONDITION ____________________ AND GO TO QUESTION 15, IF 'NO':

14.1 Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1 Do you get short of breath walking with other people of your own age on level ground? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1 Do you have to stop for breath when walking at your own pace on level ground? NO ☐ YES ☐

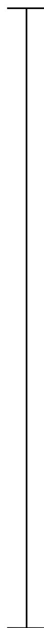
IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1.1 Do you ever have to stop for breath after walking about 100 yards (or after a few minutes) on level ground? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1.1.1 Are you too short of breath to leave the house OR short of breath on dressing or undressing? NO ☐ YES ☐

14.2 How much shortness of breath are you having right now? Please indicate by marking the height of the column. If you are not experiencing any shortness of breath at present circle the marker at the bottom of the column



Height in mm
(NB total height =100mm)

ECRHS III MAIN QUESTIONNAIRE

15. Have you ever had asthma? NO YES
    IF 'NO' GO TO QUESTION 16, IF 'YES':

15.1 Was this confirmed by a doctor? NO YES

15.2 How old were you when your asthma was confirmed by a doctor? YEARS

15.3 How old were you when you had your first attack of asthma? YEARS

15.4 How old were you when you had your most recent attack of asthma? YEARS

15.5.1-6 Which months of the year do you usually have attacks of asthma?

	NO	YES
15.5.1 January / February		
15.5.2 March / April		
15.5.3 May / June		
15.5.4 July / August		
15.5.5 September / October		
15.5.6 November / December		

15.6 Have you had an attack of asthma in the last ***12 months***? NO YES
IF 'NO' GO TO 15.9, IF YES

15.7 How many attacks of asthma have you had in the last ***12 months***? ATTACKS

15.8 How many attacks of asthma have you had in the last ***3 months***? ATTACKS

15.9 How many times have you woken up because of your asthma in the last ***3 months?***

TICK ONE BOX ONLY

- every night or almost every night 1
- more than once a week, but not most nights 2
- at least twice a month, but not more than once a week 3
- less than twice a month 4
- not at all 5

15.10. How often have you had trouble with your breathing because of your asthma in the last ***3 months?***

TICK ONE BOX ONLY

- continuously 1
- about once a day 2
- at least once a week, but less than once a day 3
- less than once a week 4
- not at all 5

15.11 Are you currently taking any medicines including inhalers, aerosols or tablets for asthma? NO YES

15.12 Do you have a peak flow meter of your own? NO YES
IF 'NO' GO TO QUESTION 15.13 , IF 'YES':

15.12.1 How often have you used it over the last 3 months?

TICK ONE BOX ONLY

- never 1
- some of the days 2
- most of the days 3

15.13 Do you have written instructions from your doctor on how to manage your asthma if it gets worse or if you have an attack? NO ☐ YES ☐

16. Has a doctor ever told you that you have chronic bronchitis? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 17, IF 'YES':

16.1 How old were you when you first had a diagnosis of chronic bronchitis? YEARS ☐☐

17. Has a doctor ever told you that you have chronic obstructive pulmonary disease (COPD)? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 18, IF 'YES

17.1 How old were you when you first had a diagnosis of COPD? YEARS ☐☐

18. Has a doctor ever told you that you have emphysema? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 19, IF 'YES':

18.1 How old were you when you first had a diagnosis of emphysema? YEARS ☐☐

19. Have you ever been diagnosed with any **other** lung disease (excluding asthma, chronic bronchitis, COPD and emphysema)? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 20, IF 'YES':

19.1 What is that lung disease called?___________________________________ CODE ☐☐

20. Do you have any nasal allergies, including hay fever? NO ☐ YES ☐

IF 'NO' GO TO Q21, IF' YES':

20.1 How old were you when you first had hay fever or nasal allergy? YEARS ☐☐

21. Have you ***ever*** had a problem with sneezing, or a runny or a blocked nose when you did not have a cold or the flu? NO ☐ YES ☐

IF 'NO' GO TO Q22, IF 'YES':

21.1. Have you had a problem with sneezing or a runny or a blocked nose when you did not have a cold or the flu ***in the last 12 months***? NO ☐ YES ☐

IF 'NO' GO TO Q22, IF' YES':

21.1.1. Has this nose problem been accompanied by itchy or watery eyes? NO ☐ YES ☐

21.1.2. In which months of the year did this nose problem occur?

	NO	YES
21.1.2.1. January/February	☐	☐
21.1.2.2. March/April	☐	☐
21.1.2.3. May/June	☐	☐
21.1.2.4. July/August	☐	☐
21.1.2.5. September/October	☐	☐
21.1.2.6 November/December	☐	☐

21.1.3 Have you had this problem for **more than 4 days in any one week** in the last 12 months? — NO ☐ YES ☐

IF 'NO' GO TO Q21.1.4, IF' YES':

21.1.3.1 Did this happen for **more than 4 weeks consecutively?** — NO ☐ YES ☐

21.1.4. For **each** of the following problems, please indicate how important it has been ***over the last 12 months***. *(SHOW A CARD WITH THE FOLLOWING OPTIONS)*

1. No problem (symptom not present)
2. A problem that is/was present but not disturbing
3. A disturbing problem but not hampering day time activities or sleep
4. A problem that hampers certain activities or sleep

CODE
Please enter code 1-4 in each of the five boxes

		CODE
21.1.4.1	a watery runny nose	☐
21.1.4.2	a blocked nose (feeling of being unable to breath through your nose)	☐
21.1.4.3	an itchy nose	☐
21.1.4.4	sneezing, especially violent and in bouts	☐
21.1.4.5	watery, red itchy eyes	☐

22. ***Since the last survey*** have you used any medication to treat nasal disorders? — NO ☐ YES ☐

IF NO GO TO Q23, IF YES

22.1 Have you used any of the following nasal sprays for the treatment of your nasal disorder? **{SHOW LIST OF STEROID NASAL SPRAYS}** — NO ☐ YES ☐

IF NO GO TO Q22.2, IF YES

22.1.1 How old were you when you first started to use **this sort of nasal spray**? — YEARS ☐☐

22.1 2 How many years have you been taking this sort of nasal spray? — YEARS ☐☐

22.1.3 Have you used any of these nasal sprays ***in the last 12 months?*** — NO ☐ YES ☐

22.1.4. Have you used this sort of nasal spray ***every year*** in the last 5 years? — NO ☐ YES ☐

IF 'NO' GO TO QUESTION 22.2 IF 'YES'

22.1.4.1 On average how many months each year have you taken them ? — MONTHS ☐☐

22.2 Have you used any of the following pills, capsules, or tablets for the treatment of your nasal disorder? **{SHOW LIST OF ANTIHISTAMINES}** — NO ☐ YES ☐

IF 'NO' GO TO Q23, IF 'YES'

22.2.1 Have you used any of these pills, capsules or tablets in the last 12 months**?** — NO ☐ YES ☐

ECRHS III MAIN QUESTIONNAIRE

23. Has your nose been blocked **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

24. Have you had pain or pressure around the forehead, nose or eyes **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

25. Have you had discoloured nasal discharge (snot) or discoloured mucus in the throat **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

26. Has your sense of smell been reduced or absent **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

27. Has a doctor ***ever*** told you that you have — NO YES
   - 27.1.1 **<u>chronic</u>** sinusitis?
   - 27.1.2 nasal polyps?

IF 'NO' TO Q27.1 and 27.2 GO TO Q 28, IF 'YES'

27.2 How old were you when a doctor told you had chronic sinusitis? — YEARS

27.3 How old were you when a doctor told you had nasal polyps?

(enter 00 if question not applicable)

28. Have you ***ever*** had eczema or any kind of skin allergy? NO YES

IF 'NO' TO Q28 GO TO Q 29, IF 'YES'

28.1 How old were you when you first had eczema or skin allergy? YEARS

28.2 Did/does your eczema or skin allergy affect your hands? NO YES

28.3 Have you noticed that contact with certain materials, chemicals or anything else **<u>in your work</u>** makes your eczema worse? NO YES DON'T KNOW

29. Have you ***ever*** had an itchy rash that was coming and going for at ***least 6 months***? NO YES

IF 'NO' GO TO QUESTION 30, IF 'YES':

29.1.. Have you had this itchy rash ***in the last 12 months?*** NO YES

IF 'NO' GO TO QUESTION 30, IF 'YES':

29.1.1. Has this itchy rash ***at any time*** affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles under the buttocks or around the neck, ears or eyes — NO YES

29.1.2 Has this itchy rash affected your hands at any time <u>in the last 12 months</u>?

30. What was the highest level of education your mother had? — TICK ONE BOX ONLY
   - a) Up to the minimum school leaving age — 1
   - b) Secondary school/technical school past the minimum age — 2
   - c) College or University — 3

31. What was the highest level of education your father had? — TICK ONE BOX ONLY
   - a) Up to the minimum school leaving age — 1
   - b) Secondary school/technical school past the minimum age — 2
   - c) College or University — 3

Appendix B

RHINESSA Adult Offspring Main Questionnaire (Interview)

Centre number : ______________
Personal number: ______________________
Sample _______
Date _____ _____ _______

Study center (answered by field worker): __________________________

I AM GOING TO ASK YOU SOME QUESTIONS. AT FIRST THESE WILL BE MOSTLY ABOUT YOUR BREATHING. WHEREVER POSSIBLE, I WOULD LIKE YOU TO ANSWER 'YES' OR 'NO'.

AIRWAYS SYMPTOMS

1. Have you had wheezing or whistling in your chest at any time in the last **12 months**? ☐NO ☐YES

IF 'NO' GO TO QUESTION 2, IF 'YES':

1.1. Have you been at all breathless when the wheezing noise was present? ☐NO ☐YES

1.2. Have you had this wheezing or whistling when you did **not** have a cold? ☐NO ☐YES

1.3. How old were you when you first had wheezing or whistling in your chest? _________YEARS

2. Have you woken up with a feeling of tightness in your chest at any time in the **last 12 months**? ☐NO ☐YES

3. Have you had an attack of shortness of breath that came on during the day when you were at rest at any time in the last **12 months**? ☐NO ☐YES

4. Have you had an attack of shortness of breath that came on **following** strenuous activity at any time in the last **12 months**? ☐NO ☐YES

5. Have you been woken by an attack of shortness of breath at any time in the last **12 months**? ☐NO ☐YES

6. Have you been woken by an attack of coughing at any time in the last **12*months***? ☐NO ☐YES

7. Do you usually cough first thing in the morning in the winter? ☐NO ☐YES
[IF DOUBTFUL, USE QUESTION 8.1 TO CONFIRM]

8. Do you ***usually*** cough during the day, or at night, in the winter? ☐NO ☐YES
IF 'NO' GO TO QUESTION 9, IF 'YES':

8.1. Do you cough like this on most days for as much as three months each year? ☐NO ☐YES

9. Do you ***usually*** bring up any phlegm from your chest first thing in the morning in the winter? ☐NO ☐YES
[IF DOUBTFUL, USE QUESTION 10.1 TO CONFIRM]

10. Do you ***usually*** bring up any phlegm from your chest during the day, or at night, in the winter? ☐NO ☐YES

IF 'NO' GO TO QUESTION 11, IF 'YES':

10.1. Do you bring up phlegm like this on most days for as much as three months each year? ☐NO ☐YES

ASTHMA

11. Have you ever had asthma? ☐NO ☐YES

IF 'NO' GO TO QUESTION 12, IF 'YES':

11.1. Was this confirmed by a doctor? ☐NO ☐YES

11.2. How old were you when your asthma was confirmed by a doctor? ________ YEARS

11.3. How old were you when you had your first attack of asthma? ________ YEARS

11.4. How old were you when you had your most recent attack of asthma? ________ YEARS

11.5. Which months of the year do you usually have attacks of asthma?

11.5.1. January / February ☐NO ☐YES
11.5.2. March / April ☐NO ☐YES
11.5.3. May / June ☐NO ☐YES
11.5.4. July / August ☐NO ☐YES
11.5.5. September / October ☐NO ☐YES
11.5.6. November / December ☐NO ☐YES

11.6. Have you had an attack of asthma in the last 12 months? ☐NO ☐YES

IF 'NO' GO TO 11.7, IF YES:

11.6.1. How many attacks of asthma have you had in the **last 12 months**? _________ATTACKS

11.6.2. How many attacks of asthma have you had in the **last 3 months**? _________ATTACKS

11.7. Are you currently taking any medicines including inhalers, aerosols or tablets for asthma? ☐NO ☐YES

NASAL ALLERGIES

12. Do you have any nasal allergies, including hay fever? ☐NO ☐YES
IF "NO" GO TO 13, IF "YES":

12.1. How old were you when you first had hay fever or nasal allergy? _________YEARS

13. Have you ***ever*** had a problem with sneezing, or a runny or a blocked nose when you did not have a cold or the flu? ☐NO ☐YES
IF 'NO' GO TO 14, IF 'YES':

13.1. Have you had a problem with sneezing or a runny or a blocked nose when you did not have a cold or the flu ***in the last 12 months***? ☐NO ☐YES
IF 'NO' GO TO 14, IF' YES':

13.1.1. Has this nose problem been accompanied by itchy or watery eyes? ☐NO ☐YES

13.1.2. In which months of the year did this nose problem occur?

January / February	☐NO	☐YES
March / April	☐NO	☐YES
May / June	☐NO	☐YES
July / August	☐NO	☐YES
September / October	☐NO	☐YES
November / December	☐NO	☐YES

14. Have you ***ever*** used any medication to treat nasal disorders? ☐NO ☐YES

IF NO GO TO 15, IF YES

14.1. Have you used any of the following nasal sprays for the treatment of your nasal disorder? **{SHOW LIST OF STEROID NASAL SPRAYS}**

IF NO GO TO 14.2, IF YES

14.1.1. Have you used any of these nasal sprays in **the last 12 months**? ☐NO ☐YES
14.1.2. Have you used any of these nasal sprays in **the last week**? ☐NO ☐YES

14.2. Have you used any of the following pills, capsules, or tablets for the treatment of your nasal disorder? {**SHOW LIST OF ANTIHISTAMINES**} ☐NO ☐YES

IF 'NO' GO TO 15, IF 'YES'

14.2.1. Have you used any of these pills, capsules or tablets in **the last 12 months**? ☐NO ☐YES
14.2.2. Have you used any of these pills, capsules or tablets in **the last week**? ☐NO ☐YES

ECZEMA AND SKIN ALLERGIES

15. Have you ever had eczema or any kind of skin allergy? ☐NO ☐YES
IF 'NO' TO 16, IF 'YES'

15.1. How old were you when you first had eczema or skin allergy? ________YEARS

15.2. Have you ever had an itchy rash that was coming and going for at least 6 months? ☐NO ☐YES
IF 'NO' GO TO QUESTION 16, IF 'YES':

15.2.1. Have you had this itchy rash in **the last 12 months**? ☐NO ☐YES

15.2.2. Has this itchy rash at any time affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles under the buttocks or around the neck, ears or eyes ☐NO ☐YES

FOOD ALLERGIES

16. Have you ever had an illness or trouble caused by eating a particular food or foods? ☐NO ☐YES
IF "NO", GO TO QUESTION 17, IF "YES"

16.1 Have you nearly always had the same illness or trouble after eating this type of food? ☐NO ☐YES

IF "NO", GO TO QUESTION 17, IF "YES":

16.1.1 What type of food was this? [List up to 3]

Food 1 ______________________________

Food 2 ______________________________

Food 3 ______________________________

<u>FOR FOOD 1:</u>

16.1.2 Did this illness or trouble include:

16.1.2.1	a rasch or itchy skin	☐NO ☐YES
16.1.2.2	diarrhoea or vomiting?	☐NO ☐YES
16.1.2.3	runny or stuffy nose?	☐NO ☐YES
16.1.2.4	severe headaches?	☐NO ☐YES
16.1.2.5	breathlessness?	☐NO ☐YES
16.1.2.6	other: ______________________________	☐NO ☐YES

16.1.3 How soon after eating this food did you get the first symptoms?

TICK ONE BOX ONLY

a)	Less than half an hour	1 ☐
b)	Half an hour to one hour	2 ☐
c)	One hour to two hours	3 ☐
d)	Two hours to four hours	4 ☐
e)	More than four hours	5 ☐

16.1.4 How old were you when you **first** had a such reaction to **food 1**? _________ YEARS

16.1.5 How old were you when you **last** had a such reaction to **food 1**? _________ YEARS

RHINESSA Adult Offspring Main Questionnaire (Interview)

ABOUT YOURSELF AND YOUR FAMILY

17. Did your father ever smoke regularly during your childhood? ☐NO ☐YES ☐DON'T KNOW

18. Did your mother ever smoke regularly during your childhood, or before you were born? ☐NO ☐YES ☐DON'T KNOW

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 18, IF 'YES':

18.1. When your mother was pregnant, in particular with you, did she

TICK ONE BOX ONLY

a) stop smoking before pregnancy? 1☐
b) cut down or stop during pregnancy? 2☐
c) smoke as usual during pregnancy? 3☐
d) don't know 4☐

19. How many brothers do or did you have? ________NUMBER (to siffer)

IF 'NONE' GO TO QUESTION 19, IF BROTHERS:

19.1. How many of your brothers ever had asthma? ________NUMBER
19.2. How many of your ***other*** brothers ever had eczema, skin or nasal allergy or hay fever? ________NUMBER
NB You can't use a number higher than XX

20. How many sisters do or did you have? ________NUMBER

IF 'NONE' GO TO QUESTION 20, IF SISTERS:

20.1. How many of your sisters ever had asthma? ________NUMBER
20.2. How many of your **k** sisters ever had eczema, skin or nasal allergy or hay fever? ________NUMBER
NB You can't use a number higher than XX

21. Did your mother ever have asthma? ☐NO ☐YES ☐DON'T KNOW

22. Did your mother ever have eczema, skin or nasal allergy or hay fever? ☐NO ☐YES ☐DON'T KNOW

23. Did your father ever have asthma? ☐NO ☐YES ☐DON'T KNOW

24. Did your father ever have eczema, skin or nasal allergy or hay fever? ☐NO ☐YES ☐DON'T KNOW

25. Did you regularly share your bedroom before the age of five years? ☐NO ☐YES ☐DON'T KNOW

26. Did you go to a school, play-school or nursery before the age of five years? ☐NO ☐YES ☐DON'T KNOW

27. How old was your mother when you were born? ________YEARS

28. Were you delivered by Caesarean section? ☐NO ☐YES ☐DON'T KNOW

29. Did your mother breastfeed you? ☐NO ☐YES ☐DON'T KNOW

29.1. If "YES", for how long? ________MONTHS

30. Did your biological parents ever suffer from any of the following?

MOTHER

		NO	YES	DK
• Chronic bronchitis, emphysema and/or COPD	30.1.1	☐	☐	☐
• Heart disease	30.1.2	☐	☐	☐
• Hypertension	30.1.3	☐	☐	☐
• Stroke	30.1.4	☐	☐	☐
• Diabetes	30.1.5	☐	☐	☐
• Chronic inflammatory bowel disease (Crohn's disease or Ulcerous Colitis)	30.1.6	☐	☐	☐

FATHER

		NO	YES	DK
• Chronic bronchitis, emphysema and/or COPD	30.2.1	☐	☐	☐
• Heart disease	30.2.2	☐	☐	☐
• Hypertension	30.2.3	☐	☐	☐
• Stroke	30.2.4	☐	☐	☐
• Diabetes	30.2.5	☐	☐	☐
• Chronic inflammatory bowel disease (Crohn's disease or Ulcerous Colitis)	30.2.6	☐	☐	☐

31. How many biological children do you have? ________NUMBER

IF ANSWER TO 30 INDICATES PARTICIPANT HAS BIOLOGICAL CHILDREN GO TO 31.1-8;
IF NO BIOLOGICAL CHILDREN GO TO QUESTION 32

	Starting with the first born	Year of birth (eg 1995)				Did this child have asthma before the age of ten years?		Did this child have asthma after the age of ten years?		Has this child ever had hayfever or allergic rhinitis?		Has this child ever had eczema or atopic dermatitis?		Was this child a boy or girl (Boy=1, Girl=2)
						NO	YES	NO	YES	NO	YES	NO	YES	Sex
31.1	Child 1													
31.2	Child 2													
31.3	Child 3													
31.4	Child 4													
31.5	Child 5													

31.6	Child 6													
31.7	Child 7													
31.8	Child 8													

YOUR OCCUPATION

32. I would like to ask you to list all jobs that you have had. I am interested in each one of the jobs that you have done for three months or more. These jobs may be outside the house or at home, **excluding homemaking or housework**, full time or part time, paid or unpaid, including self employment, for example in a family business. Please include part time jobs only if you had been doing them for 20 or more hours per week. Please start with your current or last held job.

	Job	Occupation – Job Title: *Please provide a detailed description of the job*	Industry / Branch: *What does (did) your firm or employer make or what services does (did) it provide?*	Start month/ year	End month/year *(If current job please enter CURRENT)*
32.0.1	1				
32.0.2	2				
32.0.3	3				
32.0.4	4				
32.0.5	5				
32.0.6	6				
32.0.7	7				
32.0.8	8				
32.0.9	9				
32.0.10	10				

IF JOBS ARE GIVEN, GO TO QUESTION 32.1; IF NO JOBS GIVEN GO TO 33

32.1. Have you had to change or leave any of these jobs because it affected your breathing? ☐NO ☐YES

IF 'NO' GO TO QUESTION 32.2; IF 'YES':

31.1.1 – 10 Please indicate which job(s) you had to change or leave (use numbers from question 32).

32.1.1. Job 1 ☐NO ☐YES
32.1.2. Job 2 ☐NO ☐YES
32.1.3. Job 3 ☐NO ☐YES
32.1.4. Job 4 ☐NO ☐YES
32.1.5. Job 5 ☐NO ☐YES
32.1.6. Job 6 ☐NO ☐YES
32.1.7. Job 7 ☐NO ☐YES
32.1.8. Job 8 ☐NO ☐YES
32.1.9. Job 9 ☐NO ☐YES
32.1.10. Job 10 ☐NO ☐YES

32.2. Have you had to change or leave any of these jobs because of hay fever or nasal symptoms? ☐NO ☐YES

IF 'NO' GO TO QUESTION 32.3; IF 'YES':

32.2.1-10 Please indicate which job(s) you had to change or leave (use numbers from question 32).

32.2.1. Job 1 ☐NO ☐YES
32.2.2. Job 2 ☐NO ☐YES
32.2.3. Job 3 ☐NO ☐YES
32.2.4. Job 4 ☐NO ☐YES
32.2.5. Job 5 ☐NO ☐YES
32.2.6. Job 6 ☐NO ☐YES
32.2.7. Job 7 ☐NO ☐YES
32.2.8. Job 8 ☐NO ☐YES
32.2.9. Job 9 ☐NO ☐YES
32.2.10. Job 10 ☐NO ☐YES

32.3. Have you had to change or leave any of these jobs because of eczema or skin disease? ☐NO ☐YES

IF 'NO' GO TO QUESTION 32.4; IF 'YES':

32.3.1-10 Please indicate which job(s) you had to change or leave (use numbers from question 32).

32.3.1. Job 1 ☐NO ☐YES
32.3.2. Job 2 ☐NO ☐YES
32.3.3. Job 3 ☐NO ☐YES
32.3.4. Job 4 ☐NO ☐YES
32.3.5. Job 5 ☐NO ☐YES
32.3.6. Job 6 ☐NO ☐YES
32.3.7. Job 7 ☐NO ☐YES
32.3.8. Job 8 ☐NO ☐YES
32.3.9. Job 9 ☐NO ☐YES
32.3.10. Job 10 ☐NO ☐YES

32.4. Does being at your **current workplace** ever cause breathing problems (chest tightness, wheezing, coughing)? ☐NO ☐YES

IF 'NO' GO TO QUESTION 32.5, IF 'YES':

32.4.1-5 Can you indicate what gives you breathing problems in your current workplace?

32.4.1. Physical exertion ☐NO ☐YES
32.4.2. Exposure to mist, hot or cold temperature ☐NO ☐YES
32.4.3. Exposure to vapours gas dust or fumes ☐NO ☐YES
32.4.4. Other peoples cigarette smoke ☐NO ☐YES
32.4.5. Stress ☐NO ☐YES

32.4.6. Do these breathing problems diminish or stop **during the weekend or during holidays**? ☐NO ☐YES

32.5. Do you regularly use **cleaning products** or **disinfectants** in your current job? ☐NO ☐YES

IF 'NO' GO TO QUESTION 33, IF 'YES':

In the last 12 months, on how many days a week have you used the following products at work?
(SHOW CARD WITH FOLLOWING OPTIONS)

1. Never
2. <1 day/week
3. 1-3 days/week
4. 4-7 days/week

	CODE Enter code 1-4 for all boxes
32.5.1. Bleach	☐
32.5.2. Ammonia	☐
32.5.3. Stain removers or other solvents	☐
32.5.4. Acids (including decalcifiers, liquid scale removers, vinegar, hydrochloric acid, …)	☐
32.5.5. Floor polish or floor wax	☐
32.5.6. Liquid or solid furniture polish or wax	☐
32.5.7. Furniture sprays (atomisers or aerosols)	☐
32.5.8. Sprays for mopping the floor	☐
32.5.9. Glass cleaning sprays (atomisers or aerosols)	☐
32.5.10. Degreasing sprays including oven cleaning sprays (atomisers or aerosols)	☐
32.5.11. (Ethyl) alcohol	☐
32.5.12. Soaps or foams for disinfecting hands	☐
32.5.13. Any other chemical disinfectant (for example, glutaraldehyde, formaldehyde, chloramine-T, quaternary ammonium compounds)	☐

INCIDENTS

33. Have you ever been involved in an incident at home, work or elsewhere that exposed you to high levels of vapours, gases, dusts or fumes? ☐NO ☐YES

IF 'NO' GO TO QUESTION 34, IF 'YES':

33.1. When did this occur? ________ YEAR

In case of more than one incident, please report on the most recent incident.

33.2. Could you please classify this incident — TICK ONE BOX ONLY

A fire or an explosion	1 ☐
A leakage or spill	2 ☐
An inhalation related to mixing of cleaning products	3 ☐
Something else	4 ☐

33.3. Where did this happen? TICK ONE BOX ONLY

In your own home	1 ☐
In your workplace	2 ☐
Somewhere else indoors	3 ☐
Outdoor	4 ☐

33.4. Did you experience respiratory symptoms within 24 hours following this incident? ☐NO ☐YES

IF 'NO' GO TO QUESTION 34, IF 'YES':

33.4.1. Did you seek medical treatment for these symptoms? ☐NO ☐YES

PHYSICAL ACTIVITY

34. How often do you usually exercise so much that you get out of breath or sweat? TICK ONE BOX ONLY

every day	1 ☐
4-6 times a week	2 ☐
2-3 times a week	3 ☐
once a week	4 ☐
once a month	5 ☐
less than once a month	6 ☐
never	7 ☐

35. How many hours a week do you usually exercise so much that you get out of breath or sweat? TICK ONE BOX ONLY

None	1 ☐
about ½ hr	2 ☐
about 1 hour	3 ☐
about 2-3 hours	4 ☐
about 4-6 hours	5 ☐
7 hours or more	6 ☐

36. Do you avoid taking vigorous exercise because of breathing problems? ☐NO ☐YES

RHINESSA Adult Offspring Main Questionnaire (Interview)

SYMPTOMS NEAR ANIMALS, DUSTS OR POLLEN

37. When you are near animals, such as cats, dogs or horses, do you ever

37.1. start to cough? ☐NO ☐YES
37.2. start to wheeze? ☐NO ☐YES
37.3. get a feeling of tightness in your chest? ☐NO ☐YES
37.4. start to feel short of breath? ☐NO ☐YES
37.5. get a runny or stuffy nose or start to sneeze? ☐NO ☐YES
37.6. get itchy or watering eyes? ☐NO ☐YES

IF NO TO ALL SYMPTOMS, GO TO QUESTION 38;IF YES TO ONE OR MORE SYMPTOMS

36.6.1 – 2 Do you have such symptom/s when you are near
37.6.1. cat? ☐NO ☐YES
37.6.2. dog? ☐NO ☐YES

38. When you are in a dusty part of the house, or near pillows or duvets do you ever
38.1. start to cough? ☐NO ☐YES
38.2. start to wheeze? ☐NO ☐YES
38.3. get a feeling of tightness in your chest? ☐NO ☐YES
38.4. start to feel short of breath? ☐NO ☐YES
38.5. get a runny or stuffy nose or start to sneeze? ☐NO ☐YES
38.6. get itchy or watering eyes? ☐NO ☐YES

39. When you are near trees, grass or flowers, or when there is a lot of pollen about, do you ever
39.1. start to cough? ☐NO ☐YES
39.2. start to wheeze? ☐NO ☐YES
39.3. get a feeling of tightness in your chest? ☐NO ☐YES
39.4. start to feel short of breath? ☐NO ☐YES
39.5. get a runny or stuffy nose or start to sneeze? ☐NO ☐YES
39.6. get itchy or watering eyes? ☐NO ☐YES

IF 'YES' TO ANY OF THE ABOVE:

39.7. Which time of year does this happen?
39.7.1. winter ☐NO ☐YES
39.7.2. spring ☐NO ☐YES
39.7.3. summer ☐NO ☐YES
39.7.4. autumn ☐NO ☐YES

RHINESSA Adult Offspring Main Questionnaire (Interview)

IN-DOOR ENVIRONMENT

40. When was your present home built? YEAR________

41. How many years have you lived in your current home? YEAR________

42. Has there been any water damage to the building or its contents,

for example, from broken pipes, leaks or floods? ☐ NO ☐ YES ☐ DON'T KNOW

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 43, IF 'YES':

42.1 Has there been any water damage in the last 12 months? ☐NO ☐YES ☐ DON'T KNOW

43. Has there ever been any mould or mildew on any surface, other than food, inside the home? ☐NO ☐YES ☐ DON'T KNOW

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 44, IF 'YES'

43.1 Has there ever been any mould or mildew on any surface inside the home in the last ***12 months***? ☐ NO ☐ YES ☐ DON'T KNOW

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 44, IF 'YES':

43.1 Which rooms have been affected?

	NO	YES
43.1.1. bathroom(s)	☐	☐
43.1.2. bedroom(s)	☐	☐
43.1.3. living area(s)	☐	☐
43.1.4. kitchen	☐	☐
43.1.5. basement or attic	☐	☐
43.1.6. other: ____________________	☐	☐

44. Have you noticed the odour of mould or mildew (not from food) in your home at any time in the last 12 months? ☐ NO ☐ YES

OUT-DOOR ENVIRONMENT

Is the participant currently working (answered by fieldworker)? ☐ NO ☐ YES ☐
IF NO, GO TO question 46
If YES:

45. How many days per week do you commute to work ________ (NUMBER)
IF '0' GO TO QUESTION 46; IF ONE OR MORE DAYS

45.1. On average, how much time do you spend travelling to and from work each day (total for both directions)? ________ (MINUTES)

45.2. What is your main method of commuting?

TICK ONE BOX ONLY

a) Walking or cycling	1	☐
b) In a private car	2	☐
c) Bus	3	☐
d) Train	4	☐
e) Other	5	☐

SMOKING

46. Have you ever smoked for as long as a year? ☐NO ☐YES

['YES' means at least 20 packs of cigarettes or 12 oz (360 grams) of tobacco in a lifetime, or at least one cigarette per day or one cigar a week for one year]

IF 'NO' GO TO QUESTION 47, IF 'YES':

46.1. How old were you when you started smoking? __________ YEARS

46.2. How old were you when you started smoking daily? __________ YEARS

Never smoked daily please enter 88

46.3. Do you **now** smoke, as of **one month ago**? ☐NO ☐YES

IF 'NO' GO TO QUESTION 46.4, IF 'YES':

46.3.1 – 4 How much do you now smoke on average? NUMBER

46.3.1. number of cigarettes per day ________
46.3.2. number of cigarillos per day ________
46.3.3. number of cigars a week ________
46.3.4. pipe tobacco in
a) ounces / week ________
b) grams / week ________

46.4. Have you stopped or cut down smoking? ☐NO ☐YES

IF 'NO' GO TO QUESTION 46.5, IF 'YES':

46.4.1. Did you stop or cut down due to breathing problems? ☐NO ☐YES

46.4.2. How old were you when you stopped or cut down smoking? __________ YEARS

46.4.3. 1-4 **On average** of the entire time you smoked, before you stopped or cut down, how much did you smoke? NUMBER

46.4.3.1. number of cigarettes per day ________
46.4.3.2. number of cigarillos per day ________
46.4.3.3. number of cigars a week ________

46.4.3.4. pipe tobacco in
a) ounces / week ________
b) grams / week ________

46.5. Do you or did you inhale the smoke? ☐NO ☐YES

47. Have you been **regularly** exposed to tobacco smoke in the last **12 months**?
['Regularly' means on most days or nights] ☐NO ☐YES

IF 'NO' GO TO QUESTION 48, IF 'YES'

47.1. Not counting yourself, how many people in your household smoke regularly? ________ (NUMBER)

47.2. Do people smoke regularly in the room where you work? ☐NO ☐YES

47.3. How many hours per day are you exposed to **other people's** tobacco smoke? ________ (HOURS)

47.4. How many hours per day, are you exposed to other peoples tobacco smoke in the following locations? HOURS

- at home ________
- at workplace ________
- in bars, restaurants, cinemas or similar social settings ________
- elsewhere ________

MEDICINES AND TREATMENTS

48. Have you had a course of antibiotics in the last 12 months? ☐NO ☐YES
IF NO GO TO QUESTION 49; IF YES

48.1. How many courses of antibiotics? ________ (NUMBER)
48.2. How long time ago did you last have antibiotics?
48.2.1. Last 0-1 week ☐
48.2.2. 1-2 weeks ago ☐
48.2.3. 2-4 weeks ago ☐
48.2.4. over 4 weeks ago ☐

49. Have you had a course of antibiotics in the last 12 months to help your **breathing**? ☐NO ☐YES

IF NO GO TO QUESTION 50; IF YES

49.1 How many courses of antibiotics? ________ (NUMBER)

50. Have you used antibiotics for nasal/sinus problems in the last 12 months? ☐NO ☐YES

IF NO GO TO QUESTION 51; IF YES

50.1. How many courses of antibiotics? _______ (NUMBER)

51. Are you usually vaccinated against flu? ☐NO ☐YES

IF NO GO TO QUESTION 52; IF YES

51.1. Were you vaccinated against flu in the last winter period? ☐NO ☐YES

52. Have you been vaccinated against pneumonia (Pneumovax) in the last 5 years? ☐NO ☐YES ☐DON'T KNOW

53. Have you used any inhaled medicines to help your breathing at any time in the last 12 months? ☐NO ☐YES

IF NO' GO TO QUESTION 54, IF 'YES':

Which of the following have you used in the **last 12 months**?

53.1. **Short acting beta-2-agonist (only) inhalers** ☐NO ☐YES
(Please include combinations that include beta 2 and steroids in section 53.5)

53.1.1. If used, which one?____

53.1.2. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 4 ☐

53.1.3. Number of puffs last week _______ (NUMBER)

53.2. **Long acting beta-2-agonist inhalers** ☐NO ☐YES
(Please include combinations that include long acting beta 2 and steroids in section 53.5)

53.2.1. If used, which one?____
____

53.2.2. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 4 ☐

53.2.3. Number of puffs last week _______ (NUMBER)

53.3. **Short acting or long-acting anti-muscarinic inhalers** ☐NO ☐YES

53.4. **Inhaled steroids (ONLY)** ☐NO ☐YES

(Please include combinations that include beta 2 and steroids in section 53.5)

53.4.1. If used, which one? ________________________________

53.4.2. What type of inhaler do you use? ________________________________

53.4.3. What is the dose per puff (in micrograms)? _______ (NUMBER)

53.4.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1 ☐

b) in short courses 2 ☐

c) continuously 3 ☐

d) not at all 4 ☐

If answer to 53.4.4 is <u>when needed</u>:

53.4.5. Number of puffs per month _______ (NUMBER)

If answer to 53.4.4 is in <u>short courses</u>

53.4.6. number of courses _______(NUMBER)

53.4.7. number of puffs per day _______(NUMBER)

53.4.8. average number of days per month _______(NUMBER)

If answer to 53.4.4 is <u>continuously</u>

53.4.9. number of puffs per day _______ (NUMBER)

53.4.10. How many times over the last 3 months have you temporarily increased this treatment because your symptoms became worse? _______ (NUMBER)

53.5. **Inhaled steroids and beta2 agonists <u>(combined therapy)</u>** ☐NO ☐YES

53.5.1. If used, which one? ________________________________

53.5.2. What type of inhaler do you use? ________________________________

53.5.3. What is the dose per puff (in micrograms)? _______ (NUMBER)

(Please insert the dose of the inhaled steroid)

53.5.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1 ☐

b) in short courses 2 ☐

c) continuously 3 ☐

d) not at all 4 ☐

If answer to 53.5.4 is <u>when needed</u>:

53.5.5. Number of puffs per month _______ (NUMBER)

If answer to 53.5.4 is <u>in short courses:</u>

53.5.6. number of courses _______(NUMBER)

53.5.7. number of puffs per day _______(NUMBER)

53.5.8. average number of days per month ________(NUMBER)

If answer to 53.5.4 is continuously:
53.5.9. Number of puffs per day ________ (NUMBER)
53.5.10. How many times over the last 3 months have you temporarily increased this treatment because your symptoms became worse? ________ (NUMBER)

54. Have you used any **pills, capsules, tablets** or **medicines**, other than inhaled medicines, to help your breathing at any time in the last ***12 months***? ☐NO ☐YES

IF 'NO' GO TO QUESTION 55, IF 'YES':
Which of the following have you used in the last 12 months?

54.1. **oral beta-2-agonists** ☐NO ☐YES
54.1.1. If used, which one? ________________________________

54.2. **oral methylxanthines** ☐NO ☐YES
54.2.1. If used, which one? ________________________________

54.3. **Oral steroids** ☐NO ☐YES

54.3.1. If used, which one? ________________________________

54.3.2. What dose of tablet ________ (NUMBER)

54.3.3. In the last 12 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐

If answer to 54.3.3 is when needed:
54.3.4. Number of tablets per month ________ (NUMBER)

If answer to 54.3 3 is in short courses
54.3.5. number of courses ________(NUMBER)
54.3.6. tablets per day ________(NUMBER)
54.3.7. average number of days per month ________(NUMBER)

If answer to 54.3.3 is continuously
54.3.8. Tablets per day ________ (NUMBER)

54.3.9. Have you used them in the last 3 months? ☐NO ☐YES
54.4. **Oral anti-leukotrienes** ☐NO ☐YES
54.4.1. If used, which one? ________________________________

55. Have you ever used inhaled steroids (show list, including combined therapy)? ☐NO ☐YES

IF NO GO TO QUESTION 56;IF YES

55.1. How old were you when you first started to use inhaled steroids? __________ YEARS

55.2. How old were you when you last used inhaled steroids? __________ YEARS

55.3. Have you used inhaled steroids ***every year*** since you started to use them? ☐NO ☐YES

IF 'NO' GO TO QUESTION 56; IF YES

55.3.1. On average how many months each year have you taken them? __________ MONTHS

56. Have you ever had any vaccinations or injections for the treatment of allergy or had a course of desensitization? ☐NO ☐YES

IF NO GO TO QUESTION 57; IF YES

56.1. What was this treatment? __________ CODE

56.2. Have you had this treatment in the last 12 months? ☐NO ☐YES

RHINESSA Adult Offspring Main Questionnaire (Interview)

GENERAL HEALTH

57. Which of the following dental hygiene tools do you use, and how often? (tick one box for each line)

	Never/rarely	1 /week	1/day	2/day	more than 2/day
57.1. Tooth brush					
57.2. Fluoride dental paste					
57.3. Dental floss					
57.4. Tooth picks					
57.5. Mouth wash					

58. Do you have or have you ever had any of the following illnesses?
If yes, please indicate the age you were first diagnosed with the disease

		NO		YES			YEARS	
58.1.1	Stroke				58.1.2	Age diagnosed		
58.2.1	Angina, heart attack, coronary heart disease				58.2.2	Age diagnosed		
58.3.1	Insulin dependent diabetes				58.3.2	Age diagnosed		
58.4.1	Non-insulin dependent diabetes				58.4.2	Age diagnosed		
58.5.1	Cancer				58.5.2	Age diagnosed		
58.6.1	Depression				58.6.2	Age diagnosed		
58.7.1	Hypertension				58.7.2	Age diagnosed		
58.8.1	Osteoporosis				58.8.2	Age diagnosed		
58.9.1	Crohns Disease				58.9.2	Age diagnosed		
58.10.1	Ulcerous Colitis				58.10.2	Age diagnosed		
58.11.1	Migraine				58.11.2	Age diagnosed		
58.12.1	Rheumatoid arthritis				58.12.2	Age diagnosed		
58.13.1	Ankylosing spondylitis (Mb Bechterew) or psoariatic arthritis				58.13.2	Age diagnosed		
58.14.1	Gastro-oesophagel reflux hiatus hernia or oesophagitis				58.14.2	Age diagnosed		

58.5.3	Type of cancer	

Code for 58.5.3
1= breast
2= prostate
3= lung
4= GI tract
5= other

59. Have you ever visited a hospital casualty department or emergency room (for any reason, apart from accidents and injuries)? ☐NO ☐YES

IF 'NO' GO TO QUESTION 60; IF 'YES'

59.1. Was this due at least once to ***breathing problems***? ☐NO ☐YES

59.2. Have you visited a hospital casualty department or emergency room because of breathing problems) in the last 12 months? ☐NO ☐YES

RHINESSA Adult Offspring Main Questionnaire (Interview)

60. Have you ever spent a night in hospital
(for any reason, apart from accidents and injuries)? ☐NO ☐YES

IF 'NO' GO TO QUESTION 61, IF 'YES'

60.1. Was this due at least once to **breathing problems**? ☐NO ☐YES
60.2. Have you spent a night in hospital because of breathing problems
in the last 12 months? ☐NO ☐YES

	NO	YES	HAVE NOT WORKED IN THE LAST 12 MONTHS
61. In the last **12 months** have you lost days of work because of health problems (apart from accidents and injuries)?	☐	☐	☐

IF NOT WORKED OR HAS NOT LOST DAYS OF WORK GO TO QUESTION 62; IF YES
61.1. How many days in the last 12 months? ________*(DAYS)*
61.2. Among these ones, how many because of breathing problems? ________*(DAYS)*
[Write '000' if not lost any days due to breathing problems]

62. Have you ever been forced to give up working altogether because of health
problems (apart from accidents and injuries)? ☐NO ☐YES

IF 'NO' GO TO QUESTION 63, IF 'YES'

62.1. When did this occur? ________*(MONTH)* ________*(YEAR)*
62.2. Were you forced to give up working altogether because of **breathing problems**? ☐NO ☐YES

63. In the last **12 months** have there been any days when you have had to **give up activities other than work** (e.g. looking after children, the house, studying) because of health problems (apart from accidents and injuries)? ☐NO ☐YES

IF 'NO' YOU HAVE FINISHED THE QUESTIONNAIRE, IF 'YES':

63.1. How many days **in the last 12 months?** ________*(DAYS)*

63.2. Among these ones, how many because of **breathing problems**? ________*(DAYS)*

[Write '0' if s/he has not had any days of activity lost due to breathing problems]

64. Interview type — TICK ONE BOX ONLY
1 face to face interview at clinic ☐
2 telephone ☐
3 face to face at home ☐
4 other ☐

65. Date of birth check. What is the date of birth of this participant? _____ (DAY)_____*(MONTH)* _______*(YEAR)*

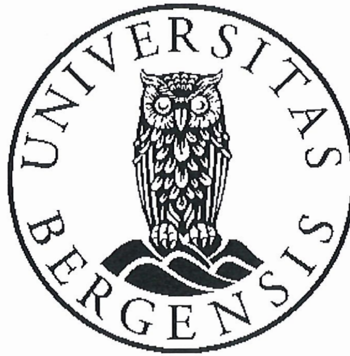
66. We would like to know where you have lived since January 1990.
Please give the address, including postcode, of all homes you have lived in **for at least one year since 1990, starting with your current address**
Fieldworkers: Check through residential history from screening questionnaire

House number	Street name	City	Postcode	Moved in	Lived there until (YEAR)
					current

All centres please note: this information will be used for your centre staff to geocode residence.

Errata for
Helminth exposure and its association with allergies and lung function in Northern European cohorts.

Nils Oskar Jõgi



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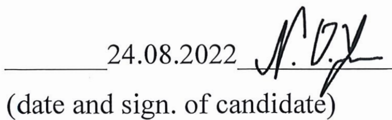
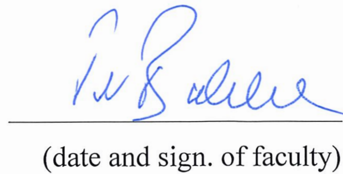
at the University of Bergen

24.08.2022

(date and sign. of candidate)

(date and sign. of faculty)

Errata

Page 41 Dilution is marked incorrectly. Instead of "1:242" it should be "1:243". Similar correction on page 6 of Paper III.

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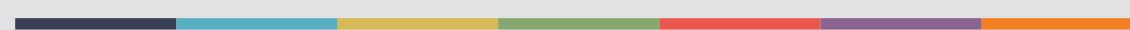

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