Atopic Diseases

Mechanisms, Exposures, and Diagnostics

DMSc thesis by

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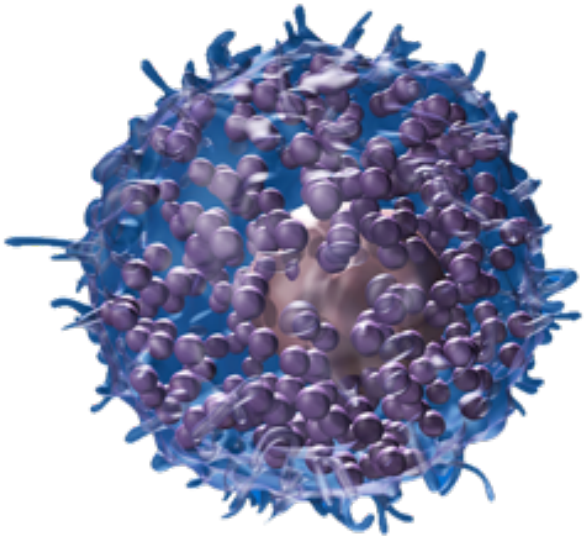


The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in Medical Science.
Copenhagen, 22 September 2023.
Dr. Bente Merete Stallknecht, Dean

Public defense will be held in the Henrik Dam Auditorium at Panum
Thursday, January 11, 2024 at 2pm.

Opponents:
Professor Nikolaos Papadopoulos, University of Manchester, 1st opponent
Professor Anna Nowak-Wegrzyn, NYU Langone Health, 2nd opponent
Professor Klaus Müller, University of Copenhagen, chair

Papers included in the thesis

The following published papers are referred to by their roman numerals in the thesis:

I. **Schoos AMM**, Hansen SM, Skov FR, Stokholm J, Bønnelykke K, Bisgaard H, et al. Allergen Specificity in Specific IgE Cutoff. JAMA Pediatr. 2020 Oct 1;174(10):993–5.

II. **Schoos AMM**, Hansen BR, Stokholm J, Chawes BL, Bønnelykke K, Bisgaard H. Parent-specific effects on risk of developing allergic sensitization and asthma in childhood. Clin Exp Allergy. 2020 Aug;50(8):915–21.

III. **Schoos AMM**, Jelding-Dannemand E, Stokholm J, Bønnelykke K, Bisgaard H, Chawes BL. Single and multiple time-point allergic sensitization during childhood and risk of asthma by age 13. Pediatr Allergy Immunol. 2019 Nov;30(7):716–23.

IV. **Schoos AMM**, Chawes BL, Bønnelykke K, Stokholm J, Rasmussen MA, Bisgaard H. Increasing severity of early-onset atopic dermatitis, but not late-onset, associates with development of aeroallergen sensitization and allergic rhinitis in childhood. Allergy. 2022 Apr;77(4):1254–62.

V. Jelding-Dannemand E, **Malby Schoos AM**, Bisgaard H. Breast-feeding does not protect against allergic sensitization in early childhood and allergy-associated disease at age 7 years. J Allergy Clin Immunol. 2015 Nov;136(5):1302-1308.e1-13.

VI. **Schoos AMM**, Chawes BL, Jelding-Dannemand E, Elfman LB, Bisgaard H. Early indoor aeroallergen exposure is not associated with development of sensitization or allergic rhinitis in high-risk children. Allergy. 2016 May;71(5):684–91.

VII. **Schoos AMM**, Bønnelykke K, Chawes BL, Stokholm J, Bisgaard H, Kristensen B. Precision allergy: Separate allergies to male and female dogs. J Allergy Clin Immunol Pract. 2017 Dec;5(6):1754–6.

VIII. **Schoos AMM**, Chawes BL, Bloch J, Hansen B, Stokholm J, Bønnelykke K, et al. Children Monosensitized to Can f 5 Show Different Reactions to Male and Female Dog Allergen Extract Provocation: A Randomized Controlled Trial. J Allergy Clin Immunol Pract. 2020 May;8(5):1592-1597.e2.

IX. **Schoos AMM**, Nwaru BI, Borres MP. Component-resolved diagnostics in pet allergy: Current perspectives and future directions. J Allergy Clin Immunol. 2021 Apr;147(4):1164–73.

Abbreviations

AD = Atopic Dermatitis

CAP = Conjunctival Allergen Provocation

COPSAC = COpenhagen Prospective Studies on Asthma in Childhood

CRD = Component Resolved Diagnostics

FeNO = Fractional exhaled Nitric Oxide

FLG = Filaggrin

GEE = Generalized Estimating Equations

ISAC = Immuno Solid-phase Allergen Chip

ISU = ISAC Standardized Units

MAAS = Manchester Asthma and Allergy Study

OR = Odds Ratio

SCORAD = SCORing Atopic Dermatitis

SD = Standard Deviation

sIgE = specific Immunoglobulin E

SPT = Skin Prick Test

TOSS = Total Ocular Symptom Score

Abstract

Epidemiological data suggest that atopic diseases (asthma, atopic dermatitis, allergic rhinitis, and food allergy) begin in early life and that most cases present clinically during early childhood. The disease incidences increase as communities adopt western lifestyles and the diseases are highly prevalent. Disentangling the pathophysiological mechanisms leading to disease debut is necessary to identify beneficial/harmful exposures so that successful prevention and treatment can be generated.

The objective of this thesis is to explore mechanisms of atopic diseases, to investigate the importance of environmental factors in early life, prior to disease development, and finally to explore new diagnostic tools to interpret allergic sensitization more accurately. The thesis is built on nine studies originating from the $COPSAC_{2000}$ and $COPSAC_{2010}$ birth cohorts investigating mechanisms of atopic disease and modifiable exposures in the environment that can affect the development of atopic diseases, and finally, examining how component resolved diagnostics can help provide a patient-tailored approach to allergy diagnostics.

First, the distribution of sIgE levels in children is investigated, as this is one of the main criteria for the definition of atopy. Thereafter, it is explored how studies of parental atopic status, sensitization patterns, and early debut and severity of atopic dermatitis have substantiated the theory of an early-life window-of-opportunity for intervention that precedes the development of atopic diseases in childhood. Then, it is examined whether early-life exposures such as breastfeeding, dogs, cats, and house dust mites in the home perinatally constitute important influencers in this crucial time of life. Finally, it is explored how component resolved diagnostics may allow patient-tailored recommendations suggesting that some dog allergic patients tolerate female dogs. The utility of molecular diagnostics is also discussed in relation to other allergen sources and future directions are proposed. Last, it is discussed how these findings could be validated in randomized controlled trials, which might prepare the ground for improved diagnostics and prevention strategies to mitigate the current atopic pandemic.

Introduction

The atopic disease pandemic

The incidence of atopic diseases, namely asthma, atopic dermatitis (AD), allergic rhinitis, and food allergy, has strikingly increased in recent decades[1–3]. The global variation of atopic diseases is considerable (**Figure 1** and **2**), and the incidence increases as communities adopt western lifestyles and become urbanized. In fact, asthma incidence in high-income countries seem to have reached a saturation level while the incidence in mid- and low-income countries is still increasing year by year, most markedly in low-income countries[4]. The parallel increase in allergic sensitization that has been observed together with the atopic diseases[5,6] argues against increased awareness and diagnostic of milder cases as an explanation for the increase. All combined, atopic diseases now affect roughly 20% of the global population[7], and for most the disease debuted in early childhood[2,8]. The diseases impact quality of life of the child and work productivity of the parents. Indeed, because of their high prevalence and economic burden, the combined overall socioeconomic burden of atopic diseases is considerable[9].

Figure 1: The global prevalence of asthma, ranging from 1,900 (dark blue) to 12,000 (red) per 100,000 in 2015, adapted from Dierick et al.[9]

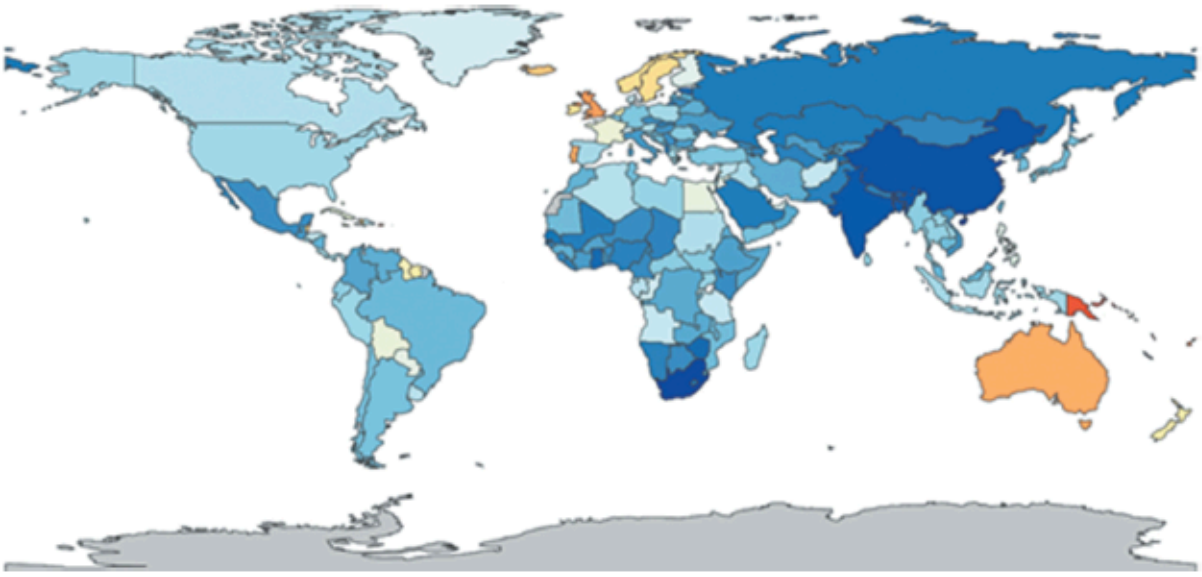
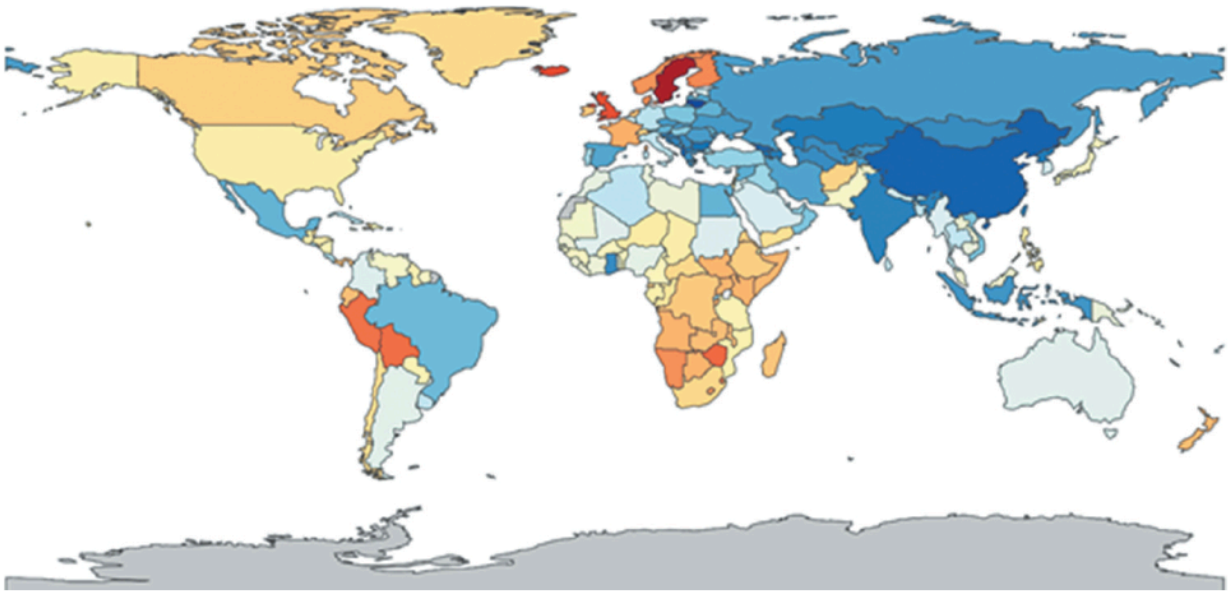


Figure 2: Global prevalence of AD, ranging from 1,200 (dark blue) to 6,400 (red) per 100,000 in 2015, adapted from Dierick et al.[9]



The atopic waltz

The increase in the incidence of asthma, AD, and allergic rhinitis has been associated with an increase in allergic sensitization[2]. The interplay between these diseases has been eagerly discussed in the literature, and for almost two decades the relationship has been referred to as "the atopic march"[10–14]. The term has been used to indicate that there is a connection between the development of first AD, then asthma and lastly allergic rhinitis[15,16]. We and others have, however, previously shown that the fraction of children with AD who follow this march (all three steps) is quite small[14,17,18], suggesting a more complicated interplay with distinct phenotypes rather than a progressive relationship. There is a general acceptance, that the diseases share a common ground with subsequent associations between them, for example, AD in early life and asthma later in life[19] or co-occurrence of asthma and allergic rhinitis[20,21]. Allergic rhinitis can also trigger an asthma exacerbation, but there is no evidence to suggest that the allergic rhinitis caused the asthma to begin with. A fundamental Th2-skewed immune system has previously been suggested to explain the associations between the atopic diseases[10,22], which entails an increased risk of both AD, asthma, and allergic rhinitis. Likewise, a genetic predisposition can also increase the risk of developing atopic diseases as shared

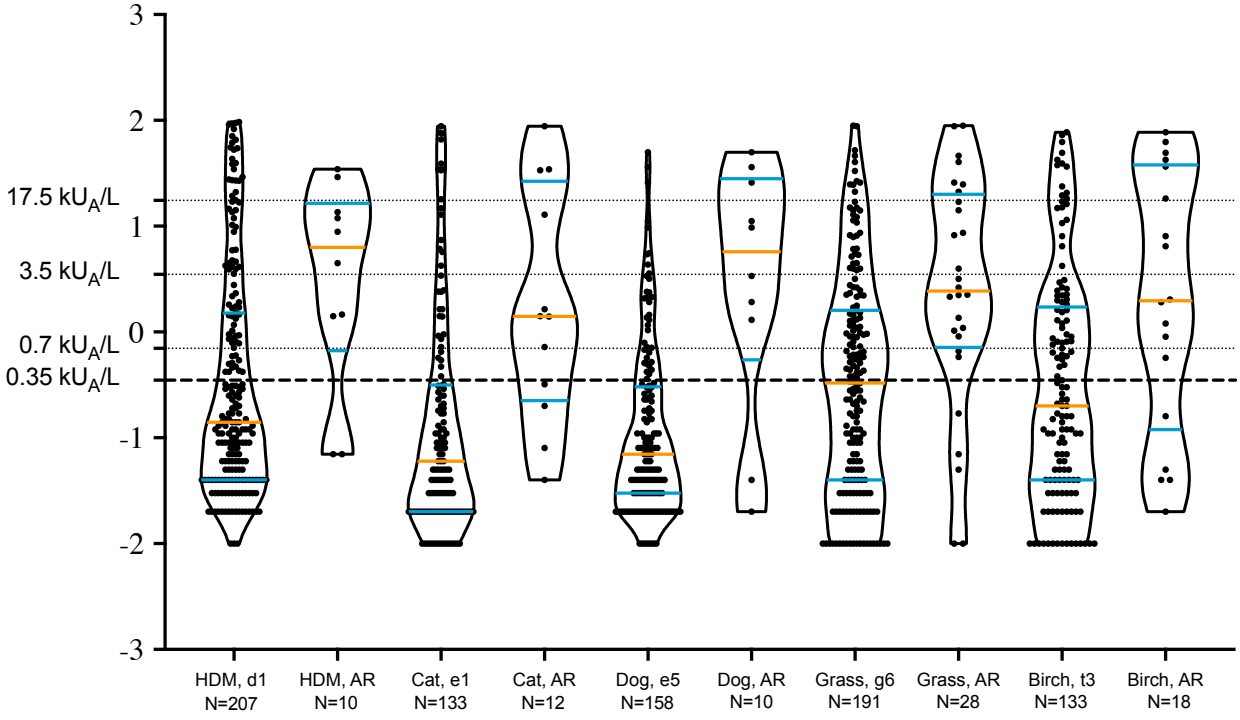
asthma, allergic rhinitis, and AD genetic variants have been identified. These variants lead to dysregulation of immune-related genes, indicating that atopic diseases may coincide because they share genetic risk loci[23]. Finally, the concept of united airways[24] argues that the upper and lower airways are connected so that any disease of the upper airway can affect the lower airway, explaining the co-existence or connection between asthma and allergic rhinitis. However, this concept is probably too simple as both diseases can be triggered by allergic and non-allergic mechanisms, and thereby present different phenotypes.

An atopic paradox

Asthma, AD, allergic rhinitis, and food allergy are often connected to the presence of specific IgE (sIgE), by referring to these diseases as "atopic". Obviously, allergic sensitization is a prerequisite for the development of allergic rhinitis and IgE-mediated food allergy, as it is part of the definition of these diseases, but the same is not true for asthma and AD. We have previously shown that the majority of preschool children (0-6 years) who suffer from either asthma or AD do not have concurrent allergic sensitization[17]. One would intuitively assume, that the definition of "atopic dermatitis" should include presence of sIgE as a mandatory criterion, however sensitization is only one out of 23 minor criteria in the Hanifin and Rajka definition of "atopic dermatitis"[25], and you can therefore easily fulfill the criteria for AD without being sensitized. The paradox of the label "atopic" is further underlined as the term is used when sensitization is present irrespective of allergen class (airborne and/or food) and test-method. Skin prick test (SPT) and sIgE have surprisingly poor agreement in children[26] and further, total IgE has poor agreement with current sIgE and is a very crude assessment of the risk of later sensitization[27,28]. Finally, the clinical relevance of the sensitization is completely disregarded, i.e., the allergen in question does not need to trigger either asthma or AD for it to be labelled "atopic", in fact it does not need to be of clinical relevance at all. Even very low levels of sIgE will deem someone atopic (sIgE ≥ 0.35 kU_A/L), and this cut-off is a paradox in itself, which was addressed in study **I**. The first commercial assay available to measure sIgE (Radio Allergo-Sorbent Test) had a detection limit of 0.35 kU_A/L and even though it was later replaced with a superior test (ImmunoCAP) with a detection limit of 0.1 kU_A/L, the cut-off remained the same. The poor concordance between sIgE and SPT may partially be explained by the uniform cut-off

value for all allergens – regardless of their allergen class or affinity. **Figure 3** illustrates the distribution of sIgE values to the most prevalent aeroallergens in 6-year-old children from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) cohorts. It is evident that the median value of sIgE varied among the different allergens, and among symptomatic children the levels were higher, but also with great variation. This calls for updated cut-offs of sIgE that are specific to each individual allergen.

Figure 3: Density plot showing the distribution of log10-transformed values of sIgE for all children with levels within the detection limit (0.1 ≤ sIgE ≤ 100 kU_A/L) and for the children with allergic rhinitis to the same allergen. The median values are marked with an orange line, the quartiles with blue lines, and the commonly used cutoff values for sIgE to grade sensitization are marked with dashed lines (modified from **I**).



HDM = house dust mite, *D. pteronyssinus*
AR = allergic rhinitis

Even though this issue with the sIgE cut-off has been well recognized among clinical specialists and researchers, a cut-off of 0.35 kU_A/L is still widely used in both settings. It is considered a biomarker for increased risk of concurrent/development of atopic disease, and while higher levels are stronger associated to a clinical reaction[29], it takes great knowledge about the normal distribution of IgE levels to interpret borderline values correctly.

The term “atopic disease” is so widely used in the literature and among clinicians, and even though it is technically imprecise in some cases (non-IgE mediated disease), it is used for simplicity throughout this thesis when referring to asthma, AD, allergic rhinitis, and food allergy.

Research challenges

As atopic diseases are becoming more and more prevalent, the seriousness of the impact on affected children’s lives has become more evident. Children with atopic diseases are less active physically[30], have lower sleep quality resulting in affected social activities during the day[31,32], more absence from school[33], impaired learning[34], and higher level of anxiety (especially children with food allergy)[35].

Pharmacological treatment of asthma is effective in controlling symptoms, and similarly, treatments exist to diminish symptom burden in AD, allergic rhinitis, and food allergy. However, once present, it is not possible to effectively alter the disease course or to cure the diseases entirely. In recent years, allergen immunotherapy[36–38] and biologics that target essential cytokines, cytokine receptors, and soluble or membrane-bound IgE[39,40] have shown promising results in treating patients with a predominant type-2 immune response. However, these treatments are very expensive, reserved for the most severe cases, and none are approved for children under 6 years, except the recently, and first ever, FDA-approved oral immunotherapy for food allergy that helps reduce the severity of allergic reactions to peanut in children aged 4-17 years[41]. Clearly, there is a high demand of prevention of atopic diseases, and despite intensive research within this field, the demand has not yet been met satisfactorily. Since evidence suggest that atopic diseases originate in early life, with possible gene-environment interactions already affecting disease development prenatally[42–44], studies of underlying pathophysiological mechanisms (endotypes) need to be conducted in early life with longitudinal follow-up to explore the natural course of the diseases. To improve prevention and treatment, in addition to endotyping, both protective and inducing factors in early life need to be identified to provide optimal personalized medical care.

Objective

The objective of this thesis is to explore mechanisms of atopic diseases and to investigate the importance of environmental exposures in early life, prior to disease development, to better understand the etiology of childhood atopy, and finally to explore new diagnostic tools to interpret allergic sensitization more accurately.

The thesis is built on 9 studies (**I-IX**) originating from the $COPSAC_{2000}$ and $COPSAC_{2010}$ birth cohorts investigating mechanisms of atopic disease and modifiable exposures in the environment that can affect the development of atopic diseases, and finally, examining how component resolved diagnostics (CRD) can help provide a patient-tailored approach to allergy diagnostics.

First, the distribution of sIgE levels in children was explored, as this is one of the main criteria for the definition of atopy, and the difference in distribution across the different allergens highlights the main issue in allergy diagnostics (**I**). Thereafter, it is explored how studies of parental atopic status (**II**), sensitization patterns (**III**) and early debut and severity of AD (**IV**) have substantiated the theory of an early-life window-of-opportunity that precedes the development of allergic sensitization and atopic diseases in childhood. Then, it is explored whether early-life exposures such as breastfeeding (**V**), dogs, cats, and house dust mites in the home during pregnancy and the first year of life (**VI**) constitute important influencers in this crucial time of life and suggest that these factors do not modify disease development in a significant way (neither as protective nor inducing factors). Finally, it is explored how CRD has a role in providing patient-tailored recommendations (**VII, VIII**) suggesting that some dog allergic patients tolerate female dogs. The utility of molecular diagnostics is also discussed in relation to other allergen sources and future directions are proposed (**IX**). Last, it is discussed how these findings could be enforced and validated via randomized controlled trials, which might prepare the ground for improved diagnostics and prevention strategies to counter the current atopic pandemic.

The COPSAC method

The birth cohorts

The $COPSAC_{2000}$ birth-cohort is a prospective study that consists of 411 at-risk mother-child pairs (all mothers with a physician-diagnosis of asthma) born between August 1998 and December 2001. The study and recruitment has previously been described in detail[45]. The children were enrolled at 4 weeks of age, excluding babies who received neonatal mechanical ventilation, with gestational age <36 weeks, lower airway symptoms at any time prior to inclusion, or severe congenital abnormality or systemic illness. The children were seen at scheduled clinical investigations at 6-monthly intervals till age 7 years and again at 12 and 18 years.

The $COPSAC_{2010}$ birth-cohort is a prospective study that consists of 700 non-selected mother-child pairs born between November 2008 and March 2011. The study and recruitment has previously been described in detail[46]. The mothers were enrolled at gestational age 22-26 weeks excluding mothers with gestational age above 26 weeks, daily intake of more than 600 IU vitamin D during pregnancy, or with any endocrine, heart, or kidney disorders. The parents attended the COPSAC research unit during pregnancy weeks 24 and 36. The children were seen at scheduled clinical investigations at 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months, and regularly thereafter till age 10 years (and ongoing). Randomized controlled trials of high-dose vitamin D and fish oil supplements were completed during pregnancy, and a trial of azithromycin for acute lung symptoms was conducted in the children with recurrent wheeze (NCT00798226 and NCT01233297 - ClinicalTrials.gov).

In both cohorts, acute visits were arranged upon occurrence of any respiratory- , skin- or allergy-related symptoms[47,48]. At each visit, the children were followed with comprehensive clinical investigations according to standard operating procedures, and clinical outcomes were diagnosed and monitored by the COPSAC physicians. The medical history was supported by daily diary cards filled from birth, capturing burden of troublesome lung symptoms between visits. Troublesome lung symptoms were defined as clinically significant cough, wheeze, or dyspnea explained to the parents as wheeze or whistling

sounds, breathlessness, or recurrent troublesome cough severely affecting the well-being of the child and recorded in the diary chart as a dichotomized daily score (yes/no)[49]. In $COPSAC_{2010}$ the parents also registered skin symptoms as active eczema and use of topical steroids. In addition, the diary cards monitored infections, fever, gastrointestinal infection, and absence from day care institution because of illness. The pediatricians employed at the COPSAC research unit, not the general practitioners, were the ones solely responsible for diagnosing and treating asthma, AD, and allergic rhinitis adherent to predefined validated algorithms[50].

Baseline characteristics of the participating children in both cohorts are outlined in **Table 1**. The two cohorts are remarkably similar except for a noteworthy reduction in mother's smoking and alcohol intake during pregnancy and a higher level of education in the most recent $COPSAC_{2010}$ cohort.

Measurements of sensitization

Specific IgE: In $COPSAC_{2000}$, sIgE levels were determined at ages 0.5, 1.5, 4, 6, 13, and 18 years using an initial screening method (ImmunoCAP, Phadiatop Infant™ and Phadiatop™, Thermo Fisher Scientific, Uppsala, Sweden)[51], followed by analysis of individual allergen sIgE levels in screening positive samples by ImmunoCAP. Sensitization was defined as values of sIgE ≥ 0.35 kU_A/L[52]. The individual allergens measured in $COPSAC_{2000}$ including both parents and children are outlined in **Table 2**.

In $COPSAC_{2010}$, sIgE levels were determined at ages 0.5, 1.5 and 6 years by using the same initial screening method. The 0.5-year blood sample was further analyzed using ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC) measuring 112 components from 51 different allergen sources. Levels ≥ 0.3 ISAC Standardized Units (ISU) were considered indicative of allergic sensitization. The 1.5-year and 6-year blood samples were analyzed for individual allergen sIgE levels in screening positive samples by ImmunoCAP. The individual allergens measured in $COPSAC_{2010}$ including both parents and children are outlined in **Table 3**.

Table 1: Baseline characteristics of the COSPAC birth cohorts (adapted from Bisgaard et al., 2004[45] and Bisgaard et al., 2013[46])

	$COPSAC_{2000}$	$COPSAC_{2010}$
Mothers enrolled, N	452	738
Babies enrolled, N	411	700
- Boys, %	49.4	51.4
- Twins, %	2	1
- Caucasian, %	97	96
Pregnancy and birth		
- Gestational age, mean (SD), weeks	39.9 (1.6)	39.9 (1.7)
- Birth weight, mean (SD), g	3517 (520)	3540 (555)
- Birth length, mean (SD), cm	52.3 (2.3)	51.9 (2.5)
- Mode of delivery, caesarean section, %	21	22
- APGAR score at 5 min, mean (SD)	9.8 (0.6)	9.9 (0.34)
- Mother's age at birth, mean (SD), years	30.0 (4.5)	32.3 (4.4)
- Father's age at birth, mean (SD), years	32.0 (5.2)	34.4 (5.6)
- Season of birth		
Winter, %	23	31
Spring, %	21	27
Summer, %	27	21
Fall, %	29	21
Exposures		
- Older children in the household, %		
0	64	43
1	24	38
2	9	15
>2	3	4
- Mother smoking during pregnancy, any, %	24	8
- Mother alcohol use during pregnancy, any, %	26	14
- Furred pets at home, any, %	30	37
- Duration of solely breastfeeding, mean (SD), days	113 (62)	105 (62)
- Age at start in daycare, mean (SD), days	349 (147)	
Socioeconomics		
- Household annual income		
- < 400.000 DKK, %	21	10
- 400.000-600.000 DKK, %	31	24
- 600.000-800.000 DKK %	30	29
- >800.000 DKK, %	17	37
- Mother with university education (> 3 years), %	13	28
- Father with university education (> 3 years), %	17	28
- Mother without occupation (unemployed or student), %	19	13
- Father without occupation (unemployed or student), %	7	8
Atopic predisposition (physician-diagnosed)		
- Mother with asthma, %	100	26
- Mother with allergic rhinitis, %	73	30
- Mother with atopic dermatitis, %	46	27
- Father with asthma, %	15	21
- Father with allergic rhinitis, %	30	27
- Father with atopic dermatitis, %	11	15
Genetics		
- Filaggrin mutation, %	13	11

Table 2: Overview of IgE and SPT measurements in the COPSAC$_{2000}$ cohort. Grey cross indicates that the allergen is part of a screening test but has not been specified on an individual allergen level.

Allergens	0.5 year		1.5 years		4 years		6 years		13 years		18 years		Parents
	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE	IgE
Foods													
Egg white (f1)	X	X	X	X	X	X	X	X	X	X		X	
Pasteurized egg	X		X		X		X		X		X		
Milk (f2)	X	X	X	X	X	X	X	X	X	X		X	
Raw milk	X		X		X		X		X		X		
Cod (f3)	X	X	X	X	X	X	X	X	X	X	X		
Wheat flour (f4)	X	X	X	X	X	X	X	X	X	X	X	X	
Rye flour (f5)	X	X	X	X	X		X		X		X		
Oatmeal (f7)	X	X	X	X					X				
Peanut (f13)	X	X	X	X	X	X	X	X	X	X	X	X	
Soybean (f14)	X	X	X	X	X	X	X	X	X	X	X		
Hazelnut (f17)										X		X	
Brazil nut (f18)										X			
Almond (f20)										X		X	
Shrimp (f24)		X		X		X		X					
Pork (f26)	X	X	X	X	X		X		X		X		
Beef (f27)	X	X	X	X	X		X						
Potato (f35)	X	X	X	X									
Coconut (f36)										X			
Grain mix (fx3)^		X		X									
Meat mix (fx23)[$]		X		X									
Airborne													
D. pteronyssinus (d1)	X	X	X	X	X	X	X	X	X	X	X	X	X
D. farinae (d2)	X	X	X	X	X	X	X	X	X	X	X		
Cat dander (e1)	X	X	X	X	X	X	X	X	X	X	X	X	X
Horse dander (e3)	X	X	X	X	X	X	X	X	X	X	X		X
Dog dander (e5)	X	X	X	X	X	X	X	X	X	X	X	X	X
Timothy grass (g6)	X	X	X	X	X	X	X	X	X	X	X	X	X
Common silver birch (t3)	X	X	X	X	X	X	X	X	X	X	X	X	X
Mugwort (w6)	X	X	X	X	X	X	X	X	X	X	X	X	X
Molds* (mx1)		X		X		X		X		X			
Penicillium notatum (m1)		X		X		X		X		X			
Cladosporium herbarum (m2)	X	X	X	X	X	X	X	X	X	X	X		X
Aspergillus fumigatus (m3)		X		X		X		X		X			
Alternaria alternata (m6)	X	X	X	X	X	X	X	X	X	X	X	X	
Total IgE		X		X		X		X		X		X	X

^ fx3 = f4, f7, f8 (corn), f10 (sesame seed) and f11 (buckwheat)
[§] fx23 = f26, f27, f83 (chicken) and f284 (turkey)
* mx1 = m1, m2, m3 and m6

Table 3: Overview of IgE and SPT measurements in the COPSAC$_{2010}$ cohort. Grey cross indicates that the allergen is part of a screening test but has not been specified on an individual allergen level. The ISAC has been crossed if components from the allergen source have been measured (more allergens have been measured, but not listed here).

Allergens	**0.5 year**		**1.5 years**		**6 years**		**Parents**
	SPT	ISAC	SPT	IgE	SPT	sIgE	IgE
Foods							
Egg white (f1)		X		X		X	
Pasteurized egg	X		X		X		
Milk (f2)		X		X		X	
Raw milk	X		X		X		
Cod (f3)		X			X	X	
Wheat flour (f4)		X			X	X	
Rye flour (f5)					X		
Peanut (f13)		X			X	X	
Soybean (f14)		X			X		
Shrimp (f24)		X				X	
Pork (f26)					X		
Blue mussel (f37)						X	
Tuna (f40)						X	
Salmon (f41)						X	
Airborne							
D. pteronyssinus (d1)		X			X	X	X
D. farinae (d2)		X			X		
Cat epithelium and dander (e1)	X	X	X	X	X	X	X
Horse dander (e3)		X			X	X	
Dog dander (e5)	X	X	X	X	X	X	X
Timothy grass (g6)		X			X	X	X
Common silver birch (t3)		X			X	X	X
Mugwort (w6)		X			X	X	X
Molds* (mx1)						X	X
Penicillium notatum (m1)						X	X
Cladosporium herbarum (m2)		X			X	X	X
Aspergillus fumigatus (m3)		X				X	X
Alternaria alternata (m6)		X			X	X	X
Total IgE		X					X

* mx1=m1, m2, m3 and m6

Total IgE levels were determined using ImmunoCAP. Measurements in both children and parents are outlined in **Table 2** and **3**. Total IgE levels were analyzed in one study (**II**) in this thesis (measurements from COPSAC$_{2010}$ at 0.5 year) using a cut-off of 50 kU/L. The cut-off for elevated total IgE in the parents was 150 kU/L[53].

Skin prick tests were performed using standard allergen extracts (ALK Abelló, Hørsholm, Denmark) at the same ages as sIgE measurements, and mostly matching the allergens measured by sIgE (**Table 2 and 3**). Histamine dihydrochloride (10mg/mL) and physiological sodium chloride (9mg/mL) were used as positive and negative controls, respectively. A drop of each of the extracts was placed on the children's volar forearm and

a lancet was used to prick through the droplet. The reaction to the positive control was read after 10 min while the reactions to the allergens and negative control were read after 15 minutes. The average of the wheal diameter and its perpendicular was noted and the test was considered positive if the wheal size was ≥ 2 mm larger than the negative control at ages ½ and 1½ years and ≥ 3 mm at ages 4, 6, 13 and 18 years[54].
Antihistamines were not allowed 72 hours before testing (the positive control was used as screening for use of antihistamines with longer half-life), mild steroid creams (group 1-2) on the arms 24h before testing, or stronger steroid creams (group 3-4) 14 days before testing. The abundance of positive tests in the two cohorts is illustrated in **Figure 4**.

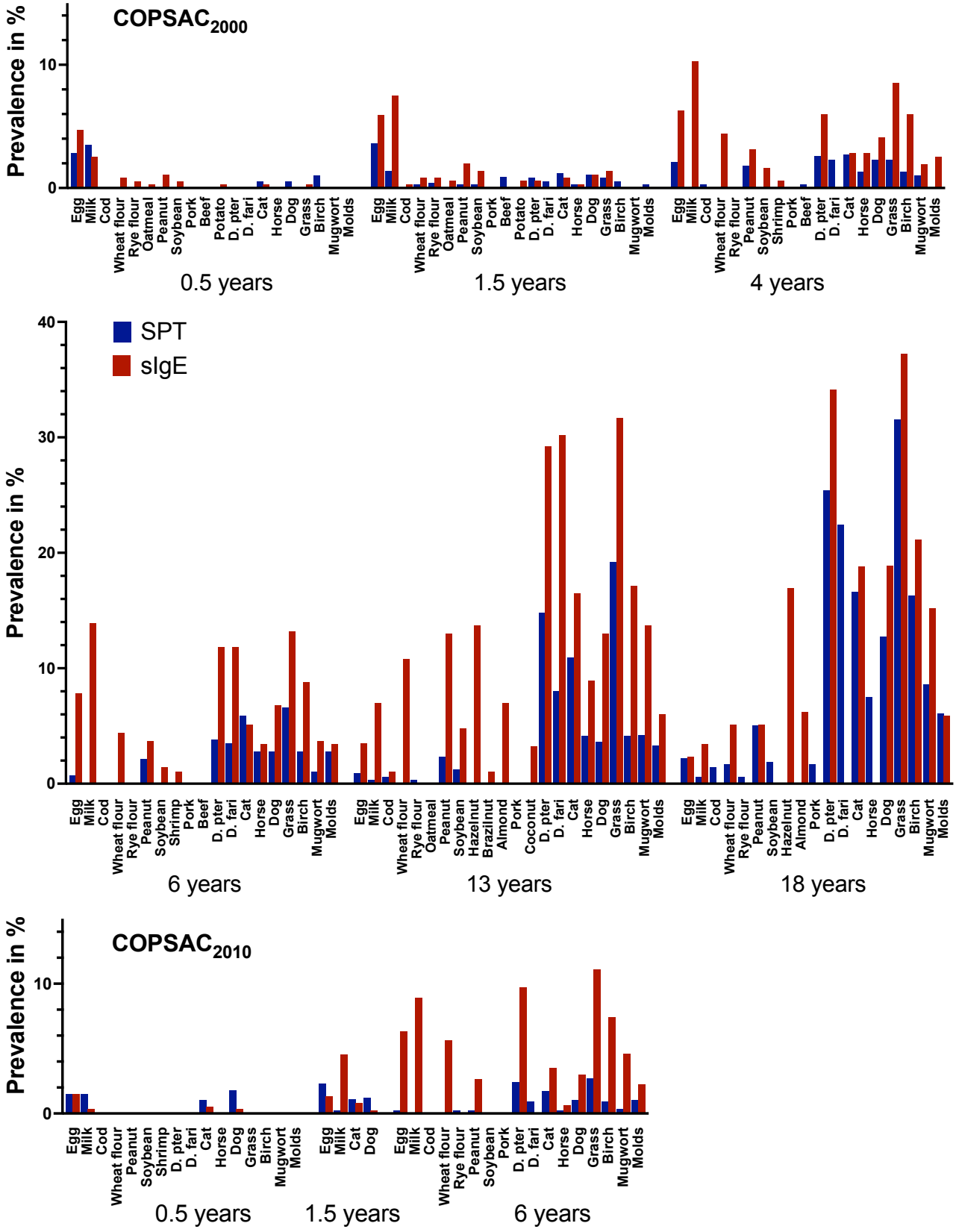
Influencers in early life

Breastfeeding was determined based on information collected prospectively by interviewing the mothers at the 1-, 6- and 12-month clinical visits on the duration of exclusive and total breastfeeding and the use of infant formula. At the time the child's diet was supplemented by anything other than breastfeeding or short periods of supplementation with formula, we considered exclusive breastfeeding as terminated. Children receiving supplementation at the maternity ward with hypoallergenic formula at for more than 7 days prior to establishing breastfeeding were considered as never having been fully breastfed[55].

Dog or cat exposure during pregnancy was determined by parental interviews at the first visit to the clinic and defined as dog or cat living in the house at any time during the 3rd trimester[56].

Dog or cat exposure in early life was determined by parental interview at the 1-year visit to the clinic and defined as dog or cat living in the house at any time during the child's first year of life[56].

Figure 4: The abundance of positive SPT (blue) and sIgE (red) in $COPSAC_{2000}$ and $COPSAC_{2010}$ through childhood



Airborne allergen levels in dust were measured in $COPSAC_{2000}$ in dust samples collected from the child's bed at age 1 year. The parents vacuumed the bedding (the pillow and mattress) for 5 min with a dust collector equipped with a Millipore filter (ALK, Copenhagen, Denmark). The dust samples were stored at -18°C for 3 days to kill possible house dust mites before sending it to the lab at Occupational and Environmental Medicine, Uppsala University Hospital, Sweden. House dust mites (*D. pteronyssinus* and *D. farinae*), cat, and dog allergen concentrations were measured using the Sandwich ELISA methodology[57] using reagents from Indoor Biotechnologies (Charlottesville, USA) expressing the allergen load in ng/g dust[56].

Clinical outcomes

Asthma was diagnosed based on predefined internationally recognized guidelines[47,58] including 4 mandatory criteria: 1) Diary-verified recurrent troublesome lung symptoms (referred to as "recurrent wheeze" in **V**) defined as 5 episodes of minimum 3 consecutive days within 6 months or 4 consecutive weeks with symptoms; 2) Symptoms judged by the COPSAC pediatricians to be typical of asthma (e.g. exercise induced symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakening at night); 3) Intermittent need of rescue inhaled β2-agonist; and 4) Response to a 3-month trial of inhaled corticosteroids initiated when the criteria for recurrent troublesome lung symptoms were met and relapse after cessation.

Allergic rhinitis was diagnosed based on parental interviews (not questionnaires) on history of symptoms performed at the COPSAC research unit. Allergic rhinitis was defined as bothersome and reoccurring sneezing, blocked, itchy or runny nose severely affecting the wellbeing of the child in the past 12 months in periods without accompanying common cold or flu, and congruence between symptoms, relevant exposure, and positive SPT and/or sIgE[50,59–61].

Atopic dermatitis was diagnosed at scheduled and acute care visits according to Hanifin and Rajka's criteria[62] capturing age of debut and age of remission as previously detailed[63,64]. A diagnosis of AD required the presence of 3 of 4 major criteria and at least 3 of 23 minor signs. The major criteria were 1) pruritus; 2) typical morphologic features and distribution; 3) chronic dermatitis; and 4) atopic history. The following 4 minor signs were

excluded: keratoconus, anterior sub-capsular cataracts, delayed blanch, and impaired cell-mediated immunity. The severity of AD was scored using the SCORing Atopic Dermatitis (SCORAD) index at scheduled and acute care visits[65], ranging from 0 to 83 points (excluding the subjective components of pruritus and sleeplessness from the modified SCORAD index).

Conjunctival allergen provocation was done according to recommended guidelines[66]. Initially, we applied a droplet of extract with a concentration of 0.25 µg/mL, and every 15 min increased the concentration until a final concentration of 25 mg/mL. Droplets were applied in the inferior-external quadrant of the bulbar conjunctiva. Before application of the allergen, one eye was used as control and installed with one drop of physiological sodium chloride (9mg/mL)[67]. A positive response was assessed according to the Total Ocular Symptom Score (TOSS)[66], evaluating itchiness (0-4), redness (0-3), and tearing (0-3) of the eye, but not evaluating chemosis, as this requires a slit-lamp examination. A total score of 4 was considered a positive response. The test was stopped when a positive response occurred or until the maximum dose was reached[67].
The children were instructed not to use antihistamines in any form within 72 hours of testing as well as systemic, nasal, or conjunctival corticosteroid 2 weeks before testing.

Inheritance

Parents' history of doctor-diagnosed asthma, allergic rhinitis, and AD was determined via a structured clinical interview with closed-response categories performed by the research physicians at the family's first visit to the COPSAC research unit[68].

IgE levels; both sIgE against the most common airborne allergens and total IgE levels were measured in both parents upon inclusion. The individual allergens are listed in **Table 2** and **Table 3**.

Filaggrin mutation in the children was determined based on genotyping for common loss-of-function mutations in Filaggrin (*FLG*) genes R501X, 2282del4, R2447X and S3247X as previously described[69]. A *FLG* mutation carrier was defined as having at least one gene mutation[70].

Mechanisms

Parental effects

Previous studies have indicated that atopic diseases are highly heritable traits and that the individual variation in the susceptibility to the disease can be attributed both to genetic risk variants and changing environmental exposures[63,71,72]. Intuitively, it would be anticipated, that both parents contribute evenly to heritability. Nevertheless, we have previously shown that atopic history of mothers, but not fathers, was associated with a repressed cytokine and chemokine signature measured in the airways of their healthy children at one month of age[73], suggesting an unequal effect of mother's and father's heritability. Likewise, a meta-analysis has shown a higher risk of childhood asthma in children born to mothers with asthma compared to fathers[74], and studies of other non-communicable diseases such as inflammatory bowel disease[75] and diabetes[76] also suggest a stronger maternal effect. Discussing this effect is interesting, as it may contribute to the understanding of atopic disease pathogenesis. The relatively higher risk that mother's disease could confer to the child than father's, would imply that a maternal-fetal interaction that occurs pre- or postnatally appears to affect the risk of atopic diseases in the child – an effect that is not tied to genetics and therefore prevention becomes possible. Moreover, this opens new possibilities for investigations into the mechanisms of disease predisposition. Previous studies on heritability of sensitization and asthma have typically focused on development of the outcome at one single time-point in childhood[77–79]. It would be plausible that the effect of the parents' sensitization and asthma not only differs, but also changes during early life. No longitudinal study has investigated this before. We aimed to address this gap in knowledge in our study of 685 parent-child trios participating in the $COPSAC_{2010}$ birth cohort investigating the parent-specific associations between a history of elevated sIgE, total IgE, and asthma in the parents and development of the same outcomes in the children during childhood (**II**). The study showed a stronger maternal effect of elevated total IgE, elevated sIgE, and asthma compared to paternal effect when analyzing appearance of the same traits in their children from 0-6 years using a Generalized Estimating Equations (GEE) model, with no noteworthy added effect when looking and mother's and father's effect combined (**Table 4**).

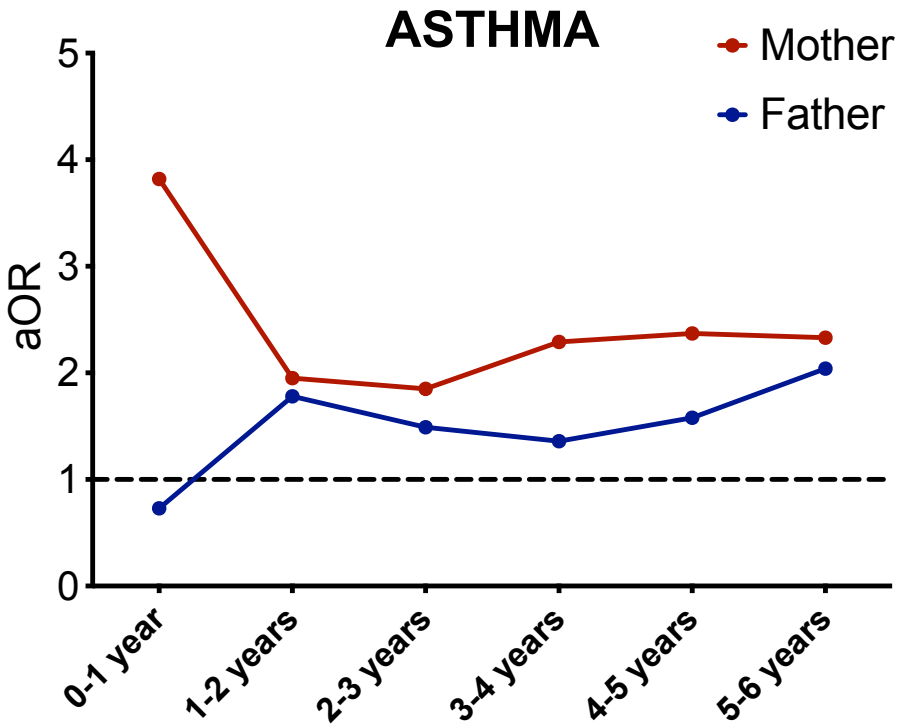
Table 4: Effect of mother and father's history of elevated total IgE, sIgE and asthma on development of corresponding conditions in early childhood (modified from **II**)

Outcome	Mother's effect		Father's effect		Mother's + father's effect	
	OR [CI]; p value	aOR^ [CI]; p value	OR [CI]; p value	aOR^ [CI]; p value	OR [CI]; p value	aOR^ [CI]; p value
Total IgE 6 months*	**4.31 [1.51;10.8]; <0.01**	**4.32 [1.51;10.8]; <0.01**	2.05 [0.78;4.85]; 0.12	2.01 [0.76;4.82]; 0.13	NA***	NA***
Specific IgE 0-6 years**	**1.47 [1.08;2.00]; 0.02**	**1.49 [1.09;2.03]; 0.01**	1.30 [0.94;1.77]; 0.11	1.32 [0.96;1.82]; 0.08	**1.52 [1.02;2.26]; 0.04**	**1.54 [1.03;2.29]; 0.04**
Asthma 0-6 years**	**2.10 [1.45;3.02]; <0.0001**	**2.11 [1.46;3.05]; <0.0001**	**1.56 [1.04;2.34]; 0.03**	**1.55 [1.03;2.33]; 0.04**	1.71 [0.85;3.45]; 0.13	1.69 [0.84;3.39]; 0.15

^ Adjusted for days of exclusive breastfeeding and maternal smoking during 3rd trimester
* Logistic regression calculating OR
** General Estimating Equations model calculating OR
*** Analysis could not be done as we only had 4 children where both parents had elevated total IgE.

When investigating the individual time-points, we also found a consistent pattern that, especially early in childhood, mother's elevated total IgE, specific IgE, and asthma were associated with the same traits in the children. Contrary, father's disease traits were only associated with an increased risk later in childhood (asthma results shown in **Figure 5**).

Figure 5: Effect of mother and father's history of asthma on the yearly risk of the child developing asthma till age 6 years, adapted from (**II**). Results are adjusted for duration of exclusive breastfeeding and maternal smoking during 3rd trimester.



Among the very few other published reports on parent-specific heritability of elevated sIgE[77–79] comparable results have been shown, however none have assessed repeated outcomes longitudinally through childhood. One study found that only elevated sIgE in the mothers imposed an increased risk of elevated sIgE in their children at 4 years of age[77], another found an equal effect from both parents in their children at age 7 years[78], and lastly, one study found that father's history of elevated sIgE imposed a higher risk than mother's history in early adulthood at a mean age of 18.4 years [79]. Combined, these studies align with our conclusions of a stronger maternal effect in early childhood and a paternal effect that increases with age.

The parental effects of elevated total IgE have also only scarcely been examined in previous studies and the results align with the pattern we found for sIgE; father's effect of total IgE was lacking in early childhood [53] and became noticeable later in childhood[77] while mother's effect was evident throughout. Unfortunately, total IgE was only measured at 6 months of age in $COPSAC_{2010}$, but our results align with the common pattern of mother's history imposing and increased risk in early childhood, and father's history imposing no risk until later in childhood.

Heritability of asthma has been investigated more thoroughly. A meta-analysis[74] gathering data from 33 parent-specific asthma studies, showed that mother's asthma imposed an increased risk of developing asthma in their children (summary OR 3.04, [2.59; 3.56]) more so than father's asthma (summary OR 2.44 [2.14; 2.79], p for comparison=0.037). Interestingly, the study found that the induced risk of both maternal and paternal history of asthma increased with the age of the child. These findings support our analyses of asthma prevalence annually (**Figure 5**), where we observed an increase in the parent-specific effect from 2-4 years of age until age 6, but a consistently stronger effect of mother's asthma. At 1 year, we found a much stronger effect of mother's asthma, and this has not been investigated previously, possibly due to the difficulty diagnosing asthma at this age.

We demonstrated a consistently stronger effect of maternal compared to paternal asthma and sensitization on the same outcomes in early life of their children. This implies that factors, other than genetics, affecting the child perinatally or *in utero* have an important role in the conduction of disease predisposition. The mechanism of these factors' influence on disease development is poorly understood. One possible explanation is placental

transport of maternal “atopic” immune molecules and thereby altering the child’s immune response. This interplay between mother and fetus’ immune system is complicated, but it has been implied that increased levels of proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α in the pregnant mother may increase the risk of autoimmune and atopic diseases in the unborn child[80]. Another mechanism could be transmission of a “pro-atopic” microbiome between mother and child during birth, breastfeeding, or other early interactions. Finally, there is an increasing focus on epigenetic changes explaining disease development including genomic imprinting, i.e., the difference in expression of homologous inherited “atopic susceptibility genes” depending on their parent-of-origin. Epigenetic changes could also explain the effect of environmental exposures on disease susceptibility[81]. In another context, we recently investigated associations between maturation of gut microbiome in early childhood and later development of allergic sensitization and asthma. We found strong associations, but only in the children where mothers had asthma and not in the ones where fathers had asthma, indicating a greater maternal effect of the vulnerability to the early-life exposome[82].

The stronger mother-specific effect on outcomes has also been described in relation to other non-communicable diseases such as type 2 diabetes[76], inflammatory bowel disease[75], rheumatoid arthritis[83] and coronary heart disease[84]. These differences in parent-specific effects, with a stronger maternal influence, support the hypothesis of a maternal intrauterine non-genetic impact on disease susceptibility.

These findings underline the possible impact of preventative advise directed at the pregnant mother (e.g. lifestyle factors, dietary supplements, avoidance of certain drugs and smoking), as the maternal-fetal interaction appears to affect the risk of atopic diseases in the child – an effect that is not tied to genetics and most prominent early in childhood.

Sensitization patterns and asthma

Once a child has been exposed to unfavorable genetics and exposome, allergen specific IgE molecules may develop, but the implication of their presence is not clear. Will the child get symptoms of food- or airborne allergy, and what about asthma and AD? We have previously found (and replicated) 7 sensitization-patterns in children from 0.5 to 6 years of age by applying an unsupervised machine learning model that clustered our sensitization

data into different patterns. The only pattern associated to asthma was the one that included sensitization to animals (dog, cat, horse). The asthma diagnosis in our cohorts was purely symptom-based and did not include allergic sensitization[85]. Almost all the sensitization patters (except the house dust mite pattern) were associated with AD. But what if the presence of IgE was just transient – does that convey the same risk for clinical disease as persistent sensitization? Does it matter if the body has made sIgE to several allergen sources or just a single one, or if the amount of IgE is very high vs. low? Some of these questions are addressed in study **III** where we analyzed measurements of sIgE and SPT longitudinally at ages 0.5, 1.5, 4, 6, and 13 years in 399 children from the $COPSAC_{2000}$ cohort. We aimed to investigate the following sensitization patterns:

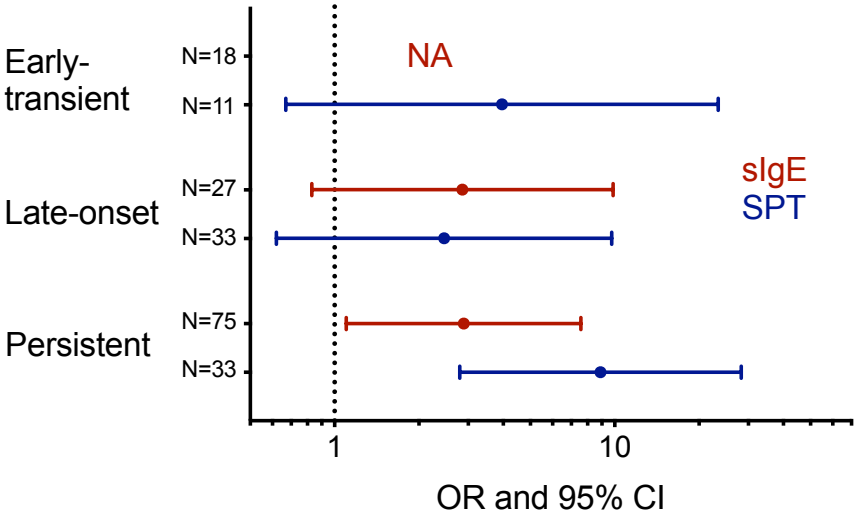
- Monosensitization = sensitization to only one allergen, single time-points
- Polysensitization = sensitization to 2 or more allergens, single time-points
- Degree of sensitization = sum of sIgE levels or SPT wheal sizes, single time-points
- Early-transient sensitization = sensitized at 0.5 years and/or 1.5 years and/or 4 years and/or 6 years, but not sensitized at 13 years
- Late-onset sensitization = sensitized at 13 years, but not earlier
- Persistent sensitization = both early sensitization and sensitization at 13 years of age

We wanted to analyze these patterns' association with asthma at age 13 and found that polysensitization and higher levels of sensitization at all time-points were associated with increased risk of asthma at age 13 (using either SPT or sIgE). Further, persistent sensitization was associated with asthma at are 13, but not late-onset or early-transient sensitization (**Figure 6**).

Our findings on polysensitization align with most recent studies on association with asthma[86–89], except one study that found no added risk of polysensitization in the association between sensitization at 1 year of age (sIgE to aero- or food allergen) and asthma at 6 years[90]. Even though our study agrees with most previous studies on polysensitization, comparing analyses that involve sensitization can be confusing as most studies do not distinguish between sIgE and SPT when defining sensitization. This may lead to diverging conclusions, as sIgE and SPT have poor agreement, especially in early childhood[54]. Further, the definition of asthma varies greatly among studies, and in general the COPSAC studies have a quite strict definition of asthma compared to other studies. Finally, the studies mentioned only included measurements of sensitization at one time-

point, and none had longitudinal measurements both SPT and sIgE. When investigating SPT monosensitization, distinguishing between allergen types, i.e. food- or aeroallergen, appeared to impact the results as monosensitization to foods during childhood was more consistently associated to asthma at 13 years than monosensitization to aeroallergens.

Figure 6: Association between temporal sensitization patterns and asthma at 13 years of age (adapted from **III**).



None of the children classified as early transient sIgE had asthma at 13 years, and the OR could therefore not be calculated. Sensitization SPT N/N_{total} 77/171. Sensitization sIgE N/N_{total} 120/215.

We observed a consistent association between polysensitization at all time-points and asthma at 13 years. Mechanistically it can be due to a high exposure to allergens leading to a state of "molecular spreading" where an excessive IgE response to one epitope can lead to the production of more IgE binding to other epitopes on the same or other allergens[91]. This phenomenon has previously been studied using CRD where sensitization to several allergen components, representing molecular spreading, was associated with increased risk of atopic disease[91], and further that polysensitization to the family of proteins called lipocalins (animal-derived) was associated with asthma severity[92].

For our analyses of mono- and polysensitization we used the low sIgE cut-off of 0.35 kU_A/L, as we were interested in using sensitization as a biomarker of predisposition to atopic disease and not necessarily current disease. However, we also investigated

quantitative sensitization score and risk of asthma to explore the meaning of bigger SPT wheal sizes and higher sIgE levels with regards to asthma development. This association was significant at all ages which proves a dose-response relationship supporting a true association between “severe forms” of sensitization (i.e., high levels of sIgE, big SPT wheal sizes, persistent sensitization, and polysensitization) and asthma. Further, these quantitative assessments of SPT and sIgE results and association to asthma was found at all in both mono- and polysensitized children at all time-points, which emphasizes the importance of considering the actual size of SPT/level of IgE when assessing the clinical impact of a positive test.

Our investigation of temporal sensitization patterns through childhood showed that children with persistent sensitization (both sIgE and SPT), but not early-transient or late-onset sensitization, were at increased risk of asthma at age 13 (**Figure 5**). Other studies have found similar results[93,94], however it is important to keep in mind that every study defines persistent sensitization differently. Our findings highlight the clinical importance of repeated measurements of sensitization when assessing if a child has increased risk of developing asthma.
Previous studies support that late-onset sensitization alone is not associated with asthma[89,93–95]. We have, however, previously found an association between any current sensitization (sIgE and/or SPT) and asthma at 13 years[17], which seems to be in contrast to our findings in **III** where late-onset sensitization (debut at 13 years) was not associated with current asthma. This implies that the group of children with persistent sensitization is primarily responsible for the previous result. It is noteworthy, however, that we in study **III** found a borderline significant association between late-onset sIgE sensitization and asthma at 13 years (p=0.10), and the result could be subject to a Type II error masking a true association as only 27 children had late-onset sensitization.

The sparse or lacking risk that early-transient and late-onset sensitization as well as monosensitization at single time-points constitute in relation to later development of asthma may indicate a generally healthier, constricted immune response when exposed to allergens and a consequent phenotype with a low asthma risk[96]. Accordingly, the children with persistent sensitization, large SPT wheal sizes and high levels of sIgE, and

polysensitization at single time-points, could signify a phenotype with a more aggressive and unhealthy immune response when exposed to allergens and subsequently an increased risk of asthma.

Atopic dermatitis and allergic sensitization – the hen or the egg

As previously discussed, allergic sensitization is part of the definition of AD, although as a minor criterion, and many studies have shown, that there is a higher prevalence of allergic sensitization among children with AD[17,97,98]. Many clinicians will also confirm that some patient's AD will get worse upon intake of certain allergenic foods such as egg, milk, and peanut[99–101] or when exposed to aeroallergens such as house dust mite[102]. Further, we have previously shown that cat ownership increases the risk of AD within the first year of life in children with *FLG* mutation[103]. So, does that conclude that *a) allergen exposure is causing AD*? On the other hand, many recent studies have spurred the hypothesis that early exposure to allergens through a defect skin barrier, before a consistent oral exposure, is a mechanism for inducing sensitization and development of atopic diseases[38,104–106] – most studies focusing on sensitization to food allergens. That supposedly concludes that *b) AD is causing allergic sensitization* and subsequent food allergy. This delayed oral exposure contrasts an early, continuous exposure, which may lead to development of immune tolerance and reduced risk of food allergy[38,105,106]. Theory *b)* has led the European Academy of Allergy and Clinical Immunology to publish updated guidelines recommending early introduction of egg, peanut, and milk (the latter after 1 week of age as cow's milk formula, if not fully breastfed) in at-risk children, e.g. children with AD[107]. A third correlation between AD and allergic sensitization is that *c) the conditions are part of a common origin with no causal effect between them*. This connection between AD and allergic sensitization is one we seek to elucidate in study **IV**. It is highly relevant as AD is the most common inflammatory skin disorder in children with a 4-6 fold increase in cumulative incidence since the 1960s to a reported prevalence of 15-18% in 1990s and still increasing[108,109]. AD is characterized by a defective skin barrier caused by an anomalous stratum corneum both in skin affected by AD and in nonaffected skin[110,111]. Most recent studies on the connection between AD and allergic sensitization have focused on this barrier dysfunction in the skin of children with AD as a part of the explanation[112,113], particularly to food allergens and later development of food

allergy[38,105,106]. A previous literature review found that up to 2/3 of AD patients will later develop allergic rhinitis[10]. This link could be explained by the development of sIgE to aeroallergens in patients with a defective skin barrier function[113]. If this theory is to be endorsed, one would assume that severe, early-onset AD compared to late-onset, less severe AD could increase the risk of later sIgE sensitization to aeroallergens and allergic rhinitis. However, this association has not previously been explored. In study **IV** we therefore investigated whether early-onset vs. late-onset AD including AD severity were associated with aeroallergen sensitization and allergic rhinitis at 6-7 and 12 years of age in the $COPSAC_{2000}$ cohort. We found that increasing severity of early-onset AD increased the risk of aeroallergen sensitization and allergic rhinitis at 6-7 and 12 years, while onset of AD after age 1 year did not (**Table 5**).

Table 5: Associations between early-onset AD (≤ 1 year) and late-onset AD (> 1 year) and allergic outcomes at 6/7-12 years. AD is either defined as a dichotomous variable (yes/no) or a continuous variable expressed as a severity score (SCORAD) for AD. The GEE model computes the overall OR using compiled data from both time points (6/7 and 12 years), adapted from **IV**.

	GEE§ 6-12 yrs, sIgE*				GEE§ 7-12 yrs, allergic rhinitis			
Debut	N^	OR	95% CI	p-value	N^	OR	95% CI	p-value
				AD yes/no				
≤ 1yr	78	1.68	[1.08; 2.62]	0.02	81	1.56	[1.01; 2.41]	0.04
> 1yr	41	1.65	[0.92; 2.94]	0.08	47	1.76	[1.00; 3.10]	0.05
				SCORAD				
≤ 1yr	67	1.08	[1.03; 1.12]	<0.001	69	1.09	[1.05; 1.13]	<0.001
> 1yr	54	1.01	[0.97; 1.06]	0.55	61	1.00	[0.96; 1.04]	0.90

^ Number of children with variable present and evaluated outcome
§ Logistic regression General Estimating Equations.
* sIgE ≥ 0.35 kUA/L to any aeroallergen
^ SPT ≥ 3 mm to any aeroallergen

Early-onset AD alone did not impose a significantly increased risk of allergic outcomes compared to late-onset ($p\text{-interaction}_{IgE}=0.93$, $p\text{-interaction}_{allergic\ rhinitis}=0.89$), but when considering the AD severity (SCORAD), early-onset AD imposed a significantly increased risk of aeroallergen sensitization (p-interaction=0.03) and allergic rhinitis (p-interaction<0.01). When stratifying for *FLG* mutation, we found that the children with *FLG* mutation had a trend of higher risk (early vs. late) of developing allergic rhinitis at 7 and 12

years with increasing severity compared to the children without a *FLG* mutation (p-interaction 0.06 and 0.055, respectively).

Previous studies have shown varying results, however with most studies showing higher frequencies of allergic sensitization and atopic disease outcomes in early-onset vs. late-onset AD[114,115], but there have also been reports of no difference[116] and higher risk with only persistent early-onset AD[117,118]. Furthermore, one study also showed that the children with late-onset AD had increased risk of aeroallergen sensitization at 18 years[118]. None of these studies assessed AD severity, which, according to our study, is a very important factor in these associations. Also, clinical outcomes were assessed at different ages (4 to 18 years) and the diagnoses were mainly based on questionnaires. To our knowledge, no previous studies have included *FLG* mutations in their assessments of this relation.

Our study supports theory *b) AD is causing allergic sensitization* and subsequent allergic rhinitis. The mechanistic justification of this theory is centering around the skin barrier defect in AD[119]. The epidermal barrier constitutes a primary defense against the surrounding milieu and when allergens are exposed to and handled by epidermal antigen-presenting Langerhans cells, they travel to the draining lymph nodes. Here, they can inter-act with naïve T cells and initiate a Th2 dominated immunological response that causes allergies[120]. This exposure of allergens through the skin is increased in AD due to the defect barrier, and it has further been implied that the response causes dermal Th2 memory cells to travel to the lymphoid tissue in the bronchi, where re-exposure to the allergen through inhalation can cause an immunological reaction in the airways, causing asthmatic symptoms and allergic rhinitis[10]. Another explanation supporting theory *b)* is that chronic lesions of AD may express proinflammatory mediators such as thymic stromal lymphopoietin, which increase the risk of allergic sensitization and inflammation in the lungs[121].

Theory *c) the conditions simply are part of a common origin with no causal effect between them* can be explained by a fundamental Th2-skewed immune system[10,22] leading to an increased risk of atopic diseases (including allergic sensitization). Additionally, shared genetic risk variants between asthma, allergic rhinitis, and AD have been identified that result in dysregulation of immune-related genes, indicating that these disorders may coincide because they share genetic risk loci[23]. Nonetheless, these theories do not explain

the role that severity of AD represent in the connection between AD and later development of aeroallergen sensitization and allergic rhinitis.

Theory *a) allergic sensitization is causing AD* has flourished because it has been observed that food allergens can act as a trigger to induce and maintain clinical manifestations of AD and specific dietary eliminations in children with allergic sensitization can significantly reduce the disease activity[99,101]. The same is also known for asthma and aeroallergens[122–124]. Most evidence does not suggest allergic sensitization as a cause of AD or asthma, but merely that the allergens serve as a trigger of disease. However, we did previously show that cat ownership increases the risk of AD within the first year of life in children with *FLG* mutation[103], suggesting a causal relationship in this subgroup of children.

Study **IV** supported theory *b)* indicating that early-onset vs. late-onset AD debut characterize distinctive endotypes. Of course, theory *a)* and *c)* are also relevant. Genetics is a big factor in both development of AD and allergic sensitization[23], and surely there will be triggers of AD in a subset of sensitized children, so all 3 theories are probably valid. Indeed it is important to remember, that there is also a large amount of children with allergic sensitization but without AD and vice versa[17].

Ealy-life exposures

Many exposures in early life have been discussed as inducing or protective of allergy in at-risk children (typically children with a family history of atopy). The Danish Health Authorities have made recommendations on exclusive breastfeeding (previously recommended duration was 6 months in at-risk children) and pets in the home (previously recommended to avoid in at-risk children). In study **V**-**VI** we challenged these recommendations and investigated associations between breastfeeding (**V**), dog, cat, and house dust mites in the home perinatally (**VI**) and development of allergic sensitization and atopic diseases in childhood.

Breastfeeding

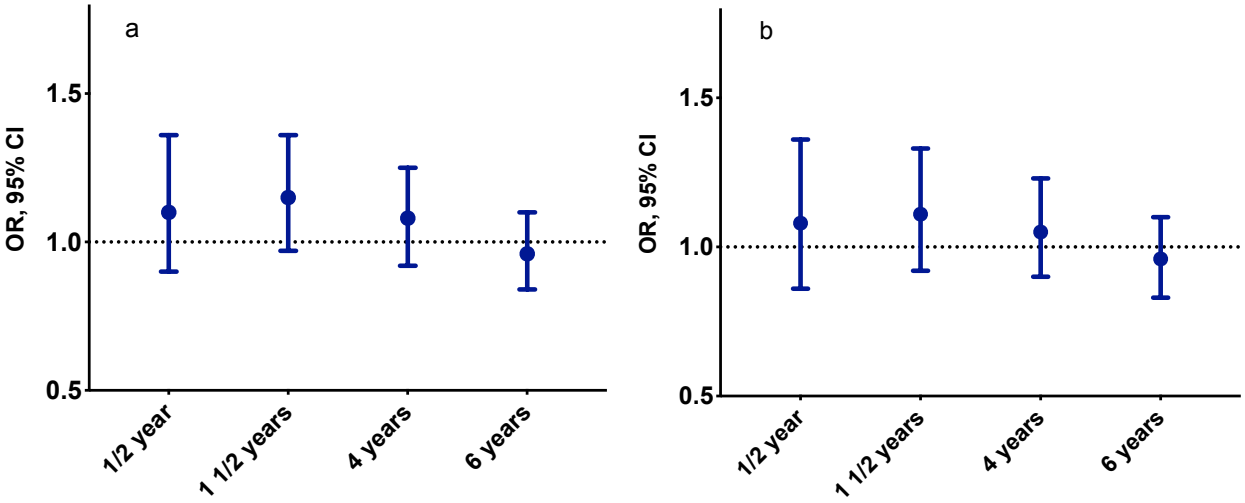
The role of food allergen exposure in allergy prevention has been eagerly discussed among researchers and clinicians, and it appears intuitively sensible to prolong exclusive breastfeeding and thereby avoid high exposure to allergenic foods in early life to avoid development of sensitization, food allergy, and perhaps even other atopic diseases. Further, evidence has shown that breastfeeding plays a central role in developing a tolerogenic immune response in early life[125]. However, evidence on associations between breastfeeding and atopic diseases has been conflicting, some studies reporting a protective effect[126–128], others reporting no effect[129,130] and some even an increased risk[131–134]. Since then, there has been a shift in this paradigm with a wide acceptance of early introduction of certain allergenic foods in at-risk children in order to avoid development of allergic sensitization and food allergy[38,105,106], which would advocate against prolonged exclusive breastfeeding in these children. In study **V** we elucidate this issue and investigate the association between duration of breastfeeding and allergic sensitization during the first 6 years of life and AD, asthma, and allergic rhinitis at 7 years of age in the at-risk $COPSAC_{2000}$ cohort.

The association between duration of exclusive breastfeeding and development of sensitization measured as SPT and/or sIgE at any time point from 0.5 to 6 years of age is shown in **Figure 8**. We found no effect of prolonged breastfeeding on the development of allergic sensitization. Adjusting for confounders such as mother's smoking, mother's age at birth, socioeconomic factors, older siblings, and father's allergy did not alter the results noteworthy.

We also investigated reverse causation, as it can mislead the interpretation of findings showing an association between prolonged breastfeeding and atopic disease, when in reality it is an early development of atopic disease which leads to prolonged breastfeeding with the intention of a protective effect[135]. We showed that the groups of children who developed wheeze, AD or had a positive SPT during exclusive breastfeeding were breastfed for a significantly longer duration compared to the group of children who developed none of these traits during exclusive breastfeeding (5.8 months vs. 4.1 months $p<0.0001$)

Therefore, it is important to consider such potential reverse causation by eliminating children who were diagnosed with AD, wheeze or had a positive SPT during exclusive breastfeeding from the analyses. In our case, however, it did not change the estimates remarkably (**Figure 8**).

Figure 8: Association between exclusive breastfeeding and any sensitization in all the children (a) and corrected for reverse causation (b). Estimates of sensitization are calculated for each month of prolonged breastfeeding (adapted from **V**).



Further, we found similar conclusions when grouping information on breastfeeding in categorical variables (</> 4 months and </> 6 months), including the 22 children who were never exclusively breastfed, sub-dividing sensitization into assessment method (SPT and sIgE separately) and type of allergen (food, aeroallergens, and egg and milk individually), analyzing only children with double-disposition to atopy (i.e. both mother and father with atopic history), children born to mothers with aeroallergen sensitization, looking at the sensitization outcome as monosensitization and polysensitization at 6 years of age, and finally, doing all of the above mentioned analyses with partial breastfeeding as the exposure variable. We therefore safely conclude that there is no association between duration of breastfeeding, neither partial nor exclusive, and sensitization in childhood. Finally, we found no association between duration of exclusive breastfeeding and AD, wheeze and/or asthma, or allergic rhinitis at 7 years of age (**Table 8**).

Table 8: Association between exclusive breastfeeding and AD, wheeze and/or asthma, and allergic rhinitis at 7 years in all the children and adjusted for reverse causation by excluding children who developed AD, recurrent wheeze, or had a positive SPT during the period of exclusive breastfeeding (adapted from **V**).

Analyses	OR [95% CI]*	P-value	N disease/total§
AD 7 yrs	1.07 [0.92-1.24]	0.373	67/304
Wheeze and/or asthma 7 yrs	0.97 [0.82-1.14]	0.689	46/310
Allergic Rhinitis 7 yrs	1.02 [0.84-1.23]	0.858	35/270
Adjusted for reverse causation			
AD 7 yrs	0.99 [0.84-1.17]	0.932	53/277
Wheeze and/or asthma 7 yrs	0.98 [0.82-1.18]	0.857	38/283
Allergic Rhinitis 7 yrs	1.00 [0.81-1.24]	0.996	27/247

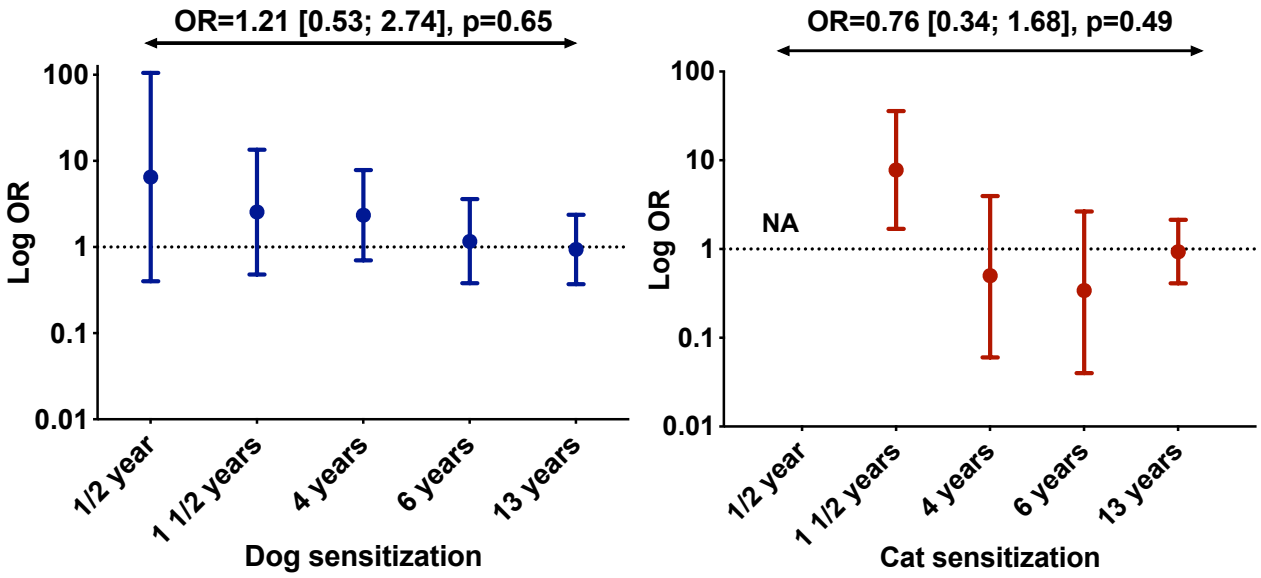
* Logistic regression was used in the analyses calculating the OR of sensitization for each month of prolonged breastfeeding.
§ Total of children with complete data on outcome and breastfeeding at a given age.

Pets and other houseguests

Aeroallergen exposure is another player in the development of sensitization which is supported by differences in sensitization patterns across the globe reflecting local exposures. However, it is not the allergen exposure alone that leads to sensitization and certain adjuvant effects, or a specific timing of the exposure may be essential for the subsequent development of sensitization. In study **VI** we address this issue and explore the association between exposures to dog, cat, and house dust mites perinatally and the development of allergic sensitization from 0.5-13 years of age and allergic rhinitis at 7 and 13 years of age in the at-risk $COPSAC_{2000}$ birth cohort.

The associations between dog and cat exposure (yes/no) in the first year of life and sensitization to dog and cat during childhood are depicted in **Figure 7**. The analyses were also done for pet exposure during 3rd trimester showing similar results. We found no effect of perinatal pet exposure on the development of sensitization to the same allergens from 0.5-13 years.

Figure 7: Plot of odds ratios (ORs with 95% CI) illustrating the association between dog exposure and cat exposure during the first year of life and allergic sensitization at the five age-points (dot with bars) and allergic sensitization at any age (bi-directional arrow), adapted from **VI**.



We also analyzed the association between loads of dog, cat, and house dust mite allergen measured in dust samples collected from the child's bed at one year of age and allergic sensitization and allergic rhinitis and found no significant associations. However, there was an indication of a protective effect of Der f 1 level in the bed dust sample on the development of allergic rhinitis to house dust mites at 13 years of age (borderline significant association, p=0.05) (**Table 6**).

We also stratified the results by AD status from 0-1 years expecting to find a stronger association in the children with early-onset AD based on the theories presented in study **IV**, but we found no noteworthy difference in the results (**Table 7**). However, in study **IV** we showed that severity of AD was more important than the presence alone, and we did not evaluate SCORAD in **VI** (published many years before **IV**).

Table 6: Allergen exposure in the first year of life and risk of sensitization to the specific allergen during childhood (0-13 years) and allergic rhinitis at 7 and 13 years of age (adapted from **VI**).

	Risk of sensitization from 0-13 years		Risk of allergic rhinitis at 7 years		Risk of allergic rhinitis at 13 years	
Exposure	**OR [CI]**	**p-value**	**OR [CI]**	**p-value**	**OR [CI]**	**p-value**
Dog allergen (Can f 1) in dust sample at 1 year	0.96 [0.88;1.04]	0.33	1.05 [0.86;1.29]	0.62	1.00 [0.87;1.16]	1.00
Cat allergen (Fel d 1) in dust sample at 1 year	0.97 [0.88;1.06]	0.51	0.99 [0.84;1.17]	0.91	1.10 [0.96;1.24]	0.19
House dust mite (Der p 1) in dust sample at 1 year	1.00 [0.93;1.06]	0.91	0.89 [0.73;1.07]	0.20	0.96 [0.88;1.05]	0.33
House dust mite (Der f 1) in dust sample at 1 year	1.01 [0.91;1.11]	0.90	0.89 [0.71;1.11]	0.30	0.89 [0.79;1.00]	0.05

Table 7: Allergen exposure prenatally and in the first year of life and specific sensitization during childhood (0-13 years) stratified by AD status during the first year of life (adapted from **VI**).

	Risk of sensitization to the specific allergen during childhood			
Exposure	**AD (N=103)**		**No AD (N=300)**	
	OR [CI]	**p-value**	**OR [CI]**	**p-value**
Dog exposure during 3rd trimester	0.74 [0.08;6.70]	0.79	1.11 [0.40;3.07]	0.84
Cat exposure during 3rd trimester	0.60 [0.16;2.30]	0.46	0.75 [0.25;2.26]	0.61
Dog exposure during 1st year of life	1.28 [0.24;6.86]	0.77	1.35 [0.52;3.50]	0.54
Cat exposure during 1st year of life	0.56 [0.15;2.10]	0.39	0.88 [0.32;2.40]	0.80
Dog allergen (Can f 1) in dust sample at 1 year	1.01 [0.86;1.19]	0.91	0.93 [0.84;1.04]	0.19
Cat allergen (Fel d 1) in dust sample at 1 year	0.89 [0.77;1.03]	0.11	1.02 [0.90;1.15]	0.77
House dust mite (Der p 1) in dust sample at 1 year	0.95 [0.84;1.07]	0.37	1.02 [0.94;1.11]	0.62
House dust mite (Der f 1) in dust sample at 1 year	0.93 [0.79;1.10]	0.41	1.05 [0.93;1.18]	0.42

Previous studies of associations between indoor allergen exposure in early life and development of sensitization during childhood have shown both a decreased risk[136–138], an increased risk[139–142], and no effect[140,142,143], and the same is true for breastfeeding where

some studies reported a protective effect on the development of atopic diseases[126–128], others an increased risk[131–134], and some reported no effect[129,130]. Many methodological difficulties can explain the diverging results within this study area. These comprise indiscriminate use of sIgE and SPT though these tests are highly incongruent[26,144,145], failure to define the exposure similarly across studies (distinguish between exclusive and partial breastfeeding, defining allergen exposure objectively by dust samples etc.), and failure to define clinical outcomes similarly across studies. Assessment of exposure and outcome are often done by interviews or retrospective questionnaires which always entails a risk of recall bias. It can be difficult to define the direction of cause and effect in retrospective studies, as the disease could have debuted before the exposure. Further, reverse causation can mislead interpretations and bias the results as already discussed with regards to breastfeeding. This is also a plausible factor in relation to aeroallergen sensitization, as dogs and cats could have been removed from the household/never acquired, or a regular, thorough action against house dust in order to diminish exposure could take place in at-risk families or when symptoms start to emerge. To ensure the direction of association between exposure and outcomes in study **VI**, we only explored dog and cat exposure perinatally prior to development of the outcomes. Further, the children participating in the $COPSAC_{2000}$ cohort are all at-risk (mothers with asthma), so the predisposition did not need to be accounted for.
It remains likely that allergen exposure is a contributing factor to the development of allergic sensitization, but perhaps it is only a spike in sensitization and thereafter tolerance can be established, or maybe other genetic or environmental factors play a role in this complex relation. Both allergen load and timing of exposure seem to be of importance, and further timing of outcome measurement is relevant.

If a clinically significant allergy already has developed, it is of course commended to mitigate exposure to the specific allergen. However, to prevent sensitization and allergic rhinitis during childhood, we found no indication that avoiding exposure to dog, cat, or house dust mites perinatally would help. Nor did we find an effect in prolonging breastfeeding to prevent development of allergic sensitization during childhood or AD, asthma, or allergic rhinitis at 7 years of age.

It is important to underline, that breastfeeding provides an important mother-child bonding[146] and has proven to protect against respiratory and gastrointestinal infections in children as well as constituting an ideal nourishment for healthy growth and development[146–148]. It contains all the nutrients and nearly all minerals and vitamins the children need[146]. Further, breastfeeding has been shown to affect the child's gut microbiota[149] and it has been speculated that it could be in a protective way with regards to development of atopic diseases[150]. However, our study has not been able to support this theory. The general message that mothers can help protect their infant against atopic disease by breastfeeding should probably be moderated to avoid mothers with at-risk children feeling distressed and guilty if they cannot breastfeed as long as recommended[151]. Based on our data and previous literature[130,132,152–154], we do not find proof of a protective effect of breastfeeding on the development of sensitization or atopic diseases. We will probably never be able to make a solid conclusion on this theme as randomized, controlled studies would be unethical to perform.
Since study **V** and **VI** were published, the Danish Health Authorities have removed their recommendations of pet avoidance and to fully breastfeed at-risk babies for 6 months with the purpose of preventing development of atopic diseases[155].

Patient-tailored allergology

Component-resolved diagnostics

Conventionally, mixed allergen extracts to measure sIgE in serum and/or SPT are used to assess allergic sensitization. While these tests are sensitive in the detection of a clinical allergy, they lack specificity[156,157]. Allergen CRD has gained substantial notice in recent years, due to the potential for a more accurate assessment of allergy. Instead of using allergen extracts consisting of mixtures of component proteins, CRD identifies sIgE responses to pure individual allergen proteins that provide more specific test results[158]. The components of each allergen source are named after their Latin name (first 3 letters of the genus name), followed by the primary letter of the species name and then an Arabic number. For example, one of the major dog components is named Can f 1 (*Canis familiaris*) and the major cat allergen is named Fel d 1 (*Felis domesticus*). This relatively

novel way of characterizing sensitization offers innovative opportunities for management of allergy. Currently, CRD is mostly used to help distinguish cross-reactivity[159–162], to identify which patients can complete an oral food-challenge, and which will most likely need immunotherapy[163–166]. CRD is also beginning to play a greater role in pet allergy diagnostics which is discussed in study **VII, VIII** and **IX**, however, this element also complicates the diagnostic process further due to cross-reactive sensitizations[158,167].
The allergen components derived from different sources can be sorted into protein families that are common across the different species, such as lipocalins, secretoglobins, serum albumins, kallikreins, and latherins (19,23). A major allergen is an allergen component that >50% of patients react to among those with sensitization to its source[168].

Male and female dogs

One of the most frequent triggers of perennial allergic rhinitis in children is dog dander[5].
The dog allergen extracts that are used to perform SPT and sIgE measurements consist of several molecular components[169] and differ in allergen content which makes the reproducibility and reliability of the results more difficult[170]. CRD can be used to individualize and refine each patent's type of dog allergy[171,172].
The lipocalins, Can f 1, 2, 4 and 6, are the most well-known dog allergens. They are produced in secretory glands and are present in urine, skin, sweat, saliva, and sebum[173–176]. Can f 1 is the most frequent allergen among patients sensitized to dog with antibodies detected in 50-90%[177].
Can f 3 is a serum albumin with low frequency of IgE reactivity among dog-sensitized patients[178]. Can f 5, however, has been identified as a major dog allergen, and in some populations, it has been reported as the most frequent dog component sensitization, even more so than Can f 1[179,180]. Can f 5 is a prostatic kallikrein secreted from the prostate, and therefore only found in male dogs[181]. From the prostate, the allergen is secreted in the urine where it is mainly present, but it can also be detected in dog dander and hair[182] and its expression is reduced in neutered males[181]. The Can f 5 amino acid sequence has no significant resemblance to other known animal-derived allergens[183] indicating no risk of cross-reactivity between animal species[182]. However, Can f 5 shares 60% sequence identity with prostate-specific antigen of human seminal plasma which indicates a risk of cross-reaction between the two[182]. Can f 5 antibodies are detected in up to 70% of

patients sensitized to dogs, and 30-60% of dog-allergic patients are monosensitized to Can f 5 (among dog-allergens)[182,184] representing a group of patients that are possible only allergic to male dogs. The extracts that are commonly used for both SPT and sIgE are manufactured based on a mixture of dander from male and female dogs of different breeds, making it impossible to distinguish an allergy to male dogs only. Therefore, it is important to investigate the clinical utility of sensitization patterns to dog allergen components, particularly monosensitization to Can f 5, which is addressed in study **VII** and **VIII**.

Can f 7 is another male-dog-only allergen[185] secreted in the epididymis of dogs, however it is a minor allergen only present in about 10-20%[185] of dog allergic patients, and therefore not explored in more detail previously.

Our knowledge of the different dog components suggests that monosensitization to Can f 5 can indicate a male-dog-only allergy, however, this this has never been explored further in previous studies. In study **VII**, we present a 54-year-old woman with a history of dog allergy, but not when exposed to female dogs. The dog allergen component pattern using CRD confirmed the theory, showing monosensitization to Can f 5. To add clinical value to our *in vitro* test, we also wanted to perform *in vivo* tests. As the extracts available for SPT are manufactured using dander from a mixture of female and male dogs, we had special extracts made for this study derived from the hair of male and female dogs separately, according to standard extraction procedures. With these extracts, we performed SPT and conjunctival allergen provocation (CAP) tests to male and female dogs separately for the first time, to verify that the patient only reacted to male dogs.

Our patient had a relatively low level of sIgE to dog dander (0.27 kU_A/L) and CRD revealed monosensitization to Can f 5 (0.31 kU_A/L). She had a positive SPT using commercial dog dander extract of 3.5 mm. Using our special extracts, the male dog SPT showed a positive reaction of 4 mm, and the female dog SPT showed no reaction. Correspondingly, she tolerated the female dog extracts during the CAP, and reacted with instant red, itchy, and tearing of the eye at the highest concentration of the male extract, in agreement with the relative low level of sIgE antibodies against Can f 5.

We thought the clinical implication of this finding was quite extraordinary as it implies that a large proportion of patients who believe they are allergic to dogs actually tolerate female dogs.

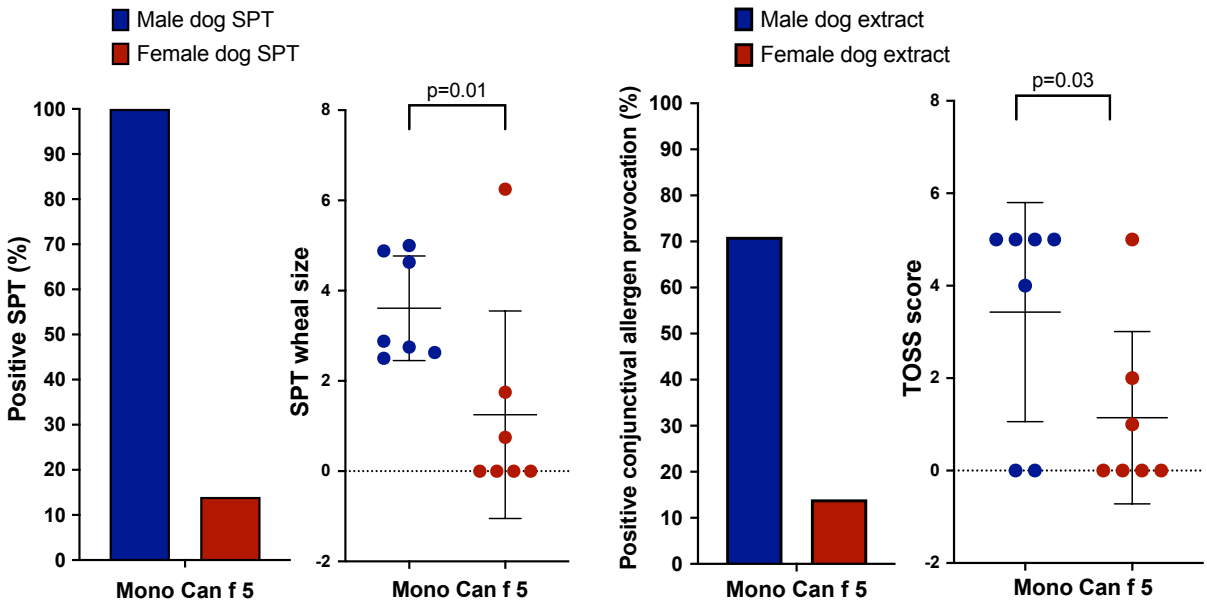
Our results called for a larger study confirming our finding, so straightaway we began to include children sensitized to dogs from $COPSAC_{2000}$ in a double-blind, randomized controlled trial (study **VIII**), where we aimed to compare reactions to SPT and CAP tests using our own male- and female dog extracts in children who were monosensitized to Can f 5 and children who were sensitized to a mix of the dog components.
We included 22 children between March and August 2017. The inclusion criteria were history of sensitization to dog dander verified based on a positive SPT, elevated sIgE or both. The exclusion criteria were daily need of antihistamine treatment, ongoing or completed allergen immunotherapy to furry animals, uncontrolled asthma, any chronic disease of the eye or eye surgery within 6 months.
We saw the included children for 2 visits; 1) at the first visit we performed SPT, CAP test in the right eye, and collected a blood sample. We did a double SPT on both volar forearms and in addition to the positive and negative control we applied 3 extracts: dog (e5), female dog, and male dog (the two latter blinded for both investigator and child). The CAP test was done in the right eye with either the male or female extract (blinded for both child and investigator) and the left eye was used as control.
2) The second visit was completed at least one week later to finalize the CAP test by applying the other extract in the left eye and using the right eye was as control.

The study was registered at ClinicalTrials.gov with identifier NCT03097094.

Of the 22 included children, 7 were monosensitized to Can f 5, 8 were sensitized to a mix of the dog components, and 7 were no longer sensitized to dog.
Of the Can f 5 mono-sensitized children, there was a significant difference between the reaction to the male and female dog extract using both SPT and CAP test (**Figure 9**), however one child reacted to the female dog extract against our expectations.

In the group of children with a mixed sensitization pattern, we found no difference between reactivity to the male and female dog extract using either test.

Figure 9: Comparison between reactions to male- and female dog allergen extracts using SPT and conjunctival allergen provocation test in children monosensitized to Can f 5 (adapted from **VIII**).



Previously, the theory of male dog allergy has only been speculated. One study found that high levels of IgE to Can f 5 as well as monosensitization to Can f 5, were associated with a frequent exposure to male dog[186], but no studies have explored reactivity to male- and female dogs. Previously, the interest in Can f 5 has evolved around its cross-reactivity to human seminal plasma and the rare cases of semen allergy[182,187,188]. Based on the high prevalences of sensitization to Can f 5 and the potential consequences in females during intercourse, one study investigated this further and included 27 women sensitized to Can f 5, who all had a male dog at home[189]. They reported a high prevalence of allergic reactions to human seminal plasma, and reactivity seemed to be related to the level of Can f 5.

Other studies of Can f 5 have demonstrated opposing results when investigating the association to clinical symptoms. One study found that children monosensitized to Can f 5 had a negative nasal challenge to dog dander extract[172] and another found no association to clinical symptoms[190], while other studies have found that sensitization to Can f 5 was strongly associated to severe, persistent allergic rhinitis, however they did explore reactivity in Can f 5 monosensitized patients[187,191]. Finally, an independent and strong

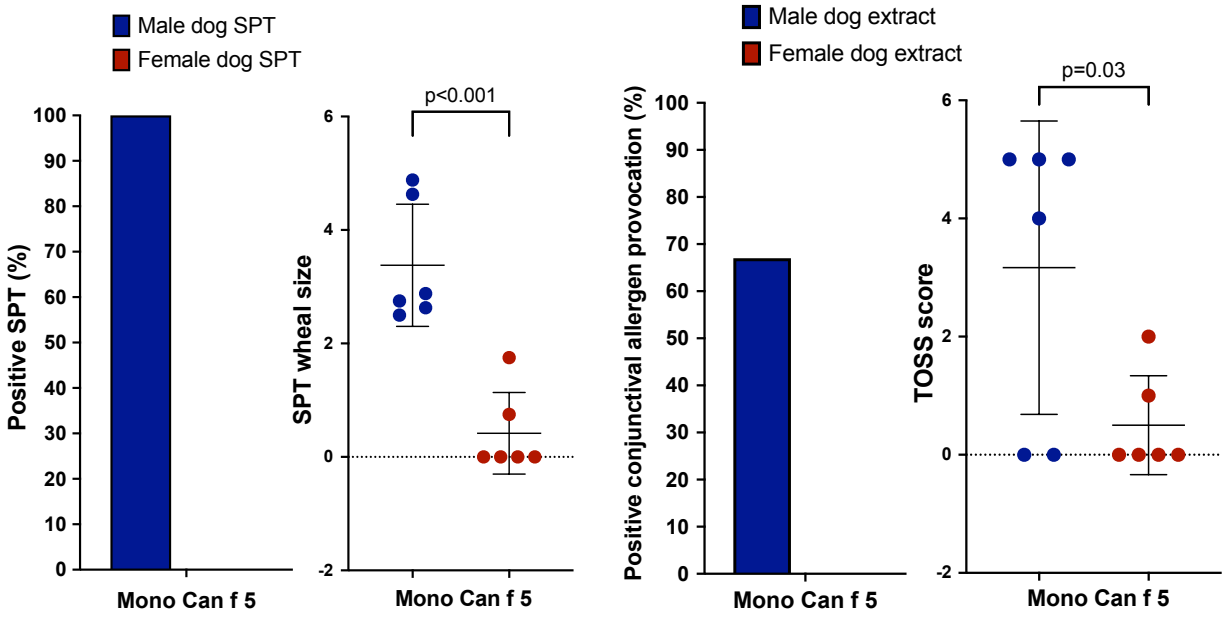
relationship between sensitization to Can f 5 IgE and asthma has been reported, but this relationship was also not explored in the context of monosensitization[192].

There are two major explanations for the conflicting results in studies on Can f 5; 1) The dog dander extracts that are available for challenge tests consist of a mixture of allergens obtained from both female and male dogs. Therefore, the fraction of female and male dog dander in these extracts may vary between batches leading to opposing results using two different batches in the same patient depending on their molecular allergen profile. 2) The definition of "monosensitization" has recently been modified as it is now possible to commercially measure sIgE to Can f 4 and Can f 6[193] which means that too many patients have been identified as monosensitized in previous studies. Until recently, a positive Can f 5 combined with negative Can f 1, 2 and 3 was enough to establish Can f 5 monosensitization. Now, a negative Can f 4 and 6 is also required, and even though these are minor allergens, it will undoubtedly cause the estimates of Can f 5 monosensitization to be lower.

Among the Can f 5 monosensitized children in our study, one showed reaction to the female dog extract using both SPT and CAP test, which we did not expect. It could be explained by the fact that he was not truly Can f 5 monosensitized, i.e., also sensitized to Can f 4 or 6. To investigate this, we called the patient back in for an extra blood sample to measure sensitization to Can f 4 and Can f 6, which had become commercially available after we had conducted the clinical visits. Interestingly, it showed that he was sensitized to Can f 4 (0.51 kU_A/L) and Can f 6 (10.5 kU_A/L), which means he belonged to the group of children with a mixed sensitization pattern and explains his reactivity to both the male and female dog extract. As this was a post-hoc analysis, it was unfortunately not feasible to conduct in all the children included in the study. **Figure 9** has been reconstructed excluding this child, as **Figure 10**.

This was the first study to show that dog-sensitized children with monosensitization to Can f 5 can tolerate female dogs with no reaction to neither SPT nor CAP test using female dog extract. It is important to underline, that Can f 5 monosensitization can only truly be identified when the complete selection of dog components have been measured (Can f 1-6)[194]. Such a precision medicine approach makes it possible for many dog-allergic persons to get a female dog in their homes.

Figure 10: Post hoc analyses comparing reactions to male- and female dog allergen extracts using SPT and conjunctival allergen provocation test in children monosensitized to Can f 5, excluding one child sensitized to Can f 4 and 6 (adapted from **VIII**).



Current perspectives of pet allergy diagnostics

Dog are not the only important perennial allergen source, but also cats and to some extend horses and other domestic mammals have been acknowledged as major risk factors for the development of allergic rhinitis and triggering asthma[195,196]. An international survey of more than 27,000 participants from 22 countries assessed that 57% of the population have at least one pet at home, most frequently cats (23%) and dogs (33%)[197]. The popularity of owning a cat and/or dog is increasing leading to a surge in dog and cat allergen exposure – not only in homes with pets, but also in places without pets such as nurseries, schools, workplaces etc., which has added to the observed increase in pet allergies in industrialized countries[5,196,198,199]. Therefore, diagnosis of pet allergy is gaining more and more importance. Cat is the most common mammalian allergy trigger[200] with up to 20% of adults worldwide being sensitized to cat[201]. Cat allergy diagnosis seems rather straightforward, possibly because the majority of patients have reactivity to one main protein, Fel d 1[202]. Consequently, the main content of cat SPT extracts is Fel d 1, but the concentrations of the other cat components might differ. Dog allergy diagnosis has proven

more complex as patient's own reports of allergic symptoms leads to misclassification in many cases[203], and the content of proteins in SPT extracts can vary up to 20-fold within different manufacturers[170].
The recognition of CRD in pet allergy diagnostics is beginning to increase, which is evident from the increasing number of molecular allergy components that have recently been discovered from both dog, cat, and horse. However, with CRD the diagnosis becomes much more complex introducing cross-reacting components where sensitization is clinically not that relevant[158,167]. In study **IX,** we provided a review of aeroallergens originating from pets focusing on dog, cat, and horse. We explored the clinical utility of CRD within this field including future potentials. We searched on PubMed to detect publications that included dog, cat, and horse allergen components published from 1997 to mid-2020.

The World Health Organization/International Union of Immunological Societies[204] lists 32 mammalian aeroallergens derived from 9 different animal sources (**Table 9**). They are classified according to their protein family.

The role of kallikreins (Can f 5) has already been discussed in study **VII** and **VIII**. Other protein families that are relevant to mention are lipocalins, secretoglobins, serum albumins, and latherins.

Lipocalins
The most essential group of mammalian aeroallergens are the lipocalins[176]. They are produced in the animal's secretory glands and are present in urine, skin, sweat, saliva, and sebum[176].
Cats contain Fel d 4 and Fel d 7 and studies have shown that these allergens are mainly present in saliva, thereby deposited onto the fur by grooming[205,206]. One study found, that the length of the cat's hair was not associated to Fel d 4 levels, however, the female cats that were neutered had significantly higher levels compared to cats that were not neutered (17.4 vs 2.2 microg/g, P=0.039)[205]. Up to 63% of cat-allergic patients have IgE reactivity to Fel d 4[207] and 38% to Fel d 7[206].

Table 9: Airborne allergen components of domestic mammals (adapted from **IX**)

Animal	Component	Protein type
Dog	Can f 1*	Lipocalin
	Can f 2*	Lipocalin
	Can f 3*	Serum albumin
	Can f 4*	Lipocalin
	Can f 5*	Arginine esterase, prostatic kallikrein
	Can f 6*	Lipocalin
	Can f 7	Epididymal Secretory Protein E1 or Niemann Pick type C2 protein
	Can f 8	Cystatin
Guinea pig	Cav p 1	Lipocalin
	Cav p 2	Lipocalin
	Cav p 3	Lipocalin
	Cav p 4	Serum albumin
	Cav p 6	Lipocalin
Domestic horse	Equ c 1*	Lipocalin
	Equ c 2	Lipocalin
	Equ c 3*	Serum albumin
	Equ c 4	Latherin
Cat	Fel d 1*	Secretoglobin (Uteroglobin, chain 1)
	Fel d 2*	Serum albumin
	Fel d 3	Cystatin
	Fel d 4*	Lipocalin
	Fel d 5	Immunoglobulin A
	Fel d 6	Immunoglobulin M
	Fel d 7*	Lipocalin (Von Ebner gland protein)
	Fel d 8	Latherin-like protein
Syrian hamster	Mes a 1	Lipocalin
Mouse	Mus m 1*	Lipocalin/ urinary prealbumin
Rabbit	Ory c 1	Lipocalin
	Ory c 3	Secretoglobin (Lipophilin)
	Ory c 4	Lipocalin
Siberian hamster	Phod s 1	Lipocalin
Rat	Rat n 1	Alpha-2u-globulin/ Lipocalin

* Currently available for CRD

Dogs contain Can f 1, 2, 4 and 6 from the lipocalin family. Sensitization to Can f 1 has been found in 50%–90% of dog-allergic patients and Can f 2 is characterized as a minor allergen with sensitization rates about 20-33%[195,208,209]. Sensitization to Can f 4 has been found in 35-46%[210,211] and Can f 6 in 56%[212] of dog-allergic patients.

Horses contain Equ c 1 and Equ c 2 from the lipocalin family, and sensitization to Equ c 1 has been found in up to 76% of horse allergic patients[213].

The lipocalins encompass a protein family with both diversity, but also with similarity leading to cross-reactivity between some components, for example between Fel d 4, Can f

6, and Equ c 1[174,214,215], Mus m 1 and Rat n 1[216], Mus m 1 and Equ c 1[213], and Can f 1 and Fel d 7[217].

Secretoglobins

Secretoglobins are produced in sebaceous and salivary glands and thereby also deposited to the fur by grooming[217]. Only two mammalian aeroallergens have been identified that belong to the secretoglobin protein family, and they are Ory c 3 from rabbit and Fel d 1 from cat[213]. This protein family is important to mention, as Fel d 1 is the most abundant cat allergen with sensitization in 90% of cat-allergic patients[218]. The sequence identity between Ory c 3 and Fel d 1 is very low with no cross-reactivity between them[216].

Serum albumins

Serum albumins are proteins with high abundancy found in blood, dander, and secretions including milk, but despite their high abundancy, they generate low frequency of IgE reactivity among dog-sensitized patients and are therefore classified as minor allergens[178]. According to the World Health Organization/International Union of Immunological Societies allergen nomenclature database[204], seven mammalian serum albumin allergens have been classified, including Bos d 6 from domestic cattle, Can f 3 from dog, Cav p 4 from guinea pig, Equ c 3 from horse, Fel d 2 from cat, and Sus s 1 from domestic pig. Serum albumins remain relevant as they have high sequence identity and thereby are responsible for species cross-reactivity (up to 87% between dog and cat)[168]. Another example is the cross-reactivity between cat and pig which is clinically relevant as cat-allergic patients can experience allergic symptoms after eating of pork meat, referred to as the *pork-cat syndrome*[219]. Vice versa, milk-allergic children with sensitization to the serum albumin Bos d 6 can experience symptoms of allergic rhinitis and asthma when exposed to animal dander[220].

Monosensitization to serum albumins is unusual, and sensitization is mainly observed in combination with sIgE directed against major allergens[214].

Latherins

The latherin Equ c 4 from horse is present in saliva, sweat, and dander[221,222] and Fel d 8 has been found in saliva of cats[206]. Equ c 4 is a major horse allergen with an IgE binding

frequency of 77% among horse-allergic patients[223]. Fel d 8, however, is a minor allergen with sensitization in only 19% of cat-allergic patients[206].

Other than current sensitization status, pet component sensitization has also been proposed as an indicator of increased atopic disease risk and severity. Children with asthma and allergic rhinitis to cat had higher levels of IgE to Fel d 1 than children with allergic rhinitis only[76]. Likewise, a study of children with allergy to cat found that symptoms of asthma triggered by exposure to cat were significantly associated with sIgE to cat allergens Fel d 1 and Fel d 4[57]. Furthermore, polysensitization to Fel d 1, 2 and 4 was found to associate with both clinical reactivity to cat[83], bronchial responsiveness and increased fractional exhaled nitric oxide (FeNO)[69]. Among dog-sensitized children, one study found that concomitant sensitization to Can f 5 and Can f 1/2 indicated the highest risk for asthma[57]. Furthermore, the study showed a dose-response pattern as asthma was associated with increasing levels of sensitization to allergen components. Sensitization to Can f 2 has also been associated with asthma[187,195]. Of horse allergens, sensitization to Equ c 1 and Equ c 3 have been associated with severe childhood asthma[191,224]. We have previously shown an association between sensitization to dog, cat, and horse (whole extracts) through childhood and asthma at 7 years[225]. That inspired another study of allergen components, where we found that sensitization to lipocalins, mostly originating from dog, cat, and horse, was associated with current asthma[226]. Polysensitization to three or more animal-derived protein families (lipocalins, secretoglobins, and kallikrein) has been associated with severity of asthma with increased bronchial inflammation and a trend towards more frequent use of oral corticosteroid treatment[227]. Recently, similar findings were reported among children with dog sensitization[172], where sensitization to an increasing number of dog allergen components was associated with a positive nasal challenge result to whole dog extract. Another study of adults analyzed sensitization patterns of cat, dog, and horse allergen components, and identified clusters associated with a markedly increased risk of asthma and/or allergic rhinitis[228]. Furthermore, polysensitization was associated with increased FeNO and eosinophil levels.
The only study so far who investigated pet components in in relation to AD showed an association between high levels of IgE to Fel d 2 and Fel d 4 and AD among children with cat allergy[229].

Comparison of allergen components and whole extracts as predictors of reactivity to the allergen source was conducted in a large longitudinal population-based study in children. At 4 years of age, sensitization to Fel d 1 and Can f 1 and polysensitization to dog or cat components was stronger associated to dog and cat allergy at 16 years than IgE to whole extracts[190]. Additionally, another study in adults showed that sensitization to a combination of whole cat extract and one or more cat components had more bronchial reactivity and higher FeNO compared to patients sensitized to the whole extract only[223]. Further, sensitization to cat components was stronger associated to allergic rhinitis and asthma over a 12-year period, than sensitization to cat extract only, highlighting the predictive value of CRD[223]. Altogether, available data indicate that sensitization to pet allergen components may offer better prediction of the progress and severity of asthma and allergic rhinitis.

Overall, when evaluating a patient with suspected dog allergy, be aware that SPT protein content varies up to 20-fold[170]. The clinical utility of CRD would be to measure all the dog components (Can f 1, 2, 3, 4, 5 and 6) to identify Can f 5 monosensitization and indicate female dog tolerance[67]. The role of Can f 7 and Can f 8 remain to be elucidated but may be part of the “dog-analysis-package” in the future. Further, polysensitization should be included in the assessment, as the probability of clinical reactivity when exposed to dog increases with the number of dog components a patient is sensitized to[172,190]. Also, higher levels of sensitization to dog components and concomitant sensitization to Can f 1/2 and Can f 5 is associated with current asthma[191].

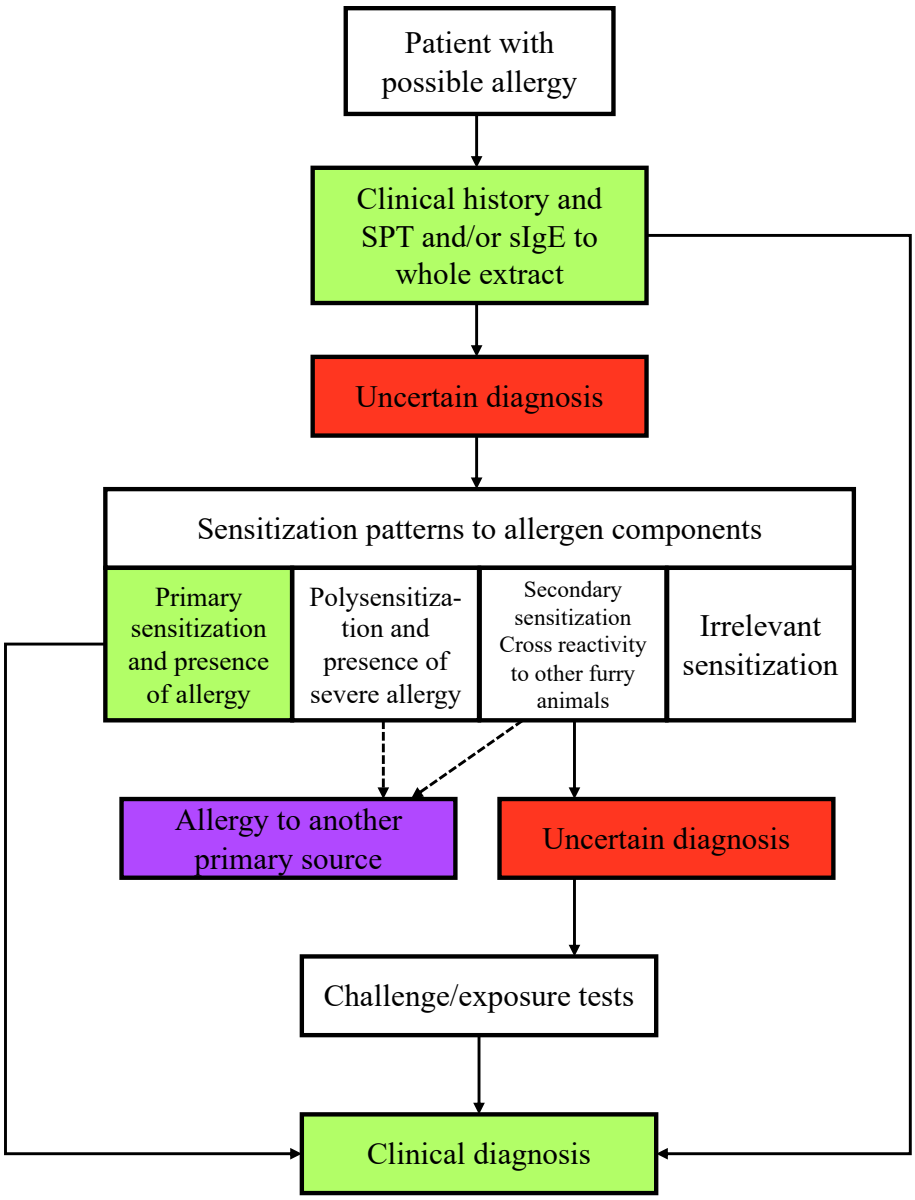
In patients with suspected cat allergy, most react to Fel d 1[202] and since the cat SPT extracts contain mainly Fel d 1, the diagnosis is more simple. However, CRD also has its place here highlighting Fel d 1, 2 and 4 as the most relevant components. Sensitization to Fel d 1 is associated with asthma[191,230], and polysensitization is associated with both allergic rhinitis to cat[190], bronchial hyperresponsiveness and increased FeNO[223]. Finally, Fel d 2 and Fel d 4 sensitization associates with AD[229].

In patients with suspected horse allergy, there are many false positive SPT[231]. Of molecular components, only Equ c 1 and Equ c 3 have been found clinically relevant as they are associated with severe childhood asthma[191,224]

CRD seems to assist in providing a detailed and personalized sensitization profile in patients, which may ultimately lead to individual treatment- and management options. However, some issues regarding the clinical utility of CRD remain to be elucidated further in patients with pet allergy, for example the role of component sensitizations as predictors and markers of disease severity and criteria to distinguish food-associated syndromes related to cat and/or dog sensitization[195]. Further the usefulness of CRD to identify patients who will respond positively to allergen immunotherapy remain to be explored including monitoring treatment responses following immunotherapy. For now, CRD should be used as an add-on to extract-based testing, not an alternative, when clinical history and conventional tests are inconclusive. CRD can differentiate abnormal sensitization patterns that include cross-reactive sensitizations or minor allergen components (**Figure 11**). CRD may also add improved sensitivity in cases where the whole allergen extract contains low levels of the relevant allergen, for example allergens like PR-10 that are present in many plant foods such as fruits and nuts. The clinical specificity of CRD has proven superior compared to whole extract testing when predicting allergic reactions to several foods, including peanut[232], hazelnut[233,234], and cashew nut[235,236], and this may also prove to be the case for aeroallergens.

Overall, CRD has the potential to develop patient-tailored diagnostics and treatments that could spare time for patients, reduce healthcare costs, reduce adverse effects, and improve quality of life of the patients.

Figure 11: Algorithm for diagnostic work-up of patients with suspected allergy to animal dander (adapted from **IX**)



Conclusions and future directions

The research presented in this thesis (**I-IX**) is based on data assembled from the $COPSAC_{2000}$ and $COPSAC_{2010}$ cohorts, which are unique due to the longitudinal design with intensive follow-up throughout childhood with a focus on early-life. This allows gathering of extensive and repetitive data during childhood, often starting before any clinical signs of disease, continuing during and, in some cases, until after the disease symptoms have ceased. The series of papers underline the complexity of the term “atopic” and that even “allergic sensitization” can insinuate a multitude of different things (**I**) making research within the field of atopic diseases challenging to conduct, interpret and compare. However, the term “atopic” is most likely here to stay. It is frequently used in the literature, among physicians and patients, however, the definition should be clarified in each content. Mostly, we are talking about diseases that are associated with development of sIgE, but a positive SPT or elevated sIgE is not a mandatory criterion in the definition of neither asthma[237] nor AD[25]. The latest European Respiratory Society guideline for the diagnosis of asthma in children aged 5-16 years[238] underlines the limited value of SPT and sIgE measurements in the diagnosis of asthma as they have too low specificity leading to over-diagnosis of asthma, especially in children with other atopic diseases, and under-diagnosis in non-allergic asthma. The guideline therefore advises against allergy testing as a diagnostic test for asthma in children. However, once an asthma diagnosis has been established, identification of sensitization can be helpful for further asthma management, particularly to establish the phenotype and to plan individualized prevention strategies. Certainly, there is evidence of common features of origin between the atopic diseases such as an underlying Th2-skewed immune system[10,22] and shared genetic risk loci[23], however we must not confuse comorbidity with endotyping[239]. A recent randomized double-blind placebo controlled study investigated whether treatment of grass pollen allergy with sublingual immunotherapy in children from age 5-12 years, with no history of asthma, could alter the natural course of atopic disease and prevent development of asthma[123]. Unfortunately, the study failed to show an effect on asthma debut, but they found reduction in symptoms of allergic rhinitis and asthma. This supports the theory of a common origin of these diseases, but not necessarily a causal link between them. Not

surprisingly, asthma symptoms will be diminished if grass pollen allergy is treated in cases where the allergy acts as a trigger of asthma, and similarly, treatment of severe allergic asthma with biologicals such as omalizumab (monoclonal antibody binding to free IgE), will also result in symptom relieve[240]. Two smaller, open randomized studies investigating grass SCIT[241] and SLIT[242] have previously shown an effect on asthma development, however the study set-ups were weaker, and results should be interpreted with caution.

Several of the studies discussed in this thesis (**II**-**IV**) point toward the early environment as an important factor in the development of atopic traits. The fact that we found a stronger effect of mother's atopic traits than father's on the child's risk of developing the same traits in early childhood (**II**) suggest that non-genetic factors play a role in early life. Further, children with the late-onset pattern of sensitization were not at increased risk of developing asthma, but only the children with early-onset, persistent sensitization (**III**). Finally, we found that children with early-onset (≤ 1 year of age) and more severe AD had an increased risk of later development of aeroallergen sensitization and allergic rhinitis compared to children with onset of AD after 1 year of age (**IV**). These conclusions suggest a window-of-opportunity in early childhood where programming occurs and that proper interventions around this time could impede the progression of AD to airway allergy, particularly in children with a combination of early-onset, more severe disease and *FLG* mutation. Along those lines, it has been hypothesized that early reinforcement of the skin barrier by applying daily emollients before the onset of AD in high-risk infants could avoid development of AD and subsequent food- and respiratory allergy, but two recent randomized controlled trials failed to prove this theory[243,244].

Another potentially effective intervention to prevent aeroallergen sensitization and allergic rhinitis in children with early-onset, severe AD may be through modulation of the immune response by controlling the route of initial exposure to the allergen, similar to what has been shown with food allergens[38,105]. The idea is that early oral exposure may led to tolerance[38,105,106] by stimulating regulatory T-cells induction and function in the gut[245] as opposed to early exposure through the skin via the environment, which may lead to allergic reactivity. This hypothesis was supported by a murine study[246], and later two human studies tested the theory by delivering a sublingual immunotherapy mixture of house dust mite, cat and grass[247] or house dust mite alone[248,249] in children around 1 year

of age to prevent development of allergic sensitization. Unfortunately, the studies did not show any effect; however, in our study (**IV**), allergic rhinitis was more influenced by early, severe AD than allergic sensitization (not necessarily clinically relevant), so maybe focus should be on allergic rhinitis as the primary outcome. Also, allergic sensitization is known to increase during the initial part of immunotherapy[250], making it more complex to interpret as a primary outcome. Further, the studies had some limitations including lack of compliance, high drop-out rates, age at inclusion, correctly taking the sublingual immunotherapy, not measuring both sIgE and SPT, and difficulty recognizing children at high risk for developing AD. To help support (or reject) the theory that development of sensitization and allergic rhinitis can be prevented in children with early-onset, severe AD through early sublingual aeroallergen exposure, randomized controlled trials taking all these factors into account are needed.

Our studies did not imply that neither prolonged breast-feeding (**V**) nor avoidance of high exposures to dog, cat, or house dust mites during pregnancy or at birth (**VI**) could contribute much as preventive factors of developing allergic sensitization or allergic rhinitis in early childhood. However, we have recently discovered that dog exposure in the home around birth reduced the risk of developing AD in the first 3 years of life in a convincing dose-dependent manner, i.e. the risk of AD decreased with increasing number of dogs in the home[251]. This study included data from both COPSAC cohorts. The protective effect of dog in relation to AD was not altered by *FLG* mutation[103,251]. Contrary, we have previously found, that perinatal cat exposure markedly increased the risk of AD in the first year of life in children with *FLG* mutation, but not in the children without[103]. This study included data from two independent birth cohorts: the at-risk $COPSAC_{2000}$ cohort and the unselected British MAAS (Manchester Asthma and Allergy Study) cohort. The latter study underlines the importance of gene-environment interactions when investigating the etiology of atopic diseases.

The microbiome is another widely discussed topic in the investigation of factors influencing the development of atopic diseases, which is suggested to be driven by microbiota-immune interaction in early infancy[252]. Multiple studies have investigated the relationship between dysbiosis of the airway microbiota and asthma and allergic rhinitis[252–256] and have found an increased microbial diversity seems to confer a risk[252,253]. We have explored the

following factors, that can influence the airway microbiota: prenatal diet[257], season of birth[258], indoor environment[259] and epigenetics[253]. The airway microbiota is established in early life[252,260], and a more detailed definition of a favorable airway microbiota is needed for a potential atopic disease prevention in early life by targeted manipulation of the developing airway microbiota.

It has also been shown that the gut microbiome plays an important role in the development of atopic traits[82,261–267] and that increased diversity seems to be protective[261,266,267] (contrary to the airway microbiota). Factors that can influence the gut microbiota in inappropriate ways are cesarian section[262,268,269], use of antibiotics[270], "westernized diet" low on fibers[271], and urban environment[263]. The observed negative influence by cesarian section has led to randomized controlled trials where oral transplantation of maternal vaginal or fecal microbiota is given to newborn infants born by cesarean section[272–276] in order to restore a gut microbiota that resembles that of children born vaginally. These studies are in the early stages (planning/phase 1) and it seems that fecal microbiota transplantation has a more promising influence on the infant gut microbiota than vaginal seeding[275]. The urban environment has for long been recognized as a risk factor for developing atopic disease – a trend that has been observed world-wide[277–283], possibly mediated, to some extent, by the gut microbiome. Consumption of unpasteurized cow's milk and exposure to farm animals and fodder have been highlighted as farm-related factors that are associated with a protective effect [284,285]. As there might be a window of opportunity during early childhood for microbial alterations in shaping gut immune maturation[286], another research group is planning to study whether consumption of a raw milk preparation (both in pregnant women, infants and small children) can prevent development of asthma and other atopic diseases in childhood[287].

Both maternal fecal transplantation and raw milk consumption are indirect ways to alter the gut microbiota. A direct approach could also be pursued where a more detailed description of the favorable gut microbiome composition is needed to produce a targeted treatment (perhaps in the form of a mixture containing either favorable microbes or bacteriophages eliminating inappropriate microbes) that could be given to children born by cesarean section or children growing up in an urban environment or maybe a combination of

genetics and other risk factors. The gut microbiome seems to be manifested in the first year of life[82,288], and most interventions should be targeted for this window of opportunity.

In a research setting, it is crucial to be accurate with the definition of atopy in order to compare results, inclusion criteria etc., because sensitization is influenced by many factors such as type of allergen(s) tested for, type of test[26], cut-off (**I**), age of the patients[26], and geography. In papers **VII-VIII,** however, the definition of allergic sensitization became very specific using CRD to distinguish male-dog-only allergy, and the findings of these studies are simple, reproducible, and highly clinically relevant as they suggest that a large proportion of dog allergic patients can get a female dog. The low cross-reactivity between Can f 5 and the other dog components[183] explains why patients monosensitized to Can f 5 showed no reactivity to the female dog extracts, and mitigates the possibility of developing an allergy to female dogs[67,289]. As this was the first time the clinical relevance of Can f 5 monosensitization was demonstrated, there is a need for studies in larger populations of children to assess the diagnostic test performance of Can f 5 monosensitization, regardless of the children's dog sensitization history. It would also be interesting to investigate if new sensitizations to the other dog components occur in children with Can f 5 monosensitization who have acquired a female dog. Finally, neutering the male dog could in theory also stop the production of Can f 5 in the prostate gland, however, this has never been tested in practice.

For cat owners, another recently investigated approach to diminish allergen load is by altering the cat food composition with anti-Fel d 1 Immunoglobulin Y. This method has shown to reduce the presence of immunologically active Fel d 1 in cat hair, dander, and saliva[290]. This provides an opportunity for cat owners sensitized to Fel d 1 to treat their cats and subsequently reduce their cat allergic symptoms. The effect of this fascinating treatment needs to be studied further.

Today, treatment recommendations for allergy to furry animals comprise avoidance, symptom-relief medication, and immunotherapy[168]. When immunotherapy is planned, CRD can be important to identify any possible cross-reactions and ascertain the main sensitizing allergen source for immunotherapy which leads to better aimed

immunotherapy. Component testing provides a patient-specific sensitization pattern, and hybridized extracts that match these patterns could provide better chances of effective therapy. Intuitively, it does not make sense to start immunotherapy with extracts that contain dog components Can f 1 and Can f 5 in a dog allergic patient who is sensitized to e.g. Can f 2 and Can f 3 only.

In conclusion, the series of papers presented in this thesis (**I-IX**) highlight the complexity of allergy diagnostics and support programming in early life suggesting that proper early interventions may impede the development of atopic diseases. These findings should be validated in randomized controlled trials, such as investigating early oral exposure to aeroallergens as a prevention of development of allergic rhinitis in line with what has been done for food allergens. The mechanism may be enforced by imposing a favorable gut microbiome composition to at-risk newborns, and indirect alterations of the gut microbiome with raw milk consumption or fecal transplantations in newborns, to reduce the risk of atopic disease development, are already being explored. Finally, if an allergy has developed, CRD can help identify patient-specific patterns of sensitization with a possibility to provide individualized extracts for immunotherapy, making treatment success more likely. Disentangling the igniting mechanisms and underlying endotypes of atopic diseases is of utmost importance to generate successful preventive measures to reduce the major global burden these diseases impose.

Summary

The incidence of the atopic diseases, namely asthma, atopic dermatitis (AD), allergic rhinitis, and food allergy, has increased in recent decades. Today, atopic diseases affect roughly 20% of the global population. The diseases impact quality of life of the child and work productivity of the parents, and because of their high prevalence, the overall socioeconomic burden is considerable. Atopic diseases mostly debut in early childhood; however, their etiology and disease mechanisms are not fully understood, which impedes successful prevention and treatment.

The objective of this thesis is to explore mechanisms of atopic diseases, to investigate the importance of environmental factors in early life, prior to disease development, and finally to explore new diagnostic tools to interpret allergic sensitization more accurately. The thesis is built on nine studies from the Copenhagen Prospective Studies on Asthma in Childhood $(COPSAC)_{2000}$ and $COPSAC_{2010}$ birth cohorts investigating how the distribution of sIgE levels in children (**I**), parental atopic status (**II**), sensitization patterns (**III**), age of debut and severity of AD (**IV**), breastfeeding (**V**), and dogs, cats, and house dust mites in the home (**VI**) are associated with the development of atopic diseases in childhood. Finally, how component resolved diagnostics (CRD) has a role in providing patient-tailored recommendations (**VII-IX**).

In study **I**, we studied the distribution of specific IgE (sIgE) levels in children, as this is one of the main criteria for the definition of atopy. We found that the median value of sIgE varied among the different allergens - also among symptomatic children - highlighting the problem of using the same sIgE cut-off value for all allergens and urging for updated cut-offs that are specific to each individual allergen.

In study **II**, we investigated the associations between parents' sIgE, total IgE, and asthma and development of the same traits in the children during the first 6 years of life. Previous studies have indicated that atopic diseases are highly heritable traits and that the individual variation in the susceptibility to the diseases can be attributed both to genetic

risk variants and changing environmental exposures. Intuitively, it would be expected, that both parents contribute equally to heritability. Study **II** showed a consistently stronger effect of maternal compared to paternal asthma and sensitization on the same outcomes in early life of their children. This implies that factors, other than genetics, affecting the child perinatally or *in utero* have an important role in the conduction of disease predisposition.

Once a child has been exposed to unfavorable genetics and exposome, allergen specific IgE molecules may develop, but the implication of their presence is not clear. In study **III**, we analyzed patterns of sIgE and skin prick test (SPT) longitudinally at ages 6 months to 13 years in relation to development of asthma at 13 years. Only the children with the early-onset, persistent sensitization pattern were at increased risk of developing asthma, and the children with the late-onset pattern of sensitization were not. Further, polysensitization and higher levels of sensitization at all time-points were associated with increased risk of asthma at age 13 (using either SPT or sIgE). These findings support the programming-in-early-life hypothesis and underline the importance of considering polysensitization and the level of IgE/size of SPT when evaluating the clinical implications of a positive test.

In study **IV** we investigated whether early-onset vs. late-onset AD and severity of AD was associated with development of aeroallergen sensitization and allergic rhinitis at 6-7 and 12 years of age. Our analyses suggested that children with early-onset (≤ 1 year of age) and more severe AD had an increased risk of later development of aeroallergen sensitization and allergic rhinitis compared to children with onset of AD after 1 year of age. The results indicate that the skin inflammation and/or skin barrier defect of AD may play an important role in the development of respiratory allergy, and that debut age can help characterize distinctive endotypes.

In study **V-VI**, we explored early-life exposures that are thought to influence development of sensitization and atopic disease. However, we could not prove an effect of prolonged breastfeeding (**V**) on the evolvement of allergic sensitization from 0-6 years or AD, asthma/wheeze, or allergic rhinitis at 7 years. Also, we found no effect of exposure to dog, cat, or house dust mites (**VI**) perinatally on the development of neither sensitization to the

same allergens nor allergic rhinitis from 0-13 years. These findings did not support recommendations to prolong exclusive breastfeeding or to avoid exposure to cat, dog, or house dust mites to prevent development of atopic disease during childhood.

As discussed in study **I**, allergy diagnostics poses many challenges, most noteworthy the high incidence of false positive results. Traditionally, allergic sensitization is assessed using mixed allergen extracts to measure sIgE in serum and/or SPT. While these tests are sensitive, they lack specificity. Allergen CRD offer a more detailed assessment of sensitization with the purpose to better anticipate clinical allergy. Instead of using allergen extracts consisting of mixtures of component proteins (whole allergens), CRD identifies sIgE responses to pure individual allergen proteins (molecular components) providing more specific test results.

In studies **VII-IX** we examined how CRD can help provide a personalized approach to pet allergy diagnostics. In dog molecular diagnostics, Can f 5 has been identified as a major dog allergen. Can f 5 is a prostatic kallikrein secreted from the prostate, and therefore only found in male dogs. This suggests that dog-allergic patients, who are monosensitized to Can f 5, can tolerate female dogs. Study **VII** describes one such patient with a history of dog allergy, but not when exposed to female dogs, and whose sensitization pattern showed monosensitization to Can f 5. To confirm our theory, we performed *in vivo* tests. As the extracts available for SPT are manufactured using dander from a mixture of female and male dogs, we had special extracts made for this study derived from the hair of male and female dogs separately. With these extracts, we performed SPT and conjunctival allergen provocation tests and thereby confirmed that the patient showed no reaction to female dogs.

In study **VIII,** we conducted a double-blind, randomized controlled trial, where we aimed to compare reactions to SPT and conjunctival allergen provocation tests using male- and female dog extracts in Can f 5 monosensitized children and children who were sensitized to a mix of the dog components. Our study showed that dog-sensitized children with monosensitization to Can f 5 can tolerate female dogs with no reaction to neither SPT nor conjunctival allergen provocation test using female dog extract. Such a precision medicine

approach makes it possible for many dog-allergic persons to get a female dog in their homes. However, male- and female dog extracts are not yet commercially available.

In study **IX**, we provided a review of aeroallergens originating from pets focusing on dog, cat, and horse. We explored the clinical utility of CRD and future potentials within this field. CRD seems to assist in providing a detailed and personalized sensitization profile in patients, which may ultimately lead to individual treatment- and management options. However, some issues regarding the clinical utility of CRD remain to be elucidated further in patients with pet allergy, for example the role of component sensitizations as predictors and markers of disease severity. Further the usefulness of CRD to identify patients who will respond positively to allergen immunotherapy remain to be explored including monitoring treatment responses following immunotherapy. For now, CRD should be used as an add-on to extract-based testing, not an alternative, when clinical history and conventional tests are inconclusive. CRD can differentiate abnormal sensitization patterns that include cross-reactive sensitizations or minor allergen components.

In conclusion, the series of papers presented in this thesis (**I-IX**) highlight the complexity of allergy diagnostics and support programming in early life suggesting that proper early interventions may impede the development of atopic diseases. These findings should be validated in randomized controlled trials, such as investigating early oral exposure to aeroallergens as a prevention of development of allergic rhinitis in line with what has been done for food allergens. The mechanism may be enforced by imposing a favorable gut microbiome composition to at-risk newborns, and indirect alterations of the gut microbiome with raw milk consumption or fecal transplantations in newborns, to reduce the risk of atopic disease development, are already being explored. Finally, if an allergy has developed, CRD can help identify patient-specific patterns of sensitization with a possibility to provide individualized extracts for allergen immunotherapy, making treatment success more likely. Unraveling the mechanisms and underlying endotypes of atopic diseases might allow improved diagnostics and prevention strategies to counter the current atopic pandemic and reduce the major burden these diseases impose.

Danish summary

Forekomsten af astma, atopisk dermatitis (AD), allergisk rhinitis og fødevareallergi (samlet kaldet atopiske sygdomme) er steget i de seneste årtier og prævalensen er aktuelt omkring 20% globalt. Sygdommene påvirker barnets livskvalitet og forældrenes arbejdsproduktivitet, og på grund af udbredelsens omfang er den samlede socioøkonomiske byrde betydelig. Atopiske sygdomme debuterer oftest i den tidlige barndom, men ætiologien og sygdomsmekanismerne er ikke fuldt ud forstået, hvilket kompromitterer vellykket forebyggelse og behandling.

Formålet med denne afhandling er at undersøge mekanismerne bag atopiske sygdomme, at undersøge betydningen af bestemte miljøfaktorer i det tidlige liv, forud for sygdomsudvikling, og endelig at udforske nye diagnostiske værktøjer til at fortolke allergisk sensibilisering mere præcist. Afhandlingen er baseret på ni studier fra Copenhagen Prospective Studies on Asthma in Childhood (COPSAC)$_{2000}$ og COPSAC$_{2010}$ fødselskohorterne, der undersøger fordelingen af sIgE-niveauer hos børn (**I**) samt hvordan forældres atopiske status (**II**), sensibiliseringsmønstre (**III**), debutalder og sværhedsgraden af AD (**IV**), amning (**V**), og hunde, katte og husstøvmider i hjemmet (**VI**) er forbundet med udviklingen af atopiske sygdomme i barndommen. Endelig, hvordan component diagnostik (CRD) har en rolle i patient-centreret diagnostik (**VII-IX**).

I studie **I** undersøgte vi fordelingen af specifikke IgE (sIgE) niveauer hos børn, da dette er et af hovedkriterierne for definitionen af atopi. Vi fandt, at medianværdien af sIgE varierede blandt de forskellige allergener - også blandt symptomatiske børn - hvilket understreger problemet med at bruge den samme grænseværdi for sIgE for alle allergener og tilskynder til at disse grænseværdier opdateres, så man får forskellige grænseværdier for hvert enkelt allergen.

I studie **II** undersøgte vi sammenhængen mellem forældres sIgE, total IgE og astma og udvikling af de samme træk hos børn i løbet af de første 6 leveår. Tidligere undersøgelser har vist, at der er en betydelig arvelig komponent ved de atopiske sygdomme, og at den

individuelle variation i udviklingen af sygdommene kan tilskrives både genetiske risikofaktorer og bestemte miljøeksponeringer. Intuitivt ville det forventes, at mor og far bidrager lige meget til arvelighed. Studie **II** viste en konsekvent stærkere effekt af mors sammenlignet med fars tilstedeværelse af allergisk sensibilisering, forhøjet total IgE og astma, især i den tidlige barndom. Disse resultater antyder, at ikke-genetiske faktorer *in utero* og/eller postnatalt kan spille en væsentlig rolle i den arvelige del af sygdomsmodtagelighed.

Når først et barn har været udsat for ugunstig genetik og miljøeksponering, kan allergenspecifikke IgE-molekyler udvikle sig, men betydningen af deres tilstedeværelse er ikke klar. I studie **III** analyserede vi mønstre af sIgE og priktest (SPT) gennem barndommen i alderen 6 måneder til 13 år i forhold til udvikling af astma ved 13 år. Kun børnene med det tidligt opståede, persisterende sensibiliseringsmønster havde øget risiko for at udvikle astma, og børnene med det sent-debuterende sensibiliseringsmønster havde det ikke. Yderligere var polysensibilisering og højere niveauer af sensibilisering på alle tidspunkter forbundet med en øget risiko for astma ved 13 år (både for SPT og sIgE). Disse resultater understøtter hypotesen om programmering i det tidlige liv og understreger vigtigheden af at overveje polysensibilisering og niveauet af IgE/størrelsen af SPT, når de kliniske implikationer af en positiv test skal vurderes.

I studie **IV** undersøgte vi, om tidlig versus sent indsættende AD og sværhedsgraden af AD var forbundet med udvikling af sensibilisering for luftbårne allergener og allergisk rhinitis ved 6-7- og 12-årsalderen. Vores resultater tydede på, at stigende sværhedsgrad af AD med tidlig debut øgede risikoen for at udvikle sensibilisering og allergisk rhinitis, mens debut af AD efter 1-årsalderen ikke gjorde det. Det kunne betyde, at hudbarrieredefekten og/eller inflammationen i huden ved AD i spædbarnsalderen kan have en programmerende effekt på udviklingen af respiratorisk allergi senere i barndommen, og at AD med tidlig debut versus sen debut kan repræsentere forskellige endotyper.

I studie **V-VI** undersøgte vi eksponeringer i det tidlige liv, der menes at påvirke udviklingen af sensibilisering og atopisk sygdom. Vi fandt dog ingen effekt af varigheden af amning (**V**) på udvikling af allergisk sensibilisering fra 0-6 år eller AD, astma/wheeze eller allergisk

rhinitis ved 7 år. Vi fandt heller ingen effekt af eksponering for hund, kat eller husstøvmider (**VI**) under graviditeten eller det første leveår på udviklingen af hverken sensibilisering over for de samme allergener eller allergisk rhinitis fra 0-13 år. Disse resultater understøttede ikke de daværende anbefalinger fra Sundhedsstyrelsen om at fuldamme længe eller at undgå eksponering for katte, hunde eller husstøvmider for at forhindre udvikling af atopisk sygdom i barndommen.

Som diskuteret i studie **I**, kan allergidiagnostik være meget udfordrende, især i kraft af den høje forekomst af falsk positive resultater. Normalt måles allergisk sensibilisering ved brug af blandede allergenekstrakter til at måle både sIgE i serum og/eller SPT. Disse tests er følsomme, men mangler specificitet. Allergen CRD tilbyder en mere detaljeret vurdering af sensibilisering med det formål bedre at forudse klinisk betydningsfuld allergi. I stedet for at bruge allergenekstrakter bestående af blandinger af komponentproteiner (hele allergener), identificerer CRD sIgE til rene individuelle allergenproteiner (molekylære komponenter), hvilket giver mere specifikke testresultater.

I studie **VII-IX** undersøgte vi, hvordan CRD kan bruges til en mere detaljeret allergidiagnostik overfor kæledyr. Ved hjælp af molekylær diagnostik af hundeallergi er Can f 5 blevet identificeret som et vigtigt hundeallergen. Can f 5 er et prostata specifikt protein, der udskilles i urinen, og som kun er til stede hos hanhunde. Dette tyder på, at hundeallergiske patienter, som kun er sensibiliserede over for Can f 5, kan tåle hunhunde. Studie **VII** beskriver en sådan patient som kun reagerede, når hun blev udsat for hanhunde, og hvor sensibiliseringsmønsteret viste monosensibilisering for Can f 5. For at bekræfte vores teori udførte vi også *in vivo* tests. De kommercielt tilgængelige ekstrakter, der anvendes til SPT og sIgE, består af en blanding af både han- og hunhunde. Til dette studie fik vi lavet specielle ekstrakter fra han- og hunhunde separat og udførte SPT og øjenprovokationstest ved hjælp af disse ekstrakter. Vi kunne derved bekræfte, at patienten kun reagerede på hanhunde.

I studie **VIII** gennemførte vi et dobbeltblindet, randomiseret kontrolleret forsøg, hvor vi ville sammenligne børn monosensibiliseret til Can f 5 med børn sensibiliseret over for en blanding af hundekomponenterne og undersøge, om de reagerede forskelligt på SPT og

øjenprovokation ved brug af han- og hunhundeekstrakt. Vores undersøgelse viste, at børn, der er monosensibiliserede over for hundeallergenet Can f 5, ikke reagerer på hverken SPT eller øjenprovokation med hunhundeekstraktet. Den slags individuel og detaljeret diagnostik gør det muligt for mange hundeallergiske personer at få en hunhund i deres husstand. Han- og hunhundeekstrakter er dog endnu ikke kommercielt tilgængelige.

I studie **IX** gav vi et overblik over de allergener katte, hunde og heste består af og undersøgte det potentielle kliniske udbytte af CRD inden for kæledyrsallergi. CRD kan være med til at lave en detaljeret og personlig sensibiliseringsprofil hos allergipatienter, hvilket i sidste ende kan føre til individualiserede behandlinger og håndteringsmuligheder. Nogle spørgsmål vedrørende den kliniske anvendelighed af CRD mangler dog at blive belyst yderligere hos patienter med kæledyrsallergi, for eksempel allergenkomponenternes rolle som prædiktorer og markører for sygdommens sværhedsgrad. Yderligere om CRD kan bruges til at identificere de patienter, der vil have mest gavn af allergen immunterapi, herunder behandlingsresponset efter immunterapi er afsluttet. Indtil videre bør CRD betragtes som en tilføjelse til de traditionelle SPT og sIgE analyser, snarere end en erstatning, når man er usikker på allergidiagnosen. CRD kan identificere atypiske sensibiliseringsprofiler, der involverer mindre hyppige allergenkomponenter eller krydsreaktive allergener.

Alt i alt fremhæver studierne præsenteret i denne afhandling (**I-IX**) kompleksiteten af allergidiagnostik og støtter teorien om programmering i det tidlige liv, hvilket tyder på, at behørige indgreb tidligt i barndommen kan hæmme progressionen af atopiske sygdomme. Disse resultater bør valideres i randomiserede kontrollerede forsøg, såsom undersøgelse af tidlig oral eksponering for luftbårne allergener som forebyggelse af udvikling af allergisk rhinitis på linje med, hvad der er blevet vist for fødevareallergener. Denne sammenhæng kan muligvis forstærkes ved at inducere et optimalt tarmmikrobiom til nyfødte i øget risiko, og man er allerede ved at udforske effekten af indirekte ændringer af tarmmikrobiomet med brug af råmælk eller fækale transplantationer hos nyfødte, med det formål at reducere risikoen for udvikling af atopisk sygdom. Hvis en allergi allerede har udviklet sig, kan CRD hjælpe med at identificere patientspecifikke sensibiliseringsmønstre med mulighed for at levere individualiserede ekstrakter til allergen immunterapi, hvilket øger

effekten af behandlingen. Ved at belyse mekanismerne og de underliggende endotyper af atopiske sygdomme kan man forbedre diagnostik og forebyggelsesstrategier for at imødegå den nuværende atopiske pandemi og reducere den store byrde, disse sygdomme inducerer.

Acknowledgements

"*We are like dwarves sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours*."

Philosopher Bernard of Chartres, 12th century

The work presented in this thesis is based on investigations performed at the COPSAC research clinic, Danish PediatricAsthma Center, Herlev and Gentofte Hospital, University of Copenhagen, from 2014 to 2021. COPSAC is based on the continuous participation and commitment of all the families included in the cohorts, and I want to say a special thanks to them and to all funding agencies, who supported the studies financially (acknowledged on www.copsac.com).

Of course, I also want to thank the founder of COPSAC, Professor Hans Bisgaard, who has guided and supported me for over a decade now. None of this would have been possible without you, and not only do I value our prosperous academic discussions throughout the years, but I have also come to cherish the personal ones, making COPSAC feel like home. Another big part of the COPSAC-vibe is all the 60+ inspiring people who work there. All of you comprise such a unique and crazy-intelligent team that makes it all worthwhile. Thanks to all my co-authors for invaluable input and support, and especially to Professors Bo Chawes and Klaus Bønnelykke, for our fruitful discussions and constant challenges, optimizing the research we do to the max.

Finally, I want to thank my family; first and foremost my husband, Mikkel, for your love and encouragement and for keeping dreams alive, our children, Lila, Oscar, Alva, and Adam, for reminding me what is important in life, my sister and brothers, Bibi, Andreas, and Willy, for being there through ups and downs, my father, Carsten, for the strength and determination you have left in me, my step-mother, Lisbeth, for your constant support, my step-father, Michael, for assuming an active role in COPSAC operating behind the scenes and taking a big part i steering the ship – thank you.

Last, but not least, I wish to dedicate this thesis to my mother; you are always there for me, and you have an infinite force in you that makes you keep going with an effortless, cheerful stamina. You have made my journey so much smoother, and I cannot thank you enough. You are completely inspiring, and I hope I make you proud.

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