Risk factors for developing atopic dermatitis

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THE THREE ORIGINAL PAPERS ARE:

I. Bisgaard H, Halkjaer LB, Hinge R, Giwercman C, Palmer C, Silveira L, Strand M. Risk analysis of early childhood eczema. J Allergy Clin Immunol 2009 June;123(6):1355-60.

II. Giwercman C, Halkjaer LB, Jensen SM, Bonnelykke K, Lauritzen L, Bisgaard H. Increased risk of eczema but reduced risk of early wheezy disorder from exclusive breast-feeding in high-risk infants. J Allergy Clin Immunol 2010 April;125(4):866-71.

III. Carson CG, Halkjaer LB, Jensen SM, Bisgaard H. Alcohol Intake in Pregnancy Increases the Child's Risk of Atopic Dermatitis. The COPSAC Prospective Birth Cohort Study of a High Risk Population. PLoS One 2012;7(8):e42710. Epub 2012 Aug 15.

GENERAL INTRODUCTION TO ATOPIC DERMATITIS IN CHILDHOOD

EPIDEMIOLOGY

Atopic dermatitis (AD) is one of the most common diseases among children in Western countries. The cumulative incidence of AD for young children in Denmark has increased 4-6 fold since the 1960s to a reported prevalence of 15-18% in 1990s and is apparently still increasing (1;2).
It usually debuts in the first year of life and is often associated with a family history of atopic diseases. AD can be a distressing condition to both the child and its parents, having a major impact on the child's and its families' quality of life, influencing the infant's well-being and social and educational development (3). Children report poorer quality of life especially due to scratching and sleep disturbances (4). Early detection of symptoms and further knowledge about possible risk factors for developing AD are therefore important.
There are significant economic costs for both the families and the society involved in the caring of children with eczema. These include both the direct costs to treatment of the child (medication, contact to physicians), but also costs related to the families loss of work (5).

APPEARANCE

AD is described as an itchy, chronically relapsing skin disease, with a typical morphology and skin localization. AD is more prevalent among girls than boys, girls being affected up to 2.6 times more often than boys (6), but boys develop AD at an earlier age than girls (7;8). Some studies, however, do not find any association to gender (9;10). AD often debuts with symptoms at the scalp, forehead, ear, and neck continuing to the extensor sides of the extremities and trunk and finally the flexor sides of the extremities (children under age 3 years) (11).
Infantile AD is typically an almost chronic dermatitis, which is followed by a more fluctuating course, with dermatitis that comes and goes over the next years (childhood dermatitis). Approximately two-thirds having infantile dermatitis will experience remission before their teenage years. However, continuation or relapse of dermatitis symptoms into early adulthood (early twenties) affects 15-25% of the children who had dermatitis. Persistence of dermatitis into later adulthood is rare, but more likely present if the symptoms have been very pronounced and persistent. Generally, a more severe cause of AD is seen, when developing early in life (12;13).

DIAGNOSIS

Uniformity and specificity are important when diagnosing AD, especially when doing clinical studies otherwise risking misclassification. However, uniformity in the use of diagnostic criteria for AD as well as validation is lacking. Often, the clinical diagnosis by a dermatologist has been used as a reference standard (14).
Today, there is no "golden test" to diagnose AD, but the diagnosis is traditionally based on a sum of criteria. Different studies choose different criteria for diagnosis, and some criteria – often the ones easier to handle - are chosen for clinical practice, whereas others are chosen for scientific studies, which is worth considering when comparing results from different studies.
An acceptable agreement has been found when comparing the The UK Working Party's Diagnostic Criteria for Atopic Eczema, Hanifin & Rajka, DARC (Danish Allergy Research Center) and Schultz criteria. In general, the use of each set of criteria will give similar frequencies of AD at least from the age of 12 months, with the mild cases of AD constituting the diagnostic problem (agreement rate 93-95 %). Results have suggested that repeated examinations provide a better certainty of diagnosing AD in small chil-

dren, with an advantage of longitudinal studies in comparison to cross-sectional studies (15).

Hanifin and Rajka diagnostic criteria

The Hanifin & Rajka criteria were used to diagnose AD in the COPSAC cohort (and thereby this thesis and its papers). The following 4 of the minor criteria were, however, excluded: keratoconus and anterior subcapsular cataracts (because they required identification by an ophthalmologist), delayed blanch (because it required an injection of methacholine) and impaired cell-mediated immunity (because it required advanced analyses). The criteria were chosen for diagnosing AD in the COSPAC cohort, because they are the most accepted international scientific diagnostic tool and marginally better in terms of sensitivity and specificity compared to the UK criteria (16;17).

The Hanifin and Rajka diagnostic criteria (18) were introduced in 1980 by Hanifin and Rajka in the absence of a clear definition of AD, mainly in order to conduct investigative studies. The diagnosis requires the presence of 3 of 4 major criteria and at least 3 of 23 minor signs (table 1).

Table 1: The Hanifin and Rajka diagnostic criteria for atopic dermatitis (18). Criteria in brackets were not included in this thesis.

Major criteria:
1. Pruritus
2. Typical morphology and distribution (erythema with vesicles/papules and/or scaling/ squamatization in a minimum of 2 regions)
3. Chronic or chronically relapsing dermatitis (physician-verified dermatitis for a minimum of 6 weeks or recurrent dermatitis within 6 months)
4. Personal or family (1st degree relative) history of atopy
Minor criteria:
a. Age debut:
Early age on onset ≤ 2 years
b. Objective clinical symptoms:
Xerosis
Ichtyosis/Keratosis pilaris/palmar hyperlinearity
Tendency towards non-specific hand- or foot dermatitis
Nipple eczema
Cheilitis
Perifollicular accentuation
Pityriasis alba
Anterior neck folds
c. Subjective clinical symptoms:
Itch when sweating
Intolerance to wool and lipid solvents
d. Immunological anomalies:
Elevated serum IgE
Immediate (Type I) skin test reactivity
Food intolerance
e. Skin infections
Tendency toward cutaneous infections/(impaired cell-mediated immunity)
f. Functional anomalies:
Facial pallor/facial erythema
White dermatographism/(delayed blanch)
Course influences by environmental/emotional factors
g. Anomalies of eyes and eye areas:
Recurrent conjunctivitis
Dennies-Morgan infraorbital fold
(Keratoconus)
(Anterior subcapsular cataracts)
Orbital darkening

SCORAD system

The SCORAD (SCORing Atopic Dermatitis) index was used to assess AD severity in the COPSAC cohort (and thereby this thesis and its papers).

For assessing AD severity several scoring-systems have been developed, but the most recommended is the SCORAD system (19;20). The SCORAD system calculates an index. When the index is below 25 (out of a maximum score of 103), AD severity is considered to be 'mild', between 25 and 50 to be 'moderate' and over 50 to be 'severe'. The index is based on information about the area of the body affected by dermatitis, the intensity of the dermatitis plaques regarding erythema, oedema, oozing, lichenification, dryness and excoriation (graduated 0-4) as well as subjective symptoms including the disturbance of sleep and degree of pruritus (graduated on a visual analog scale from 0-10). An objective SCORAD system has been established excluding the subjective components pruritus and sleeplessness from the SCORAD index leaving a possible ranging from 0 to 83 points. The severity is then categorized into mild (<15 SCORAD points), moderate (15-40 SCORAD points), and severe (>40 SCORAD points).

CO-MORBIDITY

AD is often found associated to the later development of asthma, allergic sensitization and allergic rhinitis, also known as "the atopic march". According to this, children debut with AD within the first years of life with a progression into asthma and then allergic rhinitis. The atopic march is supported by many cross-sectional and longitudinal studies highlighting AD, as a possible first step of this process (21-23).

SUMMARY OF PREVIOUS STUDIES ON RISK FACTORS FOR AD

STUDY DESIGN

Previous studies concerning this topic have often been cross sectional studies. Cross-sectional studies involve observation of a whole population, or a representative subset, at a defined time and are classified as an observational study. They can be used to describe both absolute risks and relative risks. They may be used to describe some feature of the population, such as prevalence of an illness, or they may support inferences of cause and effect, but are traditionally better suited for observations of associations. Longitudinal studies, such as prospective cohort studies, follow a group of similar individuals (cohorts), who differ with respect to certain factors, and seek to determine how these factors affect the outcome(s). Prospective cohort studies are typically ranked higher in the hierarchy of evidence than retrospective cohort studies, because the prospective study design enables a longitudinal data collection, which minimizes the risk of recall bias and gives good information about disease frequency. The prospective study is important when doing research on the aetiology of diseases and disorders in humans (cause and effect).

The COPSAC cohort, being a prospective birth cohort with close follow-ups, was designed to investigate the relationship between

environmental and genetic factors in the development of atopic diseases (asthma, allergy, allergic rhinitis and AD). The COPSAC cohort's major strength is its close follow-up using the clinic doctors instead of the patient's general practitioners, minimizing the risk of misclassification. However, as all mothers to the COPSAC children have a history of asthma, this selection of participants may limit the conclusions to involve high-risk children only, not the general population.

Inverse causality

When drawing conclusions from cross-sectional studies, it is important to be aware of the risk of inverse causality or disease-related modification of exposure. An example could be the interaction between breastfeeding and atopic disease, where debut of symptoms of dermatitis or wheezing tend to prolong the duration of exclusive breastfeeding, because of the general belief in its protective effect (24;25). Such inverse causation could be misinterpreted that longer duration of breastfeeding leads to dermatitis or wheezy disorder, when in fact the disorders led to longer breastfeeding. Cross-sectional studies with retrospective data collection cannot reliably account for the time-related relationship between disease and exposure, and are therefore prone to bias due to such inverse causation (24). Indeed, it has been suggested that observational studies may not be able to effectively reduce selection bias and inverse causation (26) and conclusions from such studies may not be valid (9;24;27;28).

GENETIC RISK FACTORS FOR AD

In a recent Swedish prospective birth cohort study following children till age 4 years, 27.1% of the children without atopic parents developed AD. In comparison, the proportion of children with single or double parental atopic history who developed AD was 37.9% and 50.0%, respectively (29). Many other studies have similarly reported the importance of hereditary factors for the development of AD (10;30;31), and is it generally considered, that AD is a polygenetic disease (32).

Filaggrin

In 2006, Palmer et al (33) were the first to show, that the two filaggrin (*FLG*) mutations, R501X and 2282del4, were associated with the development of AD. Several studies have replicated this finding, and the mutations are currently the most strongly associated genetic factors known to confer susceptibility to AD in European populations (33-37), with odds ratios varying between 3.73 and 7.1 (35;38). No negative or equivocal studies have been reported.

9-10% of the general population carry at least one null mutation in the *FLG* gene (37). Normal gene expression results in intracellular filaggrin proteins, which aggregate keratin filaments, leading to keratinocyte compaction and formation of the stratum corneum. The cornified cell envelope is crucial for the skin barrier function, as it prevents epidermal water loss and penetration of microbes, toxic chemicals and allergens (39). Heterozygous, and especially homozygous or compound heterozygous carriers of *FLG* null variants such as R501X and 2282del4, may experience dry, scaly and fissured skin (40;41). The two mutations are carried by 16.7 – 56 % of all individuals with AD (37), and are inherited in a semidominant mode (42). The high frequencies of *FLG* mutations are seen in studies investigating adults with both persisting and early onset of atopic dermatitis. The mutations are associated with asthma and allergy, early onset and persistence of AD, as well as certain phenotypic characteristics, e.g. hyperlinearity of the hands (33;35;43-45). *FLG* null defines an endotype characterized by dermatitis with predilection sites at exposed areas of the body, in particular hands and cheeks, and an upregulation of both acute and chronic morphological markers. Furthermore this endotype is characterized by an early onset of dermatitis and a more severe course, with more generalized dermatitis resulting in more frequent medical consultations (46). Clinical studies have shown that most homozygous individuals develop dermatitis very early in life, whereas heterozygous individuals may experience a milder course or no symptoms at all (42;47).

Other mutations in or nearby the *FLG* gene have been documented. In one population a third mutation, R2447X, was as strongly and independent associated to AD as the mutations R501X and 2282del4 (44). Besides R2447X, two other mutations, S3247X and 3702delG, were recently shown to be associated with AD, as well as other less frequent mutations. These mutations are qualitatively different from the old mutations, with some residual function as demonstrated by a significantly lower penetrance of dermatitis. Generally, these new mutations reported are neither as prevalent nor as strongly associated with AD, as the ones previously mentioned (44;48).

Other genes

Research on genetic causes of dermatitis, is usually divided into studies focusing on the systemic immune response (which characteristically shows a Th2 predominance and elevated IgE levels), skin-specific genes involved in cutaneous immunity, inflammation and infection and genes involved in epidermal barrier function (such as *FLG*). Associations have been found to interleukin-4 and 13, the interleukin-4 receptor (49-54), the mast cell chymase (55;56) and the gene encoding a serine protease inhibitor (kazal-type 5) which is involved in the epidermal differentiation and skin barrier formation (57-59). There is, however, a significant overlap and interplay between the function of the genes involved and a sharp division of the genes' function is not possible (60).

A recent large-scale genome-wide association study on 11,025 AD cases and 40,398 controls have identified three novel AD risk loci. Two loci, that suggest a role in epidermal proliferation and differentiation (rs479844 upstream of OVOL1 and rs2164983 near ACTL9), and one risk locus within the cytokine cluster on 5q31.1 (rs2897442 in KIF3A) (61).

Epigenetics

A few studies have been published about epigenetics and AD. Epigenetic modification means covalent modification of DNA and histones in the cells, which alters the determination of the expression of genes during the cells development and differentiation. Epigenetic modifications can be heritable, without involving a change in the DNA sequence, or can be due to environmental factors thus affecting the heritage, onset and progression of AD (62).

Focus has been on the high-affinity IgE receptor (FcεRI) which is strongly up-regulated on antigen-presenting cells of patients with AD. DNA demethylation at CpG dinucleotides in the 5′-region of the gene leads to an upregulation of the gene and an overexpression of the receptor leading to a hyperallergic response in patients with AD, thereby worsening their symptoms (63;64).

THE HYGIENE HYPOTHESIS

No clear exposure–disease relationship between allergens in early life and subsequent dermatitis has yet been found (65). Observations have been though, that more crowded houses, increased family size and the number of older siblings, might possibly increase early exposure to infections and offer protection to subse-

quent development of dermatitis (66). This has led to the formulation of the hygiene hypothesis, which seeks to explain the increased incidences in atopic diseases in developed countries to a change in early exposure to bacterial and parasitic infection (67). Maturation of the immune system and differentiation of naïve T-cells occur early in life. It is hypothesized that this maturation of the immune system is dependent on the microbial milieu, which again depends on both infections and on bacteria. The microbial burden is one major exposure in the newborn baby, who is otherwise exposed to a limited range of environmental factors. A proper balance between Th1 and Th2 responses is thought to be important in the regulation of immune responses. Newborns generally have a Th2-skewed pattern of immunity and in the absence of Th1-driving immune-maturing factors, young children remain prone to the development of Th2 responses and thus atopic disease. An unfavourable micro-flora could by this potentially skew maturation towards asthma, allergy and dermatitis (68;69).
Over the past centuries, Western living has resulted in smaller households, separated from animals, with improved household amenities and higher standards of personal cleanliness (67). According to the hygiene hypothesis, this could all lead towards more patients with AD.

ENVIRONMENTAL RISK FACTORS
Twin studies report a concordance rate for AD at 0.57-0.72 in monozygotic twins compared to 0.23 - 0.25 in dizygotic twin pairs (70;71). This supports the importance of genetic factors for the risk of developing AD, but also it indicates that environmental factors must be relevant. Generally, the aetiology of AD is considered to be multi-factorial, including interactions between genetic and environmental factors. The increase in incidences of AD over the last decades also supports the importance of environmental exposures and/or interactions with genetic factors, since genetic drift alone cannot explain the increase in AD.
The early development of AD suggests an environmental influence on disease expression early in life, maybe already intrauterine, why environmental factors triggering a genetic predisposition should be sought after, as early as the perinatal period. This is also supported by the observations of a higher risk for developing AD from the maternal line compared to the paternal line (66;72-74), although these findings are not confirmed by other studies (10;29;75).

Socioeconomics
A higher prevalence of AD has repeatedly been observed among high income families independent from household size and the number of older siblings (76-78). This was seen in physician-diagnosed dermatitis and could therefore not be explained only be differences among social classes in respect to reporting and labelling of symptoms (79).

Pets
A recent systematic review of the evidence for a role of furry pets in dermatitis found no clear evidence, that early pet exposure is associated with increased risks of subsequent dermatitis, and suggested that avoidance behaviour in high-risk families could explain such findings (inverse causality) (80). However, none of the studies reviewed accounted for *FLG* mutation and the interaction between cat exposure and *FLG* mutation. COPSAC recently reported a significant interaction between the *FLG* null mutations and cat at home at time of birth on the development of early-life AD and confirmed this in another birth cohort. COPSAC found that cat, but not dog ownership, substantially increased the risk of AD within the first year of life in children with *FLG* null mutations (HR 11.11, 95 % CI 3.79–32.60, p< 0.0001), but not amongst those without (81).

Alcohol
Alcohol consumption has been suspected as one of the factors contributing to the rise in atopic diseases (82;83) as alcohol consumption is part of the Western lifestyle and its intake has increased in the same period with an annual consumption nearly tripling in Denmark since 1955 (84;85). Also, alcohol consumption during pregnancy is frequent in westernized countries (86-89).

Smoking
The effect of parental smoking and foetal exposure to maternal smoking during pregnancy on AD is controversial. Some studies find no effect of neither prenatal nor postnatal smoking on the risk of developing AD (90;91), while others showed an increased risk of AD from maternal smoking during pregnancy (92). One study found a trend towards a positive association between maternal smoking during pregnancy and the offspring's risk of developing AD, but failed to reach statistical significance (93). The contradictory findings could be due to confounding from the effect on socioeconomic status, as lower social class is associated with a higher prevalence of smoking.

Breastfeeding
Breastfeeding is widely advocated to reduce the risk of dermatitis, sensitization and wheezy disorders particularly in high-risk families. This aligns with the understanding that the causes of these diseases should be sought in pregnancy or early infancy, since the diseases typically debut during the first months of life. The early diet is one of very few environmental exposures of the infant and avoiding non-human protein in the diet seems sensible. However, the evidence on such protective effect is ambiguous (10;26-28;94-98). AD has been related to a disturbed metabolism of polyunsaturated fatty acids (99-101), and it has previously been shown that the breast-milk from atopic mothers had significantly higher levels of n-6 and lower levels of n-3 fatty acids than non-atopic mothers (102).

Other post- and perinatal risk factors
In general, only few and in some cases contradictory findings have been published in respect to other pre- and postnatal risk factors for developing AD. Many studies have classified dermatitis from symptom questionnaires, a history of community doctors' diagnoses or have been cross-sectional observations, which hamper the results.
Higher birth weight, higher gestational age at birth and prenatal antibiotic exposure have been found positively associated with the risk of AD (9;74;78;103-105), whereas the AD rate decreased with an increasing maternal age at delivery and for children living on a farm (78;106;107). Conflicting results exist in respect to day care attendance, race/ethnicity and air pollutants (9;74;78;106;108;109). Early exposure to solid food in infancy has been shown to be associated to the later development of AD. Specifically, the introduction date for fish, milk, butter and nuts were found inverse related to the risk of AD. However, the results are conflicting (110-113).
No associations have been found in respect to mothers BMI, Paracetamol use during pregnancy, parity and antibiotic exposure through breastfeeding (78;103;104;114).

Figure 1 summarizes previously studies' findings about risk factors for developing AD.

AIMS & OBJECTIVES

The aim of this thesis was to investigate possible risk factors affecting the development of AD.

AD is a frequent disease among children and has a substantial impact on the lives of both the child and its family. A better understanding of the disease would enable better treatment, prevention and information to the families involved. Even though there have been several earlier studies concerning this topic, many of them have been hampered by an unsuitable study design (questionnaires, cross sectional observations, limited sample size, incomplete follow-up or small numbers of confounder assessments) or difficulties in a standardization of diagnosing AD (history of community doctor's diagnoses, large inter-observer variation and corresponding risk of misclassification).

The COPSAC cohort is unique in its high follow-up rate, comprehensive longitudinal data collection and close (minimum half-yearly) contact to the families, with the doctors at the COPSAC research unit treating and diagnosing the children instead of the patients' general practitioner. This is believed to be important for the understanding of AD and hopefully contribute to better diagnose and treat of this otherwise heterogenic and not well understood disease. In this thesis, we did a comprehensive risk factor analysis using the huge data material available within the COPSAC cohort. First, we tested 40 possible risk factors at once using a cross sectional approach (paper I). This traditional risk factor analysis led to two borderline significant results: duration of exclusively breastfeeding and mothers alcohol intake during pregnancy. Both possible risk factors had relatively high odds ratios (2.8 and 1.9, respectively) and could therefore not be neglected. Therefore, we secondly did two in-depth studies investigating duration of exclusively breastfeeding and mothers alcohol intake during pregnancy, respectively, and its effect on AD, using longitudinal data and data analysis instead of the traditional cross-sectional approach (paper II & III).

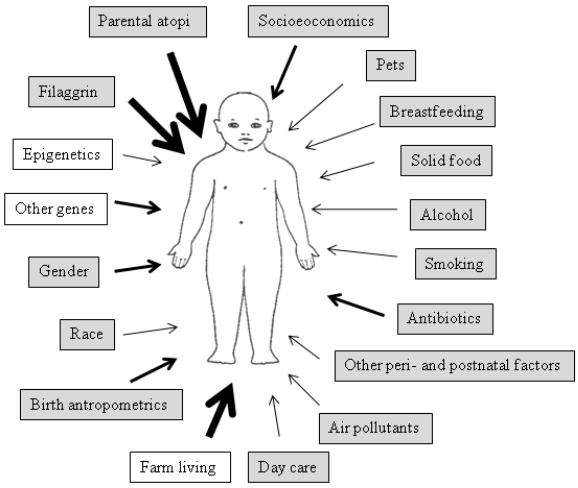

Figure 1

Summary of previous studies' findings about risk factors for developing AD. The sizes of the arrows express the level of evidence (thin/medium/thick arrow: low/medium/high level of evidence). Grey labels indicate risk factors investigated in this thesis.

METHODOLOGY

ETHICS STATEMENT

The COPSAC studies were conducted in accordance with the guiding principles of the Declaration of Helsinki, approved by the Ethics Committee for Copenhagen (KF 01-289/96) and The Danish Data Protection Agency (2002-41-2434), and in compliance with "Good Clinical Practice" (GCP) guidelines. Informed written consent was obtained from all parents.

PARTICIPANTS

The COPSAC cohort (Copenhagen Prospective Study on Asthma in Childhood) is a single-center, prospective birth cohort, following 411 children born to mothers with asthma (115) (figure 2).

In brief, pregnant women from Greater Copenhagen with a history of physician-diagnosed asthma received written information about the study (N=798), of whom 452 attended the clinic for in-depth information, and 411 accepted enrolment between August 1998 and December 2001. The cohort was comparable with the population of Greater Copenhagen, on the exception that mothers of the cohort population were less likely to have given natural childbirth, the households were slightly less affluent, with fewer children and fewer pets and Caucasians may be overrepresented. The target population of the study was defined as healthy newborns (>36 gestational weeks with no congenital abnormality, systemic illness, history of mechanical ventilation or lower airway infection). Data validity was assured by quality control procedures. History was recorded online during visits to the COPSAC clinic. Objective findings were double-checked against source data and the database subsequently locked and tracked by an audit trail, showing operations on the database after locking.

The children were enrolled at one month of age and visited the COPSAC clinic at scheduled visits every six months thereafter, until age 7 years, as well as for any acute complaints from the skin or airways. Skin examinations, diagnoses and treatment of dermatitis were handled in accordance with predefined standard operating procedures, by trained medical doctors employed for this purpose in the COPSAC clinic. The parents were told to note all skin diagnoses and use of medication prospectively between visits.

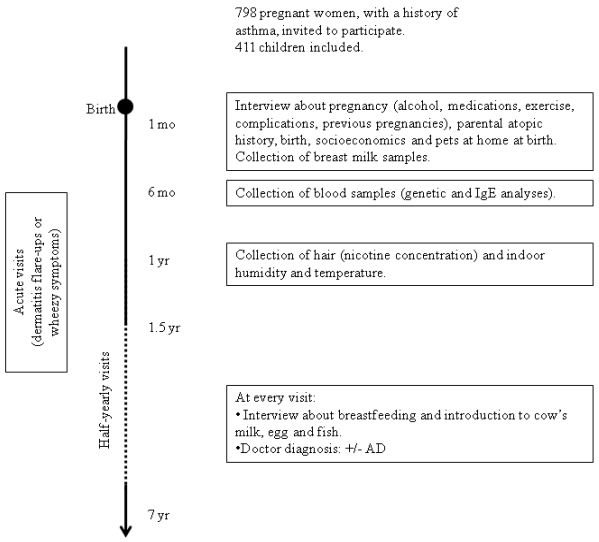

Figure 2

Study design.

The families used the clinical research unit (not the family's general practitioner) for diagnosis and treatment of any atopy-related symptoms.

RISK ASSESSMENT

Genotyping

Genotyping for R501X and 2282del4 was performed screening for short, highly specific PCR fragments as well as allelic discrimination assay, using TaqMan-based allelic discrimination assay and fluorescently labelled PCR (Applied Biosystems 3100, 3730 and 7700 sequence detection system, Foster City, CA, USA) (33). Children with either (or both) variants were classified as having the mutant allele. Call rates for both the R501X and 2282del4 mutations were 100%. The observed genotype frequencies of the two *FLG* polymorphisms did not deviate significantly from the expected frequencies under assumption of Hardy-Weinberg equilibrium in any of the study populations ($p>0.05$).

Parental history

Genetic predisposition included information on parental history of dermatitis, allergic rhinitis and/or asthma. Demographics were described by gender, race and season of birth as well as gestational age, head circumference, length and weight at birth, the latter supported by midwifes notes. Socioeconomic status was evaluated from household income (continuous variable), the educational level of parents divided into three categories (elementary or middle school, college and university) and occupational status described by four groups (professionals, non-professionals, unemployed and students) based on DISCO classification and ISCO-88 (International Standard Classification of Occupations; international Labour Organization, www.ilo.org) (115).

Pregnancy

Pregnancy was described in terms of number of previous child births, complications and exposures. Pregnancy complications during 3rd trimester were described by occurrence of pre-term contractions, preeclampsia, pneumonia or influenza, by mother's asthma status (worse, same, better), by mode of delivery (C-section, vacuum, natural birth) and by Apgar score (9-10 or <9). Exposures during pregnancy (each trimester separately) comprised information on smoking, alcohol, medications (oral antibiotics and Paracetamol) and physical exercise (0, 1 and > 1 time per week) and were assessed at the first visit (1 month after birth) in the clinic.

The average alcohol intake pr. week pr. trimester was entered online. Mothers drinking minimum one unit of alcohol in average pr. week in at least one of the 3 trimesters were defined as having an intake of alcohol during pregnancy. One unit of alcohol was defined as 15 ml or 12 g of alcohol, explained to the mothers as e.g. one beer or one glass of wine.

Postnatal exposures

Cat and dog living in the house at time of birth was assessed at the 1 month visit. Information about the childs age at introduction of egg, cow's milk and fish, as well as use of feather pillow and age at which the child started day care was assessed at every scheduled visit.

Mothers were asked about breastfeeding cessation at every scheduled and acute visit. In the database, breastfeeding was recorded as the duration of exclusive breastfeeding. Mother's milk was collected at the visit one month after birth. Milk samples (2-5 ml) were added 0.01% butylated hydroxytoluene (Sigma), frozen at -80°C and analyzed within one year after they had been taken. Lipids were extracted and separated by gas-liquid chromatography (102). The fatty acid composition of all breast-milk samples was determined in duplicate and all analyses were successful.

Sensitization

Sensitization was determined in the children at age ½, 1½ and 4 years measuring specific IgE level against the 15 most common inhalant and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, egg, milk, fish, wheat, peanut, soybean, shrimp) determined by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden). Specific IgE levels ≥ 0.35 kU/L were considered as indicative of sensitization (116) and were analyzed as a dichotomized measurement. Sensitization was determined in the parents also.

Objective assessments of air-borne exposures

Nicotine concentration was measured in hair sampled one year after birth (117). Indoor humidity and temperature was measured simultaneously in the children's bedroom using a HOBO® H8 RH/Temp Logger (www.onsetcomp.com). Measurements were logged every 10 minutes for a 4 week period within the first 2 years of life.

CLINICAL END-POINTS

All diagnoses were performed by the doctors in the COPSAC clinic, not the children's general practitioner.

Atopic dermatitis

Atopic dermatitis was diagnosed according to the criteria given by Hanifin & Rajka, as previously described ("General introduction to atopic dermatitis in childhood, diagnosis") (18).

Wheezy episodes

Wheeze was recorded by the parents in diaries starting one month after birth. The term wheeze was explained to the parents as wheeze or whistling sounds, breathlessness or recurrent, troublesome cough, severely affecting the well being of the infant. A wheezy episode was defined from the diary as an episode of 3 consecutive days with recorded wheeze. This end-point definition was described and validated in previous studies (118-120).

Wheezy exacerbations

Exacerbations were defined as wheezy symptoms treated with high-dose inhaled steroid, oral steroids or leading to hospitalization as described in previous studies (118-120).

Childhood asthma

Childhood asthma was diagnosed based on pre-asthma (recurrent episodes of troublesome wheezing, breathlessness and/or cough) and the response and subsequent relapse to a 3-month trial of inhaled corticosteroids (115).

Rhinitis

Rhinitis was defined as troublesome sneezing, blocked or running nose (minimum 1 hour pr day for 2 consecutive days) in the past 12 months in periods without accompanying cold or flu (121).

STATISTICAL ANALYSIS

Statistical analyses used in this thesis are described together with the individual papers.

PAPER I

INTRODUCTION

In this paper we analyze the effect of environmental exposures in early life and the genetic predisposition on the development of AD before age three years.

The paper is based on a comprehensive analysis of the possible association between 40 potential risk factors and AD presenting by 3 years of age. Measured confounders were adjusted for, while estimating effects of these variables on the risk of developing AD, by fitting the subset of the 40 variables showing at least moderate association with AD, in the statistical model simultaneously.

METHODS

Participants

The study cohort was the COPSAC birth cohort (see previously: "Methodology, participants").

Statistical analyses

Simple logistic regression was performed for AD at 3 years (yes/no) for each of the 40 predictor variables. For the multiple logistic regression, the following basic steps were used to achieve a final model: (i) missing values were first imputed for incomplete variables (a total of 5 imputed data sets were created); (ii) a stepwise selection process was used to include variables in successive blocks; (iii) steps i and ii were repeated for a total of five model fits; (iv) variables selected in at least 4 out of 5 models were then refit in a multiple logistic regression model (without using stepwise selection); (v) step iv was repeated for each of the five imputed data sets; (vi) composite estimates and standard errors were determined by combining results from the five models. (The standard errors of the estimates accounted for the uncertainty due to imputation of data.)

There are different approaches for model selection. When there is one key predictor variable and a few covariates, one approach to test for confounding is to simply look for changes in the effect estimate of the key predictor, when the covariates are added/removed from the model, without regard to the p-values. But in our case, we have 40 predictors and rather than to test all possible combinations of variables to see how effect estimates change, a more feasible and common approach is to use stepwise selection, which is a stepwise, p-value-based method of selecting variables. This process is more helpful in eliminating variables from the confounder list, rather than identifying confounders. By definition, confounders are related to both the outcome and the key predictor. Although our main interest was not in p-values of covariates themselves, variables with very high p-values were thus eliminated from the list of potential confounders, since they were not significantly related to the outcome. On the other hand, if a variable was significant and was added to the model, it was not necessarily a confounder just because it had a small p-value. Instead, we developed a 'final model' that included a set of variables that predict AD using stepwise selection. This way we did not build the model with a 'key predictor' and 'covariates' in mind, but rather considered predictors equally and determined those with at least marginal significance based on an ordered, stepwise selection process. However, we can still interpret this model by focusing on one predictor and determine the influence of other variables on that predictor, by noting changes in the effect estimate from the univariable to multivariable models. Since the changes in effect estimates were generally small, the variables in the final model do not appear to be strong confounders on each other.

The Markov Chain Monte Carlo method was used to impute values (122). Continuous variables were either log transformed (if right skewed) or left unchanged before the imputation analysis. Missing data for variables (either continuous or categorical) were imputed using multivariate normal theory, as described by Bernaards (123). Blocks of variables were tested successively as follows: heredity (6 variables), social status (3 variables), preconception (3 variables), prenatal (10 variables), birth (7 variables), and postnatal (11 variables). Each variable within a group was tested using the following criteria: $p<0.15$ for entry and $p<0.20$ to stay. If a variable was entered and remained in the model when its block was tested, this variable was then forced to remain in the model when subsequent blocks were tested, which allows for the possibility that $p>0.2$ for some variables in the final model. For sets of analyses involving numerous models, the false discovery rate procedure was employed using a false discovery rate of 0.05 (124).

RESULTS

Descriptive statistics

A total of 356 children (183 girls and 173 boys) completed the clinical follow-up for the first 3 years of life. Reasons for withdrawal included death from sudden infant death syndrome (one infant), family moving and refusal by parents to continue in the study or missing a scheduled visit. Descriptive statistics for 40 risk factors analyzed for association with AD is presented in Tables A1 and A2 (Appendix I).

Predictors of atopic dermatitis

Of the 40 variables, 10 reached statistical significance using simple logistic regression (Tables A3 and A4, Appendix I). Presence of *FLG* null mutations, mother's dermatitis, father's asthma and/or rhinitis, 3rd trimester alcohol use and longer duration of exclusively breastfeeding were risk factors for AD development, while raised bedroom temperature, nicotine in hair, presence of dog around time of birth and increased baby length worked protective against AD development. After applying the false discovery rate procedure and using a false discovery rate of 0.05, only *FLG* null mutations, mother's dermatitis, dog at home at time of birth and bedroom temperature remained significant among the original 10 that were significant. In the multiple logistic regression model, 10 of the 40 predictor variables were included, based on selection criteria described in the Methods section. Significance of predictors in this model decreased slightly, relative to the univariable models (Table 2), which may be at least partially explained by co linearity of predictors. However, the results show general consistency between OR's from the univariable and multivariable models. Mother's dermatitis (OR 2.80, 95% CI 1.70–4.63, $p<0.0001$) and the *FLG* null mutations (OR 3.20, 95% CI 1.46–7.02, $p=0.004$) were the most significant factors in predicting AD at 3 years. Dog at home, baby's lengths at birth and bedroom temperature were also significant factors predicting AD by 3 years of age. Families with dogs had approximately half the odds of developing AD (OR 0.44, 95% CI 0.23–0.87, $p=0.02$). Increased length at birth (OR per cm increase: 0.87, 95% CI 0.78–0.97, $p=0.01$) and increased bedroom temperature (OR per 1°C increase: 0.80, 95% CI 0.66-0.97, $p=0.02$) were also associated with reduced risk of AD. Longer duration of exclusively breastfeeding and 3rd trimester alcohol use were found to be marginally significant risk factors for AD development ($p=0.10$ and 0.07, respectively). Figure 3 displays a forest plot of predictors in the multivariable model.

Table 2: ORs for development of AD by 3 years of age using multiple logistic regression*

		Multiple regression		Simple regression	
Variable	**Comparison**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Mother's dermatitis	Yes vs. no	2.80 (1.70-4.63)	<.0001	3.10 (2.00-4.79)	<.0001
Father's rhinitis	Yes vs. no	1.91 (1.09-3.33)	.02	1.83 (1.16-2.88)	.01
FLG mutations	Yes vs. no	3.20 (1.46-7.02)	.004	2.43 (1.23-4.79)	.01
Mother's work	Student vs. nonprofessional	0.97 (0.40-2.34)	.94^	1.13 (0.54-2.36)	.75^
	Unemployed vs. nonprofessional	2.93 (1.16-7.41)	.02^	2.32 (1.07-5.04)	.03^
	Professional vs. nonprofessional	1.17 (0.65-2.11)	.59^	1.33 (0.82-2.16)	.24^
Alcohol, 3rd trimester	Yes vs. no	1.88 (0.94-3.75)	.07	1.74 (1.00-3.05)	.05
Exercise per week, 3rd trimester	Once vs. none	1.44 (0.62-3.36)	.34^	1.87 (0.90-3.88)	.09^
	More than once vs. none	0.55 (0.15-1.94)	.26^	0.56 (0.24-1.29)	.17^
Length at birth	Increase (cm)	0.87 (0.78-0.97)	.01	0.90 (0.82-0.99)	.03
Dog at home	Yes vs. no	0.44 (0.23-0.87)	.02	0.37 (0.20-0.67)	.001
Time solely breastfed	Exposure-year increase	2.80 (0.87-9.03)	.10	2.76 (1.01-7.56)	.05
Temperature first year	1°C increase	0.80 (0.66-0.97)	.02	0.77 (0.66-0.91)	.002

*See Statistical Analysis for details on model fitting. For comparison, ORs for simple regression models are given at the right.
^ Denotes a comparison of 2 levels within a categorical variable. P values for categorical variables (as a whole): mother's work, P = 0.19 for simple regression and P =0.10 for multiple regression; physical exercise, P = 0.06 for simple regression and P = 0.05 for multiple regression.

Possible selection due to parents' allergy

Homes with parents being allergic to dog, may be less likely to own a dog, even before the child is born, causing a false association between dog exposure and atopic risk in the baby. Because of this we did a sub-analysis estimating the AD rate stratified for parents' sensitization. In homes of parents sensitized to dog, the AD rate was 38% if a dog was owned at time at birth and 55% if there was no dog. Likewise, among parents without dog sensitization, the AD rate among dog owners was 18% and 47% if there was no dog. These data demonstrate that even within dog allergy strata, rates of AD were lower for those who owned a dog compared to those that did not. Running a logistic regression for AD at 3 years of age as a function of dog ownership and dog allergy yields an OR of 0.311 for the dog effect (p=0.0004) and 1.485 for dog allergy effect (p=0.09). In the model without dog allergy, the OR for the dog effect is 0.291 (p=0.0002). When not restricting the data due to missing dog allergy information, the OR for the dog exposure effect is 0.37 (p=0.001). Thus, there is no indication of strong confounding by parents allergy to dog. When this process is repeated with the full multivariable logistic regression model (i.e., adding parents dog allergy variable to the model with 10 predictors, including dog exposure), similar results are observed.

Sensitization

Of the 356 children in the study, 321 of them went through testing for sensitization. Within these 321 subjects, 134 (42%) developed AD whereof 29 (21.6%) were sensitized. In the non-AD group, 24 (12.8%) of 187 were sensitized. None of the children were sensitized to dog before the diagnosis of AD. In 2 infants the sequence could not be determined, as AD was diagnosed after a negative test, but before a subsequent positive test for sensitization.

A subset analysis was performed to explore the relationship between sensitized and non-sensitized phenotypes, within those that developed AD by 3 years of age. Sensitization was tested three times in early childhood (½, 1½ and 4 years). A subject was considered sensitized, if he or she tested positive at least one time. Of the 40 variables tested, only duration of cow's milk in diet was found to be significant in differentiating the sensitized and non-sensitized children with AD. However, this one marginal significance did not hold up, when applying the false discovery rate of 0.05.

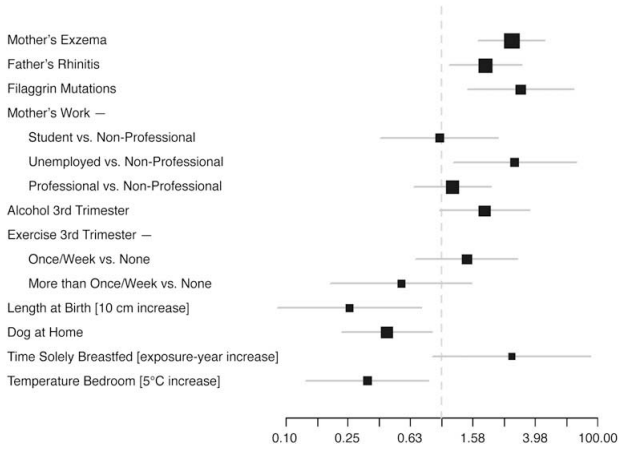

Figure 3

Forest plot of ORs and 95% CIs for predictors of development of AD by 3 years of age based on multiple logistic regression (See Statistical Analysis for details on model fitting). Variables that are risk factors for AD are greater than 1; those that are protective are less than 1. The box sizes are inversely proportional to the variances of the associated estimators.

Paper II

INTRODUCTION

In this paper, we performed a clinical study in order to assess the effect from environmental exposures, on the development of AD and wheezy disorders in our high-risk birth cohort. Our previous conventional cross-sectional analysis at 3 years of age raised suspicion, that duration of exclusive breastfeeding increased the risk of AD with an odds ratio of 2.80 (0.87-9.03), but this was not statistically significant (p=0.10) (paper I) (125). Furthermore, as in other studies, this observed trend could be biased due to "inverse causality" i.e. disease-related modification of exposure (disease manifestation in the child may motivate longer breastfeeding, due to believe of a protective effect), as well as a number of other methodological issues that often confound such complicated interrelations. This could erroneously bias the conclusion towards prolonged breastfeeding as a cause of disease. Therefore, we developed a novel statistical strategy for such analysis, taking advantage of the longitudinal information on symptom debut and duration of breastfeeding.

Furthermore, we studied the potential influence of n-3 and other fatty acids in breast milk, on the risk of AD and wheezy disorders.

METHODS

Participants

The study cohort was the COPSAC birth cohort (see previously: "Methodology, participants").

Statistical analysis

The effect of exclusive breastfeeding (breastfed yes/no at any specific age) and duration of exclusive breastfeeding on the risk of developing AD and wheezy disorder was analyzed by Poisson regression, with log person months as offset. The incidence of first AD diagnosis and first wheezy episode was estimated as cases per person month.

To avoid inverse causation, only information on exposures prior to disease onset was used. Disease onset was defined as the date for the AD diagnose, diagnosed by the doctors in the COPSAC clinic. Person months at risk were calculated for the children from birth to age of diagnosis, two years of age or first age of dropout, whichever came first. To avoid conditioning on the future, we evaluated the effect of breastfeeding duration in individuals, for whom exclusive breastfeeding ended prior to disease onset. This was done by analyzing the effect of time since end of breastfeeding, adjusting for age. The relative risk (RR) of exclusive breastfeeding and time since end of exclusive breastfeeding was assessed and interpreted as a comparison with a child of similar age still breastfed. The p-values associated with RR corresponded to Wald tests. All other p-values corresponded to likelihood-ratio tests.

The time variables (age and time since breastfeeding) entered the analysis in three-month intervals. Robustness of the results was evaluated by redoing the analysis with different groupings of time variables.

In the analysis of AD we adjusted for gender, birth weight, BMI, *FLG*, father's and mother's dermatitis, and cat and/or dog at home at time of birth. In the analysis of wheezing episodes, we adjusted for day care and mother's smoking during 3rd trimester. The confounders were placed in the regression models simultaneously. Effect modification was assessed by likelihood ratio tests, comparing models with and without interaction terms.

The correlation between the composition of fatty acids in mothers' milk and AD or wheezy disease, was examined by survival analysis. In order to take the duration of fatty acids transmission into account, we only considered the children, as long as they were exclusively breastfed. Survival analysis was performed with the use of Cox proportional-hazards regression. The dependent variable was the time to a first AD/wheeze diagnosis. The fatty acids were included as the quantity by which they were measured in the breast-milk. For this analysis, a child was censored at end of breastfeeding, or when he/she was no longer participating in the study, whichever came first. The analyses were adjusted for the same confounders, as were used in the analyses of the effects of breastfeeding. Control for functional form and proportional hazards were performed using plots and p-values based on martingales (126).

The statistical analysis was done using PROC GENMOD and PROC PHREG in SAS version 9.1 as well as R version 2.6.1. The overall significance level used was 0.05.

RESULTS

Breastfeeding

Of the 411 COPSAC children, 58 were missing information on duration of exclusive breastfeeding, 11 were non-Caucasians (excluded, since we adjusted for *FLG*) and 21 children were never breastfed and could not be analyzed in the chosen model. The remaining 321 infants were exclusively breastfeed for a mean duration of 121 days (range 1-274). 69 infants were exclusively breastfed less than 3 months, 203 for 3-6 months and 49 for more than 6 months.

Atopic dermatitis

AD was diagnosed in 122 (38%) of the 321 infants before age 2 years. The proportion of children who developed AD was 29%, 37% and 55% in children who were exclusively breastfed less than 3 months, 3-6 months or more than 6 months, respectively.

The multivariate analyses of breastfeeding as a risk factor for developing AD included 306 infants (6 missed information on *FLG* variants R501X and 2282del4, 8 missed information on father's atopic history and 1 missed information on pets at home at birth). AD was diagnosed in 116 (38%) of these infants before they either left the study or turned 2 years of age.

The effect of exclusive breastfeeding on the risk of developing AD was significant after adjustment for demographics, *FLG* variants R501X and 2282del4 status, parent's dermatitis and pets at home (RR 2.09, 95% CI 1.15-3.80, p=0.016). In addition, there was a significant effect of duration of exclusive breastfeeding (p=0.043), as the relative risk of AD increased with longer duration of breastfeeding (Figure 4). Figure 4 illustrates that children, who were still breastfed at a given age, had a RR of AD of 1.82, 3.22 and 6.67 compared to children where breastfeeding ended 0-3, 3-6 and 6-9 months earlier respectively.

Wheezy Disorders

Confounder adjusted multivariate analyses of wheezy episodes included 313 infants (8 were missing information on start in day-care). The analysis of severe wheezy exacerbations was not adjusted due to a small number of cases, and all 321 infants were therefore included. Wheezy episodes were diagnosed in 262 infants and severe wheezy exacerbation in 36 infants, before they either left the study or turned 2 years of age.

Exclusive breastfeeding reduced the risk of wheezy episodes in the multivariate analysis adjusted for mothers smoking and age at start in day-care (RR 0.67, 95% CI 0.48-0.96; p=0.021), without any further effect from duration of breastfeeding (p=0.637).

Furthermore, exclusive breastfeeding reduced the risk of severe wheezy exacerbation (RR 0.16, 95% CI 0.03-1.01; p=0.051) without any further effect from duration of breastfeeding (p=0.819).

Fatty Acid Composition of Mother's Milk

Breast-milk samples were collected from 314 women 25.2±0.9 (± SEM) days after giving birth.

Of the 321 infants described earlier, 249 were included in the confounder adjusted, multivariate analysis of fatty acid composition in relation to AD (62 children were excluded due to missing samples or technical errors, 4 had missing information on *FLG* variants R501X and 2282del4, 5 had missing information on fathers atopic history and 1 lacked information on pets at home at birth). AD was diagnosed in 23 of the infants while they were still exclusively breastfed. There was a nominal, but non-significant protective effect from the total n-3 fatty acid content in the mother's milk sample (hazard ratio 0.625, p>0.1), but no effect of the content of n-6 fatty acid (hazard ratio 0.958, p>0.1) and trans-fatty-acid (hazard ratio 1.00, p>0.1) (hazard ratios adjusted for the covariates described above).

There were no effects from the fatty acid composition of the breast-milk on wheezy disorders.

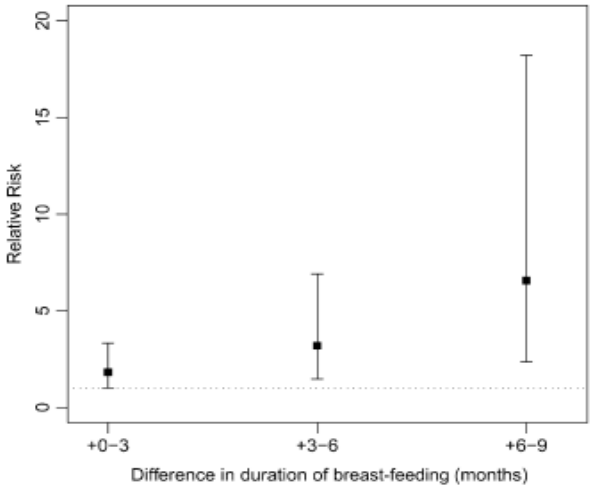

Figure 4

Relative Risk (RR) of AD in exclusively breastfed children compared to children where breastfeeding ended earlier. Children of similar age are compared by adjustment for age. The figure illustrates, that children who are still breastfed at a given age have a RR of AD of 1.82, 3.22 and 6.67 compared to children where breastfeeding ended 0-3, 3-6 and 6-9 months earlier. I.e. the risk of AD is increased with longer duration of breastfeeding.

PAPER III

INTRODUCTION

In this paper we did a 7-year follow-up study, using close longitudinal observations to re-test our initial observation of a borderline significant risk, from alcohol intake by the pregnant mother on the development of AD in the child (paper I) (125).

METHODS

Participants

The study cohort was the COPSAC birth cohort (see previously: "Methodology, participants"). The children were seen in the clinic every 6 months for planned visits, as well as for any acute flare ups of eczema.

Statistical analysis

The association between alcohol intake during pregnancy and development of AD during the first 7 years of life was examined by survival analysis. The children were kept in the analysis from birth, until age when first diagnosed with AD, drop-out or 7 years of age, whichever came first.

Kaplan-Meier curves were estimated for alcohol drinking during pregnancy. The plots were used as a descriptive presentation of the results, illustrating the cumulative risk of developing AD with respect to mother's alcohol intake during pregnancy.

Survival analyses were performed by use of Cox proportional-hazards regression, supporting the Kaplan-Meier curves with information about HR, 95 % CI and p-values. The dependent variable was the time to first event (AD diagnosis, dropout or 7 years). The confounders (mother's education, smoking habits in the 3rd trimester and AD), were placed in the regression models simultaneously. Tests for functional form and proportional hazards were based on martingales.

The overall significance level used was 0.05. The analyses were done using PROC TPHREG in SAS version 9.1 as well as R version 2.6.1.

RESULTS

COPSAC enrolled 411 infants out of which 77 were lost to follow-up before debut of AD symptoms and/or before the age of 7 years. There was no difference in alcohol consumption between the group lost to follow-up and the remaining families (table 3). 48 % of the mothers had a history of AD, 16 % and 13 % of the fathers had a history of asthma and AD, respectively. There were 16 siblings and 18 twins in the cohort, thus totalling 8.3 % of the participants. All 411 children were included in the univariate analysis. Data for mother's education were missing for 34 children, leaving 377 children for the confounder adjusted analysis (92% of the enrolled cohort).

108 mothers in the cohort (26 %) had an intake of one or more units of alcohol pr. week in minimum one of the 3 trimesters, respectively 88, 73 and 73 women in the 1st, 2nd and 3rd trimester. The quantity of alcohol intake was distributed as follows (in units of alcohol pr. week pr. trimester):

	Range	Median	Mean	IQR
1st trimester	1-7	1	1.55	1-2
2nd trimester	1-7	1	1.44	1-2
3rd trimester	1-7	1	1.51	1-2

Table 3: Baseline description in the full cohort and stratified according to drop-out status.

	Full cohort	Follow up 0-7 years	Lost to follow-up	P-value	
Numbers, N:	411	334	77	-	
Drop-out reasons, N:					
Death			2		
Disabling disease			2		
Social (removed from parents)			1		
Emotional distress to study procedures			4		
Emigration			7		
Parental lack of time			3		
No specified reason			58		
Genetics:					
Male, %	49	50	45	NS	c
Caucasian, %	97	97	95	NS	c
FLG null mutation[1], %	11	11	8	NS	c
Atopic heredity:					
Mother asthma, %	100	100	100	-	
Mother allergic rhinitis, %	76	78	67	NS	c
Mother dermatitis, %	48	51	36	0.02	c
Father asthma, %	17	20	4	0.001	c
Father allergic rhinitis, %	33	37	13	<0.001	c
Father dermatitis, %	13	14	11	NS	c
Socio-demographics:					
Household income, mean (SD), 1000 DKK	494 (207)	505 (205)	425 (207)	0.009	t
Mother's education					
Low, %	60	59	61		
Inter-medium, %	27	27	20	NS	c
High, %	14	13	18		
Mother's occupation					
Student, %	11	11	13		
Unemployed, %	10	9	17	NS	c
Non-professional, %	34	33	35		
Professional, %	46	47	35		
Urban living, %	93	94	91	NS	c
Father's age, mean (SD), yr	32 (5.2)	32.3 (5.0)	30.7 (5.7)	0.03	t
Mother's age, mean (SD), yr	30 (4.5)	30.3 (4.4)	28.9 (5.0)	0.03	t
Birth:					
Birth weight, mean (SD), Z-score	0.06 (1.12)	0.10 (1.12)	-0.13 (1.10)	NS	t
Birth length, mean (SD), Z-score	0.12 (1.14)	0.14 (1.14)	0.01 (1.18)	NS	t
Birth BMI, mean (SD), Z-score	0.00 (1.03)	0.04 (1.03)	-0.19 (1.01)	NS	t
Gestational age, mean (SD), wks	39.9 (1.6)	39.9 (1.6)	39.9 (1.4)	NS	t
Caesarean section, %	21	21	18	NS	c
Apgar score >9, %	97	96	99	NS	c
Season of birth					
Winter, %	23	23	23		
Spring, %	21	20	26	NS	c
Summer, %	27	28	25		
Fall, %	29	30	26		
Prenatal exposures:					
Alcohol in pregnancy, %	26	27	24	NS	c
Mother smoking in 3rd trimester, %	15	13	25	0.01	c
Antibiotic use in 3rd trimester, %	14	14	14	NS	c
Paracetamol use in 3rd trimester, %	14	16	8	NS	c
Postnatal exposures:					
Exclusively breastfed > 4wks, %	82	83	76	NS	c
Duration of solely breastfeeding, mean (SD), days	113 (62)	115 (62)	96 (61)	NS	t
Day care, age at start, median (IQR)	346 (246-433)	344 (245-429)	368 (260-483)	NS	w
Cat in household 1st yr, %	14	14	10	NS	c
Dog in household 1st yr, %	13	14	6	NS	c
Older siblings					
0, %	61	59	70		
1, %	28	29	20	NS	c
2+, %	11	12	9		
Nicotine in hair at 1 yr, median (IQR)	0.7 (0.3-2.5)	0.7 (0.3-2.2)	1.3 (0.5-6.1)	0.005	w

1: *FLG* genotyping was performed for R501X and 2282del4
c: chi-square test
t: unpaired T-test
w: Wilcoxon rank sum test
IQR: Inter-Quartile Range
NS*: P*>0.05

177 children of the 411 participating infants (43 %) had an AD diagnosis before age 7 years. In the group of children developing AD, 31 % (55) of the mothers had been drinking alcohol at some point during pregnancy, i.e. more than an average of one unit of alcohol pr. week in minimum one of the three trimesters. Of the 234 children who did not develop AD, 23% (53) of the mothers had been drinking alcohol at some point during pregnancy. This difference in prevalence of mother's alcohol drinking in the two groups of children (31% vs. 23%) was marginally statistically significant (p=0.055)

The age-at-onset analysis confirmed an increased risk of AD associated with alcohol intake during pregnancy, with the effect persisting throughout the whole 7 years follow-up period (Figure 5) (HR 1.44, 95% CI 1.05-1.99, p=0.024).

The hazard ratio from the univariate analysis was largely unchanged after confounder adjusted analysis, adjusting for mother's education, smoking habits in the 3rd trimester and AD (HR 1.45, 95% CI 1.04-2.02, p=0.029).

There was no association between alcohol intake during pregnancy and other atopic endpoints (wheeze episodes, asthma, allergic rhinitis, blood eosinophil count, total IgE, sensitization (specific IgE > 0.35 kU/L), nasal eosinophilia and cord blood IgE (IgE > 0.5 kU/L)). Results are not shown.

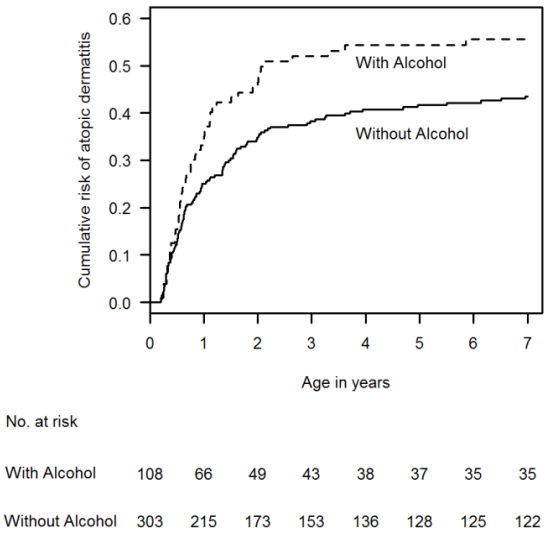

Figure 5
Kaplan-Meier plots for the effect of alcohol intake during pregnancy, on subsequent AD development in the offspring during the first 7 years of life.

DISCUSSION

MAIN FINDINGS

In this thesis, we have studied 40 possible risk factors and their role for the development of AD. We found that dog exposure around time of birth, halved the risk of developing AD during the first 3 years of life (OR 0.44, 95% CI 0.23–0.87, p=0.02). This effect was independent of parent's allergy to dog, ruling out inverse causality. We also found, that length at birth was significantly associated with the risk of developing AD, this too showing an inverse association. *FLG* null mutation and parental atopy significantly increased the risk of AD in early childhood. Lower bedroom temperature was apparently associated with increased risk of AD, but we interpret this as a case of inverse causation. In paper I, we found that breastfeeding and mother's intake of alcohol during the 3rd trimester seemed to increase the risk of AD, but the effects were only borderline significant in both the univariable and multivariable analyses. Therefore, in paper II and III, we used age-of-onset survival statistics to subsequently reanalyze these possible associations. Doing this, we found that exclusive breastfeeding was a significant risk factor for the development of AD during the first 2 years of life, and that the risk of AD increased in infants along with increased duration of breastfeeding. This dose-response relation strengthens the validity of the conclusion. In contrast, the risk of wheezy disorder and severe wheezy exacerbations was reduced, during the time the infant was exclusively breastfed. There were no significant effects from the fatty acid composition of the breast milk on the risk of AD or wheezy disorders, though we found a nominal, but non-significant, protective effect on AD from the total n-3 fatty acid content in the mother's milk sample. In paper III, we found alcohol intake during pregnancy to be associated with an increased risk of developing AD in the first 7 years of life, also after adjustment for relevant confounders (mother's education, AD and smoking habits in the 3rd trimester). The increased risk was persistent throughout the whole 7 years follow-up period.

No associations between the development of AD and gender, race, social status, previous deliveries, 3rd trimester complications (pre-term contractions, preeclampsia, pneumonia, influenza, Apgar score, mode of delivery or mother's asthma status), 3rd trimester exposures (mother's smoking, antibiotics, Paracetamol or physical exercise), anthropometrics of the newborn, birth month, diet (age at introduction of egg, cow's milk and fish), age starting day-care, cat at home, feather pillow, environmental tobacco exposure or humidity in the bedroom were seen. There was no difference between sensitized and non-sensitized AD children in respect to any of the 40 predictor variables evaluated after adjusting for multiple comparisons. This argues against the relevance of a particular phenotype of AD with or without concurrent sensitization.

STRENGTHS AND LIMITATIONS OF THE STUDIES

The major strength of our studies is the meticulous, prospective clinical monitoring, diagnosing and treatment of AD during the first 7 years of life, together with the accurate exposure assessments (127). Recall bias was reduced due to the prospective data collection, with the children in the cohort being seen regularly at 6 month intervals, as well as for any acute skin or airway diseases. Atopy related symptoms were recorded prospectively in diaries.

Misclassification of AD was reduced in these single-center studies, as the participants were diagnosed and treated according to predefined algorithms, by only a small group of medical doctors employed for this purpose at the COPSAC clinic. The risk of misclassification due to inter-observer variation is considerable, since the course of AD and wheezy disorders is capricious and the clinical presentation instrumental to making the diagnosis, which again is based on a complex algorithm of different criteria (128). Ambiguities over the definition of AD, especially during infancy and early childhood, make the estimates of prevalence unreliable and render comparisons between different studies difficult. In this cohort study, the specificity of the AD diagnosis is high, since the diagnosis, detailed phenotype and management of skin lesions were controlled solely by the COPSAC clinic physicians from predefined, standard operating procedures and included assessment of localization and severity at each visit. Wheezy symptoms were recorded prospectively in a diary made by mothers with a personal experience of asthma, thus minimizing misclassification as previously described (11;119;120). The specificity of the diag-

nosis in this study is illustrated by the observation, that the cumulative incidence of seborrhoeic dermatitis capitis was not significantly different in children with AD compared to children with skin lesions not fulfilling the AD diagnosis, suggesting that there was no misclassification between AD and seborrhoeic dermatitis capitis (11). Likewise, the clinical evaluation and perception of the terms associated with wheezy disorders are variable among practitioners and caregivers (129-132). In this way, many studies, that have classified AD and wheeze from symptom questionnaires or history of community doctor's diagnoses, are prone to misclassification.

The vigorous confounder assessments reinforce the analyses. Objective assessments included both measurements of nicotine in the hair of the child, reflecting long-term exposure of environmental tobacco exposure (117), as well as long-term logging of temperature and humidity in the child's bedroom. Perinatal data were collected from midwifes notes. Exposures assessed by history, were updated at the 6-monthly clinic visits, by clinical interviews standardized with closed response categories. The alcohol related questions were recorded before any onset of atopic diseases, including AD.

Disease-related modification of exposure (inverse causality), is a likely interaction between breastfeeding and atopic disease (paper II). Risk specific analyses have been used as an attempt to avoid such inverse causality, e.g. analyzing the risk of AD after 3 month of age (133). However, selecting the cases with a late debut after the age of 3 months, leads to a major loss of cases for analysis and a biased phenotype, excluding infants with an early onset of symptoms. Similarly, another study excluded children with symptoms during the period of breastfeeding (134). The median length of breastfeeding was 5 months, during which period a major proportion of AD had its debut (11). This highly selected analysis, excluding children with early symptoms, limits the validity of the conclusions from such studies. A recent study avoided the problem of inverse causality, by analyzing the risk rate at the time of dermatitis debut. The effect of length of breastfeeding was subsequently analyzed as the effect from 4 months of exclusive breastfeeding on the subsequent development of dermatitis, which is also prone to bias as discussed above (135). To avoid this, we developed a novel statistical approach taking the longitudinal information on symptom debut and duration of breastfeeding into account. Accurate prospective data collection of age at disease onset and end of exclusive breastfeeding, allowed us to analyze the age-adjusted risk of AD, only including information on breastfeeding prior to the time of the AD diagnosis. At any given time, we knew if the child was still being breastfed and for how long the child had been breastfed, if the breastfeeding had ended. Therefore, we substituted the duration of breastfeeding with time elapsed since end of breastfeeding for a given age. By this approach, we obtained an evaluation of the effect of breastfeeding while ongoing, as well as an unrestricted evaluation of the effect of breastfeeding duration, thereby avoiding conditioning on the future. A similar statistical approach to analyse concurrent exposure and endpoint is known from the literature of e.g. smoking vs. asthma (136). Many studies have used infants never breastfed as the comparator (133). There are however, indications that such infants are distinctly different from breastfed children (137), why we have chosen to analyze the effect from varying duration of breastfeeding, excluding infants who were never breastfed (21 of 411 newborns). Lifestyle factors have a strong influence on both breastfeeding practice and the risk of atopic disease, acting as potential confounders (138). For this reason, we prepared the current analysis by analyzing a comprehensive set of risk factors (paper I), including gene-environmental interaction (81) on the development of AD in the COPSAC birth cohort (11). In the current analysis, the covariates included, were the risk factors which had shown relevant within this cohort, instead of selecting covariates based on studies from different settings. Also, maternal and paternal heritage may modify the effect of breastfeeding on disease development (24;133;134;139). All mothers in the birth cohort had a history of asthma, but in addition we included parents' dermatitis and more specifically, we also used non-functional mutations in the major risk gene, i.e. *FLG* (33), as covariate in our analyses, as well as testing for any effect modification.

The limitations of the studies are related to the generalizability of our results, because of the high-risk nature of the cohort. All children were born of mothers with a history of asthma and all newborns had a gestational age above 35 weeks with no congenital abnormality, systemic illness, history of mechanical ventilation or lower airway infection and were predominantly Caucasians. The risk factors identified may therefore be particular to high-risk populations and need replication in unselected populations.

In paper III, we used predefined questions concerning alcohol consumption, recording average alcohol intake per week per trimester. We are aware that alcohol histories are notoriously unreliable and that the data can be prone to recall bias, as the mothers were interviewed about drinking habits during pregnancy, after giving birth. However, this unreliability is not biased, as it must be expected to be the same among mothers to children later developing AD and not. Therefore, it can only result in a type 2 and not a type 1 error, i.e. it could have obscured an effect, but it could not cause a false effect to be seen. We have chosen to present data as a binary variable to determine whether alcohol intake during pregnancy correlates to AD at all. We found similar estimates in sub-analyses stratifying for alcohol amount or onset/duration of alcohol intake, but since the number of individuals in each group was very small, these results were not included in this thesis. This narrow definition of alcohol intake limits our results and future studies designed to determine quantity of alcohol ingested should be sought.

In paper I, we used multiple regression analyses of the cumulated incidence of AD by age 3 years, not accounting for the time-order, i.e. including data on exposures that may have occurred after disease onset. This approach (opposed to longitudinal analyses used in paper II and III) was necessitated by the large number of variables analyzed. Although 40 variables were included in the analysis, there is the potential risk that unmeasured confounders could still play a role in the observed results. Multivariable logistic regression analysis was performed in addition to simple logistic regression in order to find a set of variables that collectively predict AD at 3 years. Since predictors were fit simultaneously, the effect of any one predictor was adjusted for the other predictors in the model, hence minimizing effects of confounders on the results. The consistency of the results between the multivariable and univariable models suggests that there were no major confounders. Here we take a conservative approach and report the primary results, as those from the multivariable model. However, it is possible that the univariable results are meaningful in the absence of confounders, which is why they are also presented. Hosmer-Lemeshow statistics indicated strong goodness of fit across the logistic regression models, including the multivariable model ($p>0.3$ across fit multivariable models for 5 imputed data sets). The use of imputation was important in the multivariable analysis in order to make complete records, and boost power for the analysis. The uncertainty due to imputing values was ac-

counted for in the final estimates. The conclusions of the study are limited by the power of the study. Due to the comprehensive clinical follow-up, the number of subjects included was limited to 356. This is particularly a concern for the risk variables showing borderline significance (length of exclusively breast feeding and mothers use of alcohol). Therefore, in paper II and III, we used age-of-onset survival statistics to subsequently reanalyze these possible associations.

INTERPRETATION

COPSAC recently discovered the *FLG* null mutations to be a substantial determinant of AD (33), and replicated this finding in a population based study (140). In paper I we confirmed a 3-fold increased risk of AD from *FLG* null mutation after confounder adjustment. Still, mother's dermatitis and father's allergic rhinitis remained strong risk factors beyond the *FLG* null mutation, confirming the role of other genetic mutations. Notably, our analyses show a considerable heritability from mother's dermatitis and none from father's dermatitis. This suggests a stronger penetrance of maternal than paternal dermatitis, though it may be confounded from the selection of mothers with asthma. Despite this very substantial heritability of AD, the increasing prevalence in recent decades suggests a strong environmental influence. Recently, COPSAC also demonstrated a significant interaction between *FLG* null mutations and cat ownership at time of birth on the development of early-life AD. Cat exposure at time of birth substantially increased the risk of AD within the first year of life in children with *FLG* null variants, but not amongst those without. Dog exposure was protective independent of *FLG* genotype. The observations were replicated in a birth cohort from the UK (81). Paper I confirms a robust protective effect from perinatal exposure to dog in the home after comprehensive confounder adjustment. A recent systematic review of the evidence for a role of furry pets in AD, found no clear evidence that early pet exposure is associated with increased risk of subsequent AD, and suggested that avoidance behaviour in high-risk families could explain such findings (inverse causality) (80). However, none of the studies reviewed accounted for *FLG* mutation and the interaction between cat exposure and *FLG* mutation, recently demonstrated (81). It seems unlikely, that avoidance behaviour explains these observations in our study of protection from dog, inasmuch as cat was found to be a risk factor in the same study. Importantly, in our study we recorded cat and/or dog exposure from around time of birth (i.e. before AD occurrence), thus eliminating uncertainty of the direction of association between exposure and disease. The effects of dog and cat exposures were therefore not prone to inverse causality from symptom development in the child, since the indicator was presence at birth. Avoidance behaviour on behalf of the parent was accounted for, by including parent's allergic rhinitis, dermatitis and asthma in the model. In addition, we incorporated parents' specific allergy to dogs, which did not reduce the observed dog effect. In particular, there were no considerable changes in estimates when an indicator variable for dog sensitization (for mother, father or both) was added to the final multivariable model. It is thus unlikely, that the protective effect of dog is caused by parents avoiding dog due to allergy. We only assessed animals living in the household, though other exposures outside the home may be relevant, but this would only have biased the risk estimates toward the null, potentially underestimating the true risk. The mechanism by which exposure to dog around time of birth protects against AD is unknown. Specific IgE would not be expected to be involved as the effect is protective, and indeed specific IgE was not present before AD in exposed children. This suggests that dog exposure may act through other mechanisms than specific IgE, or it may be a surrogate marker for other yet unknown environmental influences. Early colonization of airways with pathogenic bacteria was recently shown to be a significant determinant of the development of childhood asthma and raised total-IgE (120). In analogy with this observation, we speculate that the particular microbiology carried by pets may be mediating their effect on disease expression. Alternatively, having a dog is a surrogate measure of other particular life-style factors, which needs to be explored in future studies.

The association between higher bedroom temperature and a lower risk of AD is probably an example of inverse causality. The temperature was determined at age 1 year, after debut of the majority of AD cases, i.e. parents may have reduced bedroom temperature in infants with AD, because of the common observation that itching worsens in warm environments. Therefore, we also examined the final multivariable model without bedroom temperature (data not shown). Generally, odds ratios did not change by more than 5% and p-values did not change from significant to non-significant (or vice versa) at the 0.05 level. We kept temperature in the reported model, as it could affect other risk factors in the model, even if it is not a (forward) causal variable for AD itself.

Length at birth exhibited an inverse association to the risk of AD development after confounder adjustment, i.e. including adjustment for birth weight and gestational age. In contrast, gestational age and weight were previously reported to be individual risk factors, but length at birth was not included in these analyses (9;74;103). A recent twin study showed the opposite of our findings, with a positive association between birth length and risk of developing AD. The outcome variables were, however, collected by telephone interviews (78). Our observation suggests an association between intrauterine growth and AD, and lends support to the theory of intra-uterine programming as a determinant of the risk of AD. Factors affecting the intrauterine programming could be either genetic factors, factors specific to the fetus (e.g. individual nutrition or oxygen supply) or factors dependent on the family (e.g. maternal nutrition, BMI, smoking or socio-economic factors). There was no effect on the risk of AD from surrogate markers of exposure to infections (time spent in day-care and older siblings), social status, season of birth or environmental tobacco exposure (concentration of nicotine in hair).

Duration of breast feeding was positively associated with an increased risk of AD in our simple regression model (p=0.05) (paper I). The OR was essentially unchanged after confounder adjustment in the multiple regression model, but decreased slightly in significance (OR 2.80, 95% CI 0.87–9.03, p=0.10). As the possibility of reverse causation could not be excluded, we developed a novel analytical approach taking the longitudinal information on symptom debut and duration of breastfeeding into account, thus avoiding the risk of inverse causality (paper II). Our finding of a significant increased risk from longer duration of exclusive breastfeeding, is in line with other studies reporting an increased risk of dermatitis from breastfeeding (10;27;28;98) and a reduced risk of asthma (96), but generally the results from previous studies have been contradictory. Systematic reviews with meta-analysis on the risk of dermatitis (95) and asthma (96) reported a reduced risk from breastfeeding, particular in high-risk populations. A cluster randomized trial including 13,889 infants found no protective effect against asthma or dermatitis (26). Other studies have reported increased risk of wheezy disorder, asthma and sensitization (94;97) from breastfeeding, a small protective effect on dermatitis (134) and dual effect protecting

high-risk infants from developing AD, but increasing the risk in infants without such heredity (135). The indiscriminate use of AD, sensitization and asthma as end-points has added to the confusion and lack of consensus. Though these diseases are genetically linked, the risk factor profiles are different (as seen in the present study), and extrapolation of evidence on the role of breastfeeding between these diseases is erroneous. Accordingly, our study found a dual effect from exclusive breastfeeding, increasing the risk of AD, while protecting against wheezy disorders in infants with maternal heredity for asthma, suggesting two different mechanisms. The increased risk of AD from breastfeeding was proportional to the increasing length of the period, that the child had been exclusively breastfed. This observation suggests the transmission of a risk factor for AD in mother's milk, the nature of which may be e.g. cytokines (141), immune cells, antibodies or specific fatty acids, especially the content of n-3 fatty acids. In line with this, we found a non-significant trend, suggesting protection against the risk of AD (but not wheezy disorders) from n-3 fatty acid in mother's milk. Previous studies on the role of polyunsaturated fatty acids in the development of dermatitis and wheezy disorders have been contradictory. Some reported that the n-3 to n-6 fatty acid ratio was protective against dermatitis and asthma in at-risk infants (101;142;143), while others suggested n-3 polyunsaturated fatty acids to be a risk factor for allergy in infants of atopic mothers (144). Even though the COPSAC cohort is the largest cohort studying the effect of fatty acid in mother's milk, the power for these analyses was limited by the number of infants that got AD while being exclusively breastfed. Furthermore, the breast-milk content of polyunsaturated fatty acids show a large day-to-day variation (145).

As shown, breastfeeding protected infants from wheezy disorders. This is consistent with previous studies reporting a protective effect in cross-sectional populations (141). Previously, such apparent association was found in an unselected, prospective birth cohort study to be confounded (by strong confounders) (146) . These were adjusted for in the current analysis. The mechanism of a possible protection may be due to protection against infections, the main driver of wheezy symptoms in early life. Other studies have found a protective effect from breastfeeding on infant wheeze in the first year of life and associated this with the level of TGF-β1 (143).

Alcohol showed a positive association with the development of AD in the simple regression model (p=0.05), with marginal significance in the multiple regression model (OR 1.88, 95% CI 0.94–3.75, p=0.07) (paper I). Again, complex factors may confound such analyses and raise caution to any general conclusion. For this reason, we again used age-of-onset survival statistics in subsequent analyses to show, that not only breastfeeding (paper II), but also mother's alcohol intake in pregnancy were both significant risk factors for AD in the child. AD has increased dramatically in Western countries since the 1960´s (103;147-149) and genetic factors alone, cannot explain the recent rapid increase in incidence. The exposure to early life style factors is thought to be crucial for the later development of AD, and suggestions about such factors influencing the intrauterine milieu, have been made (150-156). A questionnaire based cohort study, reported a significant and dose-dependent effect from the mother's intake of alcohol during pregnancy on the risk of developing AD during the first 2 months of the child's life, but not beyond this period. This effect was mainly seen in high-risk infants (two parents with allergic disease) (157). The authors interpreted their observations to be indirectly supported by a study investigating the influence of environmental factors on levels of total IgE in cord blood of 2631 newborn infants (158). They found an independent, positive dose–response relationship between maternal alcohol consumption during pregnancy and the levels of cord blood IgE, and hypothized that the increased IgE synthesis may contribute to the immunologic sensitization. Increased IgE is generally not, however, believed to be directly associated to AD, but rather to illustrate a common mechanism for the atopic diseases. This is also supported by the paradox, that alcohol consumption is found to be associated with an increased risk of developing perennial allergic rhinitis, but not seasonal allergic rhinitis (159). In our study we did not find any association to other atopic diseases, including cord blood IgE, allergic rhinitis, total IgE and sensitization and can therefore not support the hypothesis, that the association between alcohol and AD is caused by a higher level of IgE. We have adjusted for reasonable confounders, including mother's education, smoking habits in 3rd trimester and mother's AD. These confounders were chosen, because we believe them to be the only factors both affecting the child's risk of developing AD and the mother's alcohol intake. Other factors, such as prenatal behaviour, pets in home at birth and breastfeeding, were not included in the analysis as confounders, because we do not believe them to affect both the exposure (mother's alcohol intake during pregnancy) and the outcome (AD). We detected an association between alcohol intake during pregnancy and the child's risk of developing AD, but the underlying causal relationship is not clear and further studies are needed to both confirm our findings, and to explore the reasons for our observed associations. It has been suggested that factors, which influence cytokine production by the feto-placental unit, may be important determinants of atopic disease (160). This is further supported by findings, that the effect of the maternal line on childhood AD is greater than that of the paternal line (66;72-74), which are, however, not confirmed in all studies (29;75). Alcohol may trigger hypersensitivity reactions, i.e. allergic, asthmatic or dermatitis symptoms. The mechanisms underlying these reactions are unknown, but are hypothesized to be due to a histamine-releasing effect of acetaldehyde (161-166). Gonzalez-Quintela et al (167) have reported that alcohol intake may induce changes in the cytokine profile, including increasing levels of some Th2-associated cytokines and impairment of the Th1 lymphocyte response, this is also supported by other studies (165;166). However, such differences in cytokine profiles are most often found in alcohol abusers, and caution should be taken, in transferring these findings to our cohort's pregnant women. Still, it could be worth considering as a possible explanation for our observed association, between mother's alcohol intake and the offspring's risk of developing AD, although further studies are needed to clarify this.

CONCLUSION

In this thesis the focus has been on investigating possible risk factors for developing atopic dermatitis. The analyses used, have been both a conventional cross-sectional analysis at 3 years of age, as well as longitudinal analyses to explore two border-line findings. All analyses included adjustment for relevant confounders. We found a strong heredity of AD and confirmed earlier findings about the non-functional *FLG* allele mutations and their association with development of AD. Otherwise, remarkably few of the very comprehensive range of pre-, peri- and postnatal exposures, including those related to the hygiene hypothesis (40 possible risk factors were included in the analyses), had any significant influence on disease expression. Perinatal exposure to dog was the only environmental exposure significantly decreasing

the disease manifestation, suggesting other as yet undisclosed environmental factors driving the increasing prevalence of AD in children. In our two-in-depth studies, using longitudinal data, we found an increased risk of AD, but a protective effect on wheezy disorders in infancy from exclusive breastfeeding. Although there are many other beneficial effects from breastfeeding, high-risk populations should not necessarily be recommended extended breastfeeding only for the prevention of AD in the child. The risk associated with exclusive breastfeeding was not explained by the fatty acid composition of mother's milk, though a trend showed higher risk of AD if the mother's milk had low concentrations of n-3 fatty acids. Furthermore, we found that alcohol during pregnancy was associated with a significantly higher risk of developing AD in the offspring. The increased risk was still significant after confounder adjustment for other known risk factors. However, the underlying mechanism was not clear.

The risk factor profile for AD was different from what has been reported on sensitization and asthma, emphasizing the independency of these disorders. AD, asthma and sensitization are common co-morbidities, but the general use of the term atopic disease and indiscriminate extrapolation of risk factors between these disorders, may not be justified.

FUTURE RESEARCH

The increased cumulative incidence of AD during recent decades (147;148) remains unexplained, despite this and other comprehensive analyses indicate environmental influence on disease expression. Further studies are therefore needed, especially studies exploring gene-environmental interactions, like the previous COPSAC publication regarding *FLG* interaction with cat at home as risk factors for developing AD (81). Epigenetic factors also need to be explored, although they are difficult to investigate.

The finding of dog exposure as a factor decreasing the risk of developing AD, may be a surrogate for other exposures associated with having a dog at home, such as a particular microbiology milieu or alternative life-style factors. This should be explored, as it may hold the key to an understanding of what environmental exposures in general, that triggers genetically susceptible individuals and are responsible for the observed increased disease prevalence.

Because the COPSAC cohort consists of high-risk children, our findings cannot be expanded to apply to the general population, why prospective unselected birth cohorts are needed to generalize our findings. In COPSAC we are currently conducting a large-scaled randomized, controlled trial of supplement with n-3 polyunsaturated fatty acids during 3rd trimester in an unselected pregnancy cohort (the COPSAC2010 cohort). This can hopefully help the re-testing of our obtained results, including a clarification of n-3 polyunsaturated fatty acids potential preventive effect on AD and wheezy disorders (paper II). It is plausible that the significance of the association would be improved, by use of a pool of breast milk samples or a biomarker of the habitual intake in the infants, e.g. erythrocyte fatty acid composition, rather than a single breast milk sample.

Other interesting studies to explore our findings about duration of breastfeeding and the risk of developing AD, might involve the breast milks content of cytokines, chemokines and phthalates. A previous COPSAC study has shown neonatal bacterial airway colonization to be associated with childhood asthma (168). Unpublished data have found a similar association to the development of AD, why it could be interesting with an investigation of the breast milks content of antibiotics and/or the breast milks antibiotic effect.

In regard to our finding of mother's alcohol intake as a risk factor for the offspring's development of AD, future research could focus on a possible dose-response relationship. A positive finding would strengthen our observation. This would require more detailed information about the women's alcohol intake than we were able to collect. However, in the ongoing COPSAC2010 cohort, only 2% of the women have reported an alcohol intake during pregnancy (unpublished data) vs. 26 % in this cohort (paper III). Therefore, at least in Denmark, it can be difficult to repeat the analysis.

For a better understanding of the mechanisms behind the observations it could be interesting with studies exploring Th1/Th2 cytokine profiles in child, mother, and cord-blood.

Another approach to better understand AD and possible risk factors for its development, would be to try to subgroup the AD patients and look for individual risk factors depending on the AD-subgroup, e.g. by use of the *FLG* mutations, as different subgroups of AD might have different risk profiles.

SUMMARY

The aim of this thesis was to investigate possible risk factors affecting the development of AD. AD is a frequent disease among children and has a substantial impact on the lives of both the child and its family. A better understanding of the disease would enable better treatment, prevention and information to the families involved. Previous risk factor studies have been hampered by an unsuitable study design and/or difficulties in standardization when diagnosing AD, which limit their conclusions.

In paper I, we conducted a traditional cross-sectional analysis testing 40 possible risk factors for developing AD at 3 years of age. Our data suggested a strong heredity of AD and confirmed the risk associated with the non-functional *FLG* allele mutations after adjustments for confounders. Besides this mother's dermatitis and father's allergic rhinitis were found to increase the risk of AD. Perinatal exposure to dog was the only environmental exposure that significantly reduced the disease manifestation, suggesting other, yet unknown environmental factors affecting the increasing prevalence of AD in children. Length at birth was shown to be inversely associated with the risk of later developing AD. This traditional risk factor analysis led to two borderline significant results: duration of exclusive breastfeeding and mother's alcohol intake during the 3rd trimester. Since these possible two risk factors could neither be rejected nor accepted, we decided to do two in-depth studies, further investigating these, using longitudinal data information and data analysis instead of the traditional cross-sectional approach (paper II & III).

In paper II, we investigated the risk of developing AD and wheezy symptoms until age 2 years depending on duration of breastfeeding. We found an increased risk of AD, but a protective effect on wheezy disorders in infancy from exclusive breastfeeding. The effect of exclusive breastfeeding on the risk of development of AD was significant after adjustment for demographics, *FLG* variants R501X and 2282del4 status, parent's AD and pets at home (RR 2.09, 95% CI 1.15-3.80, p=0.016). In addition, there was a significant effect of duration of exclusive breastfeeding (p=0.043), as the relative risk of AD was increased in proportion to increased duration of breastfeeding. The risk associated with exclusive breastfeeding was not explained by the fatty acid composition of mother's milk, though a trend showed higher risk of AD if mother's milk had low concentrations of n-3 fatty acids.

In paper III, we found that alcohol intake during pregnancy was associated with a significantly higher risk of developing AD in the offspring, with the effect persisting throughout the whole 7 years follow-up period (HR 1.44, 95% CI 1.05-1.99, p=0.024). The increased risk was still significant after confounder adjustment for mother's education, AD and smoking habits during the 3rd trimester. There was no association between alcohol intake during pregnancy and other atopic endpoints (wheeze episodes, asthma, allergic rhinitis, blood eosinophil count, total IgE, sensitization, cord blood IgE and nasal eosiniphilia). However, the underlying explanation was not clear.

The thesis is based on data collected as part of the ongoing COPSAC cohort. The cohort is a longitudinal, prospective birth cohort following 411 children born to mothers with asthma. This selection of high-risk children restricts the interpretation of the results and they cannot necessarily be expanded to apply to the general population.

LIST OF ABBREVIATIONS

AD	Atopic dermatitis
BMI	Body mass index
COPSAC	COpenhagen Prospective Study on Asthma in Childhood
FLG	Filaggrin gene
HR	Hazard ratio
IgE	Immunoglobulin E
IQR	Interquartile range
kU/L	Kilo units per liter
n-3/n-6	Omega 3/omega 6
OR	Odds ratio
PUFA	Polyunsaturated fatty acids
RR	Relative risk
TGF-β1	Transforming growth factor beta 1

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APPENDIX I – ADDITIONAL RESULTS

Table A1: Total sample size and number (%) of cases for categorical variables.

		No.		No. of cases (%)
Heredity				
Filaggrin mutation		352		40 (11.4)
Mother's dermatitis		356		172 (48.3)
Mother's allergic rhinitis		356		263 (73.9)
Father's asthma		347		42 (12.1)
Father's dermatitis		346		49 (14.2)
Father's allergic rhinitis		347		112 (32.3)
Demographics				
Sex: male		356		173 (48.6)
Race: white		356		346 (97.2)
Season of birth		356	Spring	73 (20.5)
			Summer	101 (28.4)
			Fall	104 (29.2)
			Winter	78 (21.9)
Social status	Mother's education	350	Elementary/medium	218 (62.3)
			College	85 (24.3)
			University	47 (13.4)
	Mother's work	355	Unemployed	34 (9.6)
			Student	39 (11.0)
			Nonprofessional	118 (33.2)
			Professional	164 (46.2)
Pregnancy				
Previous deliveries		347	0	213 (61.4)
			1	100 (28.8)
			More	34 (9.8)
3rd trimester complications	Preterm contractions	347		230 (66.3)
	Pre-eclampsia	350		19 (5.4)
	Pneumonia	356		20 (5.6)
	Influenza	356		31 (8.7)
	Asthma status	356	Worse	72 (20.2)
			Same	186 (52.3)
			Better	98 (27.5)
	Mode of delivery	356	Natural	231 (64.9)
			Caesarean section	78 (21.9)
			Vacuum	47 (13.2)
	Apgar score	351	9 or 10	338 (96.3)
3rd trimester exposures	Smoking	356		51 (14.3)
	Alcohol	356		60 (16.9)
	Antibiotics	356		49 (13.8)
	Paracetamol	356		55 (15.5)
	Physical exercise	212	None	143 (67.5)
			Once	37 (17.5)
			More than once	32 (15.1)

Table A2: Summary statistics for distributions of continuous variables.

			Percentile				
		No.	Min.	25th	50th	75th	Max.
Demographics at birth							
Gestational age (wks)		356	36	39	40	41	44
Head circumference (mm)		343	305	340	350	360	390
Length (cm)		356	43	51	52	54	59
Weight (kg)		356	1.62	3.20	3.52	3.85	5.16
Social status (family income, €1000)		355	100	380	500	600	1300
Postnatal exposures							
Diet, duration:	Solely breastfed (d)*	355	0	91	131	180	534
	With eggs in diet (d)*	344	0	696	760	828	953
	With cow's milk in diet (d)*	354	334	791	881	913	996
	With fish in diet (d)*	355	33	804	858	906	1005
Day care	Time in day care (d)*	348	0	660	748	841	955
Ambient air at age 1 y							
Environmental tobacco exposure	Nicotine in hair (ng/mg)	339	0.03	0.32	0.72	2.45	103.84
Temperature (°C)		300	15.22	19.57	20.44	21.33	24.95
Humidity (%)		300	31.81	47.35	54.42	61.82	76.62

* Up to 3 years of age

Table A3: Prenatal risk factors: ORs for development of AD by 3 years of age using heredity, demographic, and pregnancy predictors with simple logistic regression models*.

		OR comparison	OR (95 % CI)	P value
Heredity				
Filaggrin mutation		Yes vs. no	2.43 (1.23-4.79)	.01
Mother's dermatitis		Yes vs. no	3.10 (2.00-4.79)	<.001**
Mother's allergic rhinitis		Yes vs. no	1.57 (0.97-2.57)	.07
Father's asthma		Yes vs. no	2.01 (1.04-3.87)	.04
Father's dermatitis		Yes vs. no	1.38 (0.75-2.52)	.30
Father's allergic rhinitis		Yes vs. no	1.83 (1.16-2.88)	.01
Demographics				
Sex		Males vs. females	1.08 (0.71-1.64)	.72
Race		Non-white vs. white	0.86 (0.24-3.11)	.82
Season of birth		Summer vs. fall	0.86 (0.50-1.50)	.60^
		Winter vs. fall	1.21 (0.67-2.18)	.52^
		Spring vs. fall	0.71 (0.39-1.31)	.28^
Gestational age		Week increase	0.94 (0.83-1.08)	.38
Head circumference at birth		Increase (mm)	0.99 (0.98-1.01)	.37
Length at birth		Increase (cm)	0.90 (0.82-0.99)	.03
Weight at birth		Increase (kg)	0.71 (0.47-1.05)	.09
Social status	Family income	Increase (€ 10,000)	1.00 (0.99-1.01)	.68
	Mother's education	College vs. Elementary/Medium	1.40 (0.85-2.32)	.19^
		University vs. Elementary/Medium	1.57 (0.83-2.96)	.16^
	Mother's work	Student vs. nonprofessional	1.13 (0.54-2.36)	.75^
		Unemployed vs. nonprofessional	2.32 (1.07-5.04)	.03^
		Prof. vs. nonprofessional	1.33 (0.82-2.16)	.24^
Pregnancy				
Previous deliveries		1 vs. none	0.96 (0.59-1.54)	.85^
		2 or more vs. none	1.00 (0.48-2.07)	1.00^
3rd trim. complications	Preterm contractions	Yes vs. no	1.03 (0.66-1.62)	.89
	Preeclampsia	Yes vs. no	1.83 (0.72-4.67)	.21
	Pneumonia	Yes vs. no	0.54 (0.20-1.43)	.22
	Influenza	Yes vs. no	1.43 (0.68-2.99)	.34
	Asthma status	Worse vs. same	0.52 (0.30-0.93)	.03^
		Better vs. same	0.91 (0.56-1.48)	.70^
	Mode of delivery	Caesarean section vs. natural	1.08 (0.65-1.82)	.76^
		Vacuum vs. natural	0.72 (0.37-1.37)	.31^
	Apgar score	<9 vs. 9-10	1.57 (0.52-4.78)	.43
3rd trim. exposures	Smoking	Yes vs. no	0.67 (0.36-1.24)	.20
	Alcohol	Yes vs. no	1.74 (1.00-3.05)	.05
	Antibiotics	Yes vs. no	0.65 (0.35-1.22)	.18
	Paracetamol	Yes vs. no	1.30 (0.73-2.32)	.37
	Physical exercise times per week	1 vs. none	1.87 (0.90-3.88)	.09^
		> 1 vs. none	0.56 (0.24-1.29)	.17^

*Sample sizes available for analysis for each variable are the same as those given in Tables A1 and A2, Appendix I.
**Significant after applying the false discovery rate procedure set at 0.05.
^Denotes a comparison of 2 levels within a categorical variable. P values for categorical variables (as a whole): season of birth, P = 0.41; mother's education, P = 0.22; mother's work, P = 0.19; previous deliveries, P = 0.98; asthma status, P = 0.08; mode of delivery, P = 0.53; physical exercise, P = 0.06.

Table A4: Post-natal risk factors: ORs for development of AD by 3 years of age, using postnatal exposure and ambient air by 1 year of age predictors with simple logistic regression models*.

		OR comparison	OR (95% CI)	P value
Postnatal exposures				
Cat at home at birth		Yes vs. no	1.28 (0.74-2.22)	.37
Dog at home at birth		Yes vs. no	0.37 (0.20-0.67)	.001**
Diet, duration:	Solely breastfed (d)^	Exposure-year increase	2.76 (1.01-7.56)	.05
	With eggs in diet (d) ^	Year increase	0.94 (0.59-1.51)	.79
	With cow's milk in diet (d) ^	Year increase	0.88 (0.41-1.90)	.75
	With fish in diet (d) ^	Year increase	0.94 (0.43-2.03)	.87
Pillow		Use vs. none	2.10 (0.22-20.48)	.40
Day care	Time in day care (d)		0.71 (0.46-1.11)	.13
Ambient air at age 1 y				
Environmental tobacco exposure	Nicotine in hair (ng/mg)	1 ng/mg increase	0.95 (0.91-0.99)	.02
Temperature (°C)		1 °C increase	0.77 (0.66-0.91)	.002**
Humidity (%)		1% increase	1.01 (0.98-1.03)	.52

*Sample sizes available for analysis for each variable are the same as those given in Tables A1 and A2, Appendix I.
**Significant after applying the false discovery rate procedure set at 0.05.
^Up to 3 years of age.