

Deep phenotyping of the unselected COPSAC₂₀₁₀ birth cohort study

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Summary

Background We hypothesize that perinatal exposures, in particular the human microbiome and maternal nutrition during pregnancy, interact with the genetic predisposition to cause an abnormal immune modulation in early life towards a trajectory to chronic inflammatory diseases such as asthma and others.

Objective The aim of this study is to explore these interactions by conducting a longitudinal study in an unselected cohort of pregnant women and their offspring with emphasis on deep clinical phenotyping, exposure assessment, and biobanking. Exposure assessments focus on the human microbiome. Nutritional intervention during pregnancy in randomized controlled trials are included in the study to prevent disease and to be able to establish causal relationships.

Methods Pregnant women from eastern Denmark were invited during 2008–2010 to a novel unselected 'COPSAC₂₀₁₀' cohort. The women visited the clinic during pregnancy weeks 24 and 36. Their children were followed at the clinic with deep phenotyping and collection of biological samples at nine regular visits until the age of 3 and at acute symptoms. Randomized controlled trials of high-dose vitamin D and fish oil supplements were conducted during pregnancy, and a trial of azithromycin for acute lung symptoms was conducted in the children with recurrent wheeze.

Results Seven hundred and thirty-eight mothers were recruited from week 24 of gestation, and 700 of their children were included in the birth cohort. The cohort has an overrepresentation of atopic parents. The participant satisfaction was high and the adherence equally high with 685 children (98%) attending the 1 year clinic visit and 667 children (95%) attending the 2 year clinic visit.

Conclusions The COPSAC₂₀₁₀ birth cohort study provides longitudinal clinical follow-up with highly specific end-points, exposure assessments, and biobanking. The cohort has a high adherence rate promising strong data to elucidate the interaction between genomics and the exposome in perinatal life leading to lifestyle-related chronic inflammatory disorders such as asthma.

Keywords Asthma, eczema, allergy, child, birth cohort, microbiome, vitamin D, fish oil, randomized clinical trial

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Background

Asthma, eczema, and allergy are the most common chronic diseases in childhood and the major cause for healthcare utilization [1]. These diseases are rarely life-threatening, but severely affect the quality of life of

children and their families, in particular families with young children.

The prevalence of childhood asthma, eczema, and allergy has increased several fold in recent decades in westernized cultures, suggesting a strong influence of environmental factors on disease expression [2, 3]. Dis-

eases with such dramatic increase despite a generally improved welfare and health care represent a significant societal and scientific challenge. With a stable genetic pool, the changing geographical and temporal incidences offer evidence of important, yet unknown, lifestyle changes causing a new category of welfare diseases. Particularly, environmental exposures in pre-natal and perinatal life, including the early microbiome [4, 5] and maternal vitamin D [6] and fish oil [7, 8] intake during pregnancy, have been hypothesized to affect the risk of asthma, eczema, and allergy. Risk factors and pathways seem to be shared with other chronic inflammatory diseases later in life [8] meaning that understanding of the mechanisms causing asthma, eczema and allergy may have implications for a wider range of lifestyle related inflammatory disorders.

The mission of the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) is to develop new knowledge on the inception of asthma, allergy, and eczema in order to provide a basis for primary and secondary preventive measures, novel diagnostic tests, and therapeutics for these chronic diseases. COPSAC is fundamentally a prospective clinical study of pregnancy cohorts following the children with an intense focus on longitudinal deep phenotyping in disease and health together with genotyping and objective assessments of the exposome.

The first COPSAC birth cohort was built around year 2000 (COPSAC₂₀₀₀) of 411 children of mothers with a history of asthma [9]. This at-risk cohort has generated substantial new insights into the origins of asthma, eczema, and allergy. Ten years later, COPSAC has built a second birth cohort (COPSAC₂₀₁₀) on the routines and experiences of the first COPSAC₂₀₀₀ birth cohort. The aim is to replicate and explore key findings from COPSAC₂₀₀₀ in an unselected population. Randomized controlled trials of high-dose vitamin D and fish oil supplements were conducted during pregnancy, and a trial of azithromycin for acute lung symptoms was conducted in the children with early asthma symptoms. These interventions were included to prevent and treat disease and to be able to establish causal relationships behind previously observed associations. The main hypotheses are as follows:

- Chronic inflammatory disorders like asthma are programmed in the perinatal life as the results of complex gene–environment interactions.
- The early human microbiome is an important environmental exposure, where an unbalanced composition may cause deregulation of the immature immune system leading to a trajectory towards chronic inflammatory diseases such as asthma.
- Maternal diet during pregnancy influence maturation and regulation of neonatal immune responses and provide an opportunity for preventive supplementa-

tion with vitamin D and n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) in pregnancy.

- Asthma, eczema, and allergies represent several specific endotypes, each associated with divergent underlying molecular mechanisms, distinct clinical features, and individual treatment responses. Identifying these endotypes is essential for establishing effective preventive measures and determining appropriate individualized treatment.

This article describes the recruitment, baseline, clinical study programme, and the standard operating procedures built for the COPSAC₂₀₁₀ birth cohort. The aim is to share methods and hypotheses of the COPSAC resource that will generate important scientific discoveries in the future and to invite international collaborators to explore this vast biobank and metadata.

Materials and methods

COPSAC₂₀₁₀ pregnancy cohort

Recruitment. The study catchment area was Zealand, an island in the eastern part of Denmark, including the capital Copenhagen. Pregnant women were recruited by a monthly surveillance of reimbursement to general practitioners for the mandatory pregnancy visit. They received an invitation by posted mail to contact the clinic during 2008–2010. Exclusion criteria were gestational age above week 26; daily intake of more than 600 IU vitamin D during pregnancy; or having any endocrine, heart, or kidney disorders. Women who contacted the COPSAC clinic by phone received detailed verbal information. Those who were still interested and qualifying for the study received comprehensive study information by posted mail. Finally, the women attended the clinical research unit within pregnancy weeks 22–26 for a visit in the research clinic with detailed information and enrolment into the pregnancy cohort.

Power calculation. The cohort was powered for replication of the association between neonatal airway colonization and early asthma symptoms as discovered in COPSAC₂₀₀₀ [4]. The main outcome was 'recurrent wheeze', based upon an algorithm of recurrent troublesome lung symptoms as previously described [10]. Power calculations were based on survival analysis (log-rank test). Based on the findings from COPSAC₂₀₀₀, we assumed that 12% of children would develop 'recurrent wheeze' in the first 3 years of life (16.5% in the COPSAC₂₀₀₀ high-risk cohort). Furthermore, the following were assumed: the prevalence of colonization in infancy to be 20% (21% in COPSAC₂₀₀₀); a hazard ratio of 2 associated with colonization (2.40 in COPSAC₂₀₀₀); an exponential survival curve; and a dropout rate of 5%

of recruited pregnant women and of further 15% of children by age 3 years. With a significance level of 0.05 and a power of 80%, the required sample size would be 660 recruited pregnant women. Statistical power for the randomized trials was calculated based on the available numbers. Six hundred and ninety-eight newborns were included in the fish oil study and 587 in the vitamin D study. With an assumed hazard ratio of 0.5 in the treatment arms and expected frequency of 'recurrent wheeze' of 12% in the control arm, this resulted in a power of 73% for detection of a statistically significant difference with a significance level of 0.05. Statistical power in the vitamin D study following similar calculations was 65%.

Selection analysis. Characteristics of the women not included in the pregnancy cohort were analysed from letters sent to 1000 women, who had not responded to the primary invitation, inviting them to answer a number of general health questions. Reminder letters were sent on two occasions to non-responders.

COPSAC₂₀₁₀ birth cohort

Neonates born to the women from the COPSAC₂₀₁₀ pregnancy cohort were included in the COPSAC₂₀₁₀ birth cohort.

Scheduled visits were planned at 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months, and regularly thereafter until age 7.

Acute visits were arranged whenever parents considered the need for medical attention for significant troublesome lung symptoms or at the onset or exacerbation of eczema. Asthma, eczema, allergic rhinitis and respiratory infections were diagnosed and treated by the doctors in the research clinic according to predefined algorithms (detailed in the Supporting information).

Database. All information was obtained by personal interview at clinical visits by medical doctors (MDs) and research assistants both with paediatric training. Medical, familial, environmental, and socio-economic histories were assessed by predefined questions and closed response categories and entered online into a dedicated database running an audit trail (Oracle database on a Novel SQL server). Objective information and measurements were entered into the database, subsequently double-checked against source data, and finally locked for further editing. Quality assurance followed 'Good Clinical Practice' guidelines (detailed in the Supporting information).

Standard operating procedures (SOPs) were predefined for all study and database registration procedures similar to those used in the ongoing at-risk COPSAC₂₀₀₀ birth cohort study (see Supporting information) and were available online and in binders in every clinic room.

Participant communication. Telephone calls, posted mails, emails, text messages, and Internet survey (www.esurveyspro.com) were used to keep a close and continuing communication with the participating families between the visits.

Nested, blinded, controlled, randomized clinical trials

Vitamin D supplement during third pregnancy trimester. The pregnant women were randomized 1 : 1 to a daily dose of 2400 IU cholecalciferol D₃ daily supplement or matching placebo (Camette, Denmark A/S) from pregnancy week 24 to 1 week after delivery. All participants were instructed to continue supplementation of 400 IU cholecalciferol D₃ currently recommended by the Danish National Board of Health; that is, daily doses were 400 IU vs. 2800 IU. Serum vitamin D level was assessed at the time of randomization and at completion allowing for stratification of habitual vitamin D intake and assessment of adherence to the treatment plan. Counting returned capsules complemented assessment of compliance. The primary end-point is the development of 'recurrent wheeze', based upon an algorithm of episodes of troublesome lung symptoms, and will be analysed when all the participating children reach 3 years of age.

Fish oil supplement during third pregnancy trimester. From pregnancy week 24 to 1 week after delivery, the pregnant women were randomized 1 : 1 to daily fish oil supplement (four 1-g capsules (Incomomega TG33/22) supplying a total of 2400 mg/day of n-3 LCPUFA; 1320 mg/day eicosapentaenoic acid (EPA), and 880 mg/day docosahexaenoic acid (DHA) contributing to an estimated 10-fold increase relative to the normal daily intake of n-3 LCPUFA or a matching placebo (four 1-g capsules of olive oil (Pharmatech A/S, Norway) containing 72% n-9 oleic acid and 12% n-6 linoleic acid, which contributes an estimated 3% of normal daily intake of PUFA. Whole-blood PUFA levels were assessed at the time of randomization and at completion allowing for stratification of habitual n-3 LCPUFA intake and assessment of adherence to the treatment plan. Compliance was assessed by counting returned capsules. The primary end-point is the development of 'recurrent wheeze', based upon an algorithm of episodes of troublesome lung symptoms and will be analysed when all participating children reach 3 years of age.

Azithromycin treatment of acute severe episodes of troublesome lung symptoms. The children aged 12–36 months with recurrent episodes of troublesome lung symptoms lasting at least 3 days were offered participation in a double-blinded, randomized, placebo-controlled treatment with azithromycin or placebo at

acute episodes of troublesome lung symptoms. Children were recruited from the main cohort study if they had recurrent 'wheezy episodes' (five episodes of three consecutive days of troublesome lung symptoms within a period of 6 months or 4 weeks of continuous troublesome lung symptoms or acute severe asthma). At every subsequent episode, treatment was administered at acute clinical visits, after excluding suspected pneumonia (tachypnoea, RF ≥ 50 ; fever $\geq 39^\circ\text{C}$; CRP ≥ 50). The study was designed as a double-blind, placebo-controlled, randomized, cross-over comparison of azithromycin in a dose of 10 mg/kg/day (TEVA, Kgs. Lyngby, Denmark A/S) for 3 days or matching placebo (the Pharmacy of Glostrup, Copenhagen). Randomization of the trials was performed at the Pharmacy of Glostrup, Copenhagen, and copies of the randomization code were kept in sealed envelope at the research site as well as at the pharmacy. The primary end-points are symptom burden and duration of the asthmatic episode after randomization and will be analysed when a total of 250 treatments have been completed or all children turned 3 years of age.

Figure S9 schematically shows the relationship between the trials on nutritional intervention during pregnancy and the trial on azithromycin.

Influenza A H1N1v vaccination. During the H1N1v pandemic winter 2009–2010, the women were invited from gestational week 20 until 8 months after birth to a phase IV randomized, participant-blinded comparison of monovalent influenza A/California/2009 (H1N1v) surface antigen vaccine (Focetria®, Novartis Vaccines and Diagnostics GmbH, Marburg, Germany) in both MF59-adjuvanted and non-adjuvanted forms [11]. The study consisted of four groups: pregnant women receiving: (1) 7.5 µg H1N1pnd09 antigen with a full complement of MF59 adjuvant (P7.5 µg); (2) 3.75 µg antigen, with half the usual content of MF59 (P3.75 µg); (3) 15 µg antigen, unadjuvanted vaccine (P15 µg); and (4) non-pregnant women receiving 7.5 µg antigen with full MF59-adjuvanted vaccine (NP7.5 µg). After recruitment, the pregnant women were randomized by a computer-generated permuted block randomization table, in blocks of 45 women with allocation rate of 1 : 1 : 1. The P3.75 µg group was closed after 4 months due to low recruitment rate.

Clinical assessments

Daily diary cards were used from birth to monitor significant troublesome lung symptoms as previously analysed in detail [12] including components of cough, wheeze, and dyspnoea, and use of β_2 -agonists, inhaled corticosteroids, and montelukast. Skin symptoms were monitored as active eczema and use of topical steroids. In addition, the diary cards monitored infections, cate-

gorized into common cold, pneumonia, pharyngitis, otitis, fever, gastrointestinal infection, and absence from day care institution because of illness (Figure S1). The diary cards were reviewed with the family by the research MD at each visit to validate symptom definitions. All information were subsequently entered into the online database and double-checked.

Physical examination was performed by the research MD at all scheduled and acute visits, including lung and heart stethoscopy and examination of skin [13, 15], ear, nose, and throat.

CRP measurement was performed at acute visits or when needed using QuickRead 101 (QuickRead Instrument, Orion Diagnostica, Espoo, Finland).

Tympanometric evaluation of the middle ear pressure was performed at yearly visits or when needed (MT10, Interacoustics, Denmark) on both ears.

Spontaneous physical activity was assessed by age 2 years using an omnidirectional accelerometer worn on the ankle for 2 weeks as previously described [16, 17].

Blood pressure was assessed yearly from age 3 years (Welch Allyn Connex: ProBP 3400).

Lung function by multiple-breath washout (EcoMedics: Exhalyzer D) was assessed from the age of 3 years.

Airway resistance was measured from 3 years of age by whole-body plethysmography (Master Screen Body; Erich Jaeger GmbH; Würzburg, Germany) [18, 19].

Airflow was measured by spirometry (Vitalograph: Spirotrac II) before and after inhalation of a standard dose of β_2 -agonist from age 5 years.

Fractional nitric oxide (FeNO) was measured using an Aerocrine NO system (CLG77AM chemiluminescence analyzer from Ecophys AG, Duernten, Switzerland) and assessed from age 5 years.

Anthropometrics

Gestational age and fetal biometrics were determined by an ultrasound nuchal translucency scan in week 13 of the pregnancy (biparietal diameter and occipito-frontal diameter, abdominal circumference, length of femur, and fetal weight estimated by the Hadlock equation for fetuses <2500 g) [20].

Length was measured at every visit until age 24 months by the infantometer, Kiddimetre® (Raven Equipment Limited, Essex England).

Height was measured at every visit from age 24 months (i.e. overlapping with length at age 24 months) by Harpenden stadiometer (Holtain Ltd, Crymych, Dyfed, Wales).

Parental height was measured at recruitment to the study with Harpenden stadiometer.

Weight was measured at every visit using calibrated digital weight scales.

Head circumference was measured three times at each visit to the closest millimetre at the frontal and occipital protuberance positions.

Abdominal circumference was measured three times at each visit at the level of the umbilicus, in the horizontal parallax.

Whole-body dual-energy X-ray absorptiometry (DXA, GE Healthcare, Fairfield, CT, USA) was measured at age 3 years for detailed body composition.

Motor and cognitive development

Milestones of motor development were monitored prospectively by the parents using a registration form based on The Denver Development Index and WHO milestones registration [21, 22].

Language development was assessed at 12 and 24 months of age by a web-based parent report instrument (MacArthur CDI form) translated into Danish [23].

Cognitive development was assessed at 30 months using the cognitive part of Bayley Scales of Infant and Toddler Development, third edition [24]. The tests were performed by trained examiners, and all sessions were video recorded allowing reviewing by a single observer.

Immune function

Mucosal lining fluids were sampled from the upper airways at 1 and 24 months of age as well as at all acute airway visits for assessment of cytokines and chemokines as previously described [25, 27].

At 18-months, whole blood samples were stimulated with Toll-like receptor (TLR) ligands, cell type-specific ligands, and a NLRP3 inflammasome ligand. Whole-blood pheno-typing by flow cytometry was performed at 18 months and in a random subset of cord blood samples.

Skin prick tests with milk, egg, dog, and cat extracts were performed at 6 and 18 months, using standard extracts from ALK-ABELLO with both a positive control and a negative control.

Specific IgE was determined at 6 and 18 months by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) against 15 common inhalant and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen's egg, cow's milk, fish, wheat, peanut, soybean, and shrimp).

Genomics

Deoxyribonucleic acid was purified from peripheral blood cell fraction from plasma of the infant collected at 6 months and from both parents at recruitment and stored at -80°C .

Genome-wide genotyping was performed using the Illumina OmniExpress and Exome beadchip and

imputed using both the Hapmap and 1000 genomes reference populations [28, 29].

RNA was stabilized in the blood sample collected at 18 months and stored at -80°C .

Biobanking

Cord blood was collected by needle puncture from the umbilical cord immediately after birth (collected by midwives) and transported to the laboratory by courier, where cells, plasma, and serum were separated and stored.

Neonatal dried blood spots were sampled as part of the neonatal screening programme and stored in the Danish Neonatal Screening Biobank for further analyses of metabolomics, epigenetic alterations [30] and cytokine levels [31].

Exhaled volatile organic compounds (VOCs) were assessed at 1 and 18 months of age and at acute visits. Breath samples collected were analysed by an 'Electronic nose' (Cyranose 320) with a 32-carbon sensor array for assessment of fingerprints, and specific VOCs were measured by gas chromatography-mass spectrometry.

Urine was sampled at 1 and 12 months of age and stored at -80°C for biomarker analyses.

Peripheral blood was collected by 6 and 18 months of age and stored as plasma and serum and as peripheral blood mononuclear cells at 18 months.

Environmental exposures

History of exposures was recorded yearly from clinical interviews according to predefined categories including variables on social status (educational and occupational status of the parents and household income), siblings, and home environment (e.g. exposure to pets, passive smoking, and fireplace at home).

Adherence to asthma medication and use of antibiotics and other medications was verified from the online national register on medications filed at Danish pharmacies.

Hair samples were obtained at 12, 24, and 36 months for nicotine and cotinine assessment [32].

Dust samples from bedding were collected at 6 months of age by the parents. Settling dust was collected from the child's bedroom.

Human microbiome

Vaginal swaps were collected at 24 weeks and 36 weeks of pregnancy. Wet smears and conventional culture for bacteria were performed, and material was stored for full sequencing of the microbiome.

Hypopharyngeal aspirates were collected at 1 week, 1 month, and 3 months after birth, and at every acute

visit with lung symptoms [33, 34]. The samples were cultured for conventional bacteria and stored for sequencing of the microbiome.

Nasopharyngeal aspirates were collected at 1 week, 1 month, 3 months, and at every acute visit with lung symptoms. Samples were stored for analysis of respiratory viruses.

Fecal samples were collected at 1 week, 1 month, and 12 months. The samples were cultured for conventional bacteria and stored for sequencing of the microbiome.

Skin swaps were performed at 12 months of age and again at 36 months from the volar forearm and the axilla and stored for sequencing of the microbiome.

Diet

Mother's diet was assessed from a 360-item questionnaire with detailed information on dietary habits for the first month prior to recruitment [35].

Breast milk samples were obtained from the mother 1 month post-partum. 0.01% 2,6-di-*tert*-butyl-4-methylphenol was added to the milk and stored at -80°C for later fatty acid analysis [36].

Maternal blood fatty acid levels. EDTA blood samples were collected from the women in pregnancy week 24 and 1 week post-partum, 0.01% 2,6-di-*tert*-butyl-4-methylphenol was added, and the sample was stored at -80°C . Fatty acid analyses were performed within a year from sampling by gas chromatography [37, 38].

Approvals

The main study protocol was approved by the Ethics Committee (H-B-2008-093) and the Danish Data Protection Agency (2008-41-2599).

Vitamin D supplement randomized controlled trial in pregnant women: Ethics Committee (H-B-2009-014); Danish Data Protection Agency (2008-41-2599); EudraCT number 2008-007871-26; ClinTrial.gov ID NCT00798226.

n-3 LCPUFA supplement randomized controlled trial in pregnant women: Ethics Committee (H-B-2008-093); Danish Data Protection Agency (2008-41-2599); ClinTrial.gov ID NCT00798226.

Azithromycin randomized controlled trial: Ethics Committee (H-3-2010-065); Danish Data Protection Agency (2010-41-5023); EudraCT number 2010-018592-16. ClinTrial.gov ID NCT01233297.

Influenza A H1N1v vaccination randomized controlled trial: Ethics Committee (H-B-2008-093); Danish Data Protection Agency (2009-41-4031); EudraCT number 2009-016877-14; ClinTrial.gov ID NCT01012557.

Results

Pregnancy cohort recruitment

Brief invitations were posted to 54 358 women. 1876 women responded and received further information. 733 women attended the clinical research unit for extensive information and were all included in the pregnancy cohort as illustrated in the flow chart of the study recruitment (Fig. 1). The 733 women participated during a total of 738 pregnancies as five women participated during two pregnancies. Questionnaires were mailed to 1000 women, who had not responded to the study invitation, of whom 870 (87%) women responded to the questionnaire.

Table 1 compares the populations of 690 mothers participating in the birth cohort with the group of 870 women choosing not to participate. Women who chose to participate in the cohort were characterized by higher prevalence of asthma, eczema, and hayfever. They were more frequently employed as professional and less frequently unemployed, and had higher income levels. There were no differences with respect to tobacco smoking, alcohol intake, pets at home, or mode of delivery.

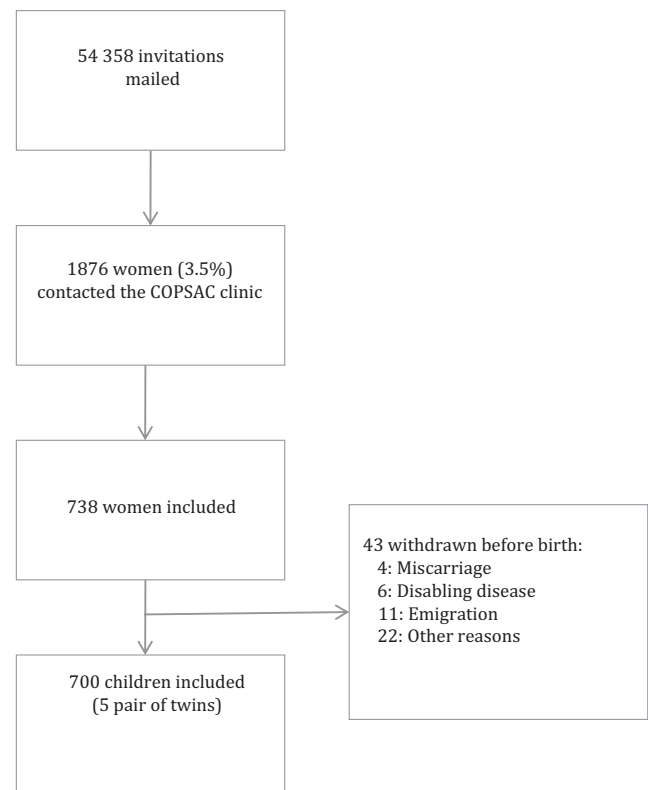


Fig. 1. Flow of subjects from invitation to the first visit to the COPSAC clinic.

Table 1. Dropout analysis. Results from questionnaires mailed to 1000 women who had received an invitation during pregnancy, but chosen not to participate in the COPSAC₂₀₁₀ pregnancy cohort, compared with mothers of children participating in the study. The questionnaire response rate was 86%.

	Non-participants, <i>N</i> = 870*	COPSAC ₂₀₁₀ <i>N</i> = 690†	<i>P</i> -value
Natural birth	82%	78%	0.098
Furred pets in the home	36%	38%	0.458
Smoking during pregnancy, more than one cigarette per week	9%	7%	0.256
Alcohol during pregnancy, more than one unit per week	2%	2%	0.697
Asthma (self-reported)	11%	30%	<0.001
Eczema (self-reported)	12%	21%	<0.001
Allergy (self-reported)	27%	34%	<0.0001
Hayfever (self-reported)	20%	38%	<0.0001
Education level, university education longer than 3 years	33%	36%	0.18
Employment			0.006
Unemployed	10%	6%	–
Student	8%	8%	–
Non-professional	8%	9%	–
Professional	73%	78%	–
Household income			<0.001
Below 400 000 DKK	22%	12%	–
400 000 DKK–600 000 DKK	27%	22%	–
600 000–800 000 DKK	27%	29%	–
Above 800 000 DKK	24%	37%	–

*The response rate for the different variables varied from 849 to 870 of 870 non-participants.

†The response rate for the different variables varied from 620 to 690 of 690 participants.

Significant associations (*P* < 0.05) were marked in bold.

Birth cohort

Forty-three children were withdrawn before enrolment into the birth cohort (Fig. 1; six disabling diseases, four deaths, 11 emigrations, 22 unspecified reasons). The birth cohort enrolled a total of 700 newborns at 1 week of age (born between 3 November 2008 and 17 March 2011), including five pairs of twins and five pairs of siblings born of a total of 690 mothers (Fig. 1). Baseline characteristics of the 700 children in the COPSAC₂₀₁₀ birth cohort are presented in Table 2 and compared with the previous COPSAC₂₀₀₀ birth cohort. The two cohorts are quite similar (the predefined difference in asthma risk aside), except for a remarkable reduction in mothers' smoking and intake of alcohol during pregnancy, and a higher level of education in the recent COPSAC₂₀₁₀ cohort.

Adherence

The adherence to the study was high with 685 children (98%) attending the 1 year visit and 667 children (95%) attending the 2 year visit.

Cohort satisfaction with study participation. Table 3 shows results from the Internet survey of participant's satisfaction with the study. The main complaint was difficult parking access (27%) and, to a lesser extent, blood sampling in the infants (9%).

Discussion

The aim of COPSAC is to understand the origins of chronic inflammatory diseases with a primary focus on asthma, eczema and allergy in young children and to translate this into improved disease prevention, diagnosis, and treatment; to identify endotypes of diseases in early life from their disease mechanism; and specifically to understand the impact of the early human microbiome and maternal diet on the early immune maturation, disease development, and the interaction between the exposures and the genome.

Clinical birth cohorts

Information based on diagnoses and treatments of young children decided by community doctors and hospital records is of very low accuracy and precision. This is partly due to the fact that clinical assessments, diagnoses, and treatments are more difficult and imprecise in young children than later in life. This is of particular importance in young children with asthma, eczema, and allergy where there is very little standardization of diagnoses and treatments in the general medical community both nationally and internationally [1]. Therefore, the COPSAC cohort studies of pregnant women and their children were planned as longitudinal clinical monitoring of symptoms, objective end-points, and environmental exposures at a clinical research site with a standardized approach to diagnosis and treatment [9]. The deep phenotyping includes prospective daily symptom characteristics, lung function tests, biomarkers, and other objective measurements performed at regular visits to the clinic from birth till adulthood. Analysis of biomarkers was planned in accordance to NIH guidelines [39]. COPSAC serves as the *de facto* primary health centre for the cohorts of approximately 1100 children with regular and acute visits to the clinic. The cohorts use the COPSAC research clinic for all airway- and skin-related diagnoses and treatments, avoiding the inconsistent practice in the community. This close adherence of the cohort to our research unit allows us to standardize all procedures

Table 2. Baseline characteristics of the COPSAC₂₀₁₀ cohort and comparison with COPSAC₂₀₀₀

Probands	COPSAC ₂₀₁₀	COPSAC ₂₀₀₀
Mothers enrolled, <i>n</i>	738*	452
Number of newborns, <i>n</i>	700†	411‡
Birth cohort		
Boys	51.4%	49.4%
Twin pairs	1%	2%
Sibling pairs	1%	2%
Caucasian	96%	97%
Mother's age at birth, mean (SD), years	32.2 (4.4)	30.0 (4.5)
Father's age at birth, mean (SD), years	34.4 (5.6)	32.0 (5.2)
Season of birth		
Winter	31%	23%
Spring	27%	21%
Summer	21%	27%
Fall	21%	29%
Pregnancy and birth		
Assisted reproductive technology	10%	
Gestational age, mean (SD), weeks	39.9 (1.7)	39.9 (1.6)
Birthweight, mean (SD), kg	3.54 (0.555)	3.52 (0.519)
Birth length, mean (SD), cm	51.9 (2.5)	52.3 (2.3)
Head circumference at 1 week, mean (SD), cm	35.7 (1.4)	35.2 (1.6)
Apgar at 5 min., mean (SD)	9.9 (0.34)	9.8 (0.6)
Mode of delivery, caesarean section,	22%	21%
Acute	12%	12%
Elective	9%	9%
Newborns hospitalized in relation to labour		
Preterm birth (<36 weeks)	3%	Excluded
In need of assisted ventilation	2%	Excluded
Other reasons	4%	2%
Mothers hospitalized in relation to labour	12%	
Exposures		
Parity		
1	46%	46%
2	38%	26%
3 or more	16%	28%
Older children in household		
0	43%	64%
1	38%	24%
2	15%	8%
3 or more	4%	3%
Mother smoking during pregnancy	8%	24%
Alcohol use during pregnancy (any)	14%	26%
Antibiotics during pregnancy (any)	37%	
Antibiotics during birth (any)	33%	
Furred pets at home (any)	37%	30%
Duration of solely breastfeeding, mean (SD), days	105 (62)	113 (62)
Duration of breastfeeding, mean (SD), days	240 (144)	246 (156)

(continued)

Table 2 (continued)

Probands	COPSAC ₂₀₁₀	COPSAC ₂₀₀₀
Socio-economic variables		
Household annual income		
Index 2010. National average household income for families in 2010: 458 527 DKr		
Below 400 000 DKK	10%	21%
400 000–600 000 DKK	24%	31%
600 000–800 000 DKK	29%	30%
Above 800 000 DKK	37%	17%
Mothers with university education (more than 3 years)	28%	13%
Fathers with university education (more than 3 years)	28%	17%
Mothers without occupation (unemployed or student)	13%	19%
Fathers without occupation (unemployed or student)	8%	7%
Atopic disposition (diagnosed by doctor)		
Mothers with asthma	26%	100%
Mothers with allergic rhinitis	30%	73%
Mothers with eczema	27%	46%
Fathers with asthma	21%	15%
Fathers with allergic rhinitis	27%	30%
Fathers with eczema	15%	11%

*Mothers were recruited during 738 unique pregnancies, but these only represent 733 women as five women participated with more than one child (five sibling pairs participating in the cohort).

†The response rate for the different variables varied from 680 to 700 of 700 children.

‡The response rate for the different variables varied from 385 to 411 of 411 children.

including objective assessments, diagnostic criteria, and medical treatments.

Proposed dietary risk factors, such as vitamin D and fish oil intake during pregnancy, are likely to be subject to confounding from lifestyle factors, which can only partly be controlled for in observational studies. We therefore chose to study these hypothesized risk factors in embedded randomized controlled trials to be able to standardize these possible confounders as well as establish any causal effects. The choice of applying several randomized trials in the same cohort carries the risk of interaction between treatments that must be accounted for in the statistical analyses. The argument for doing this is that the applied interventions are environmental factors that all pregnant women are normally exposed to and often use as supplementations. Vitamin D and fish oil levels vary markedly between individuals as a result of different lifestyles in terms of sun exposure, diet, and intake of supplements, and antibiotics are commonly used as part of the treatment of asthma exacerbations [1]. The randomized trials will allow us to assess potential causal effects and effect modification from these exposures that would otherwise still be present as confounding factors without standardization.

Table 3. Internet-based survey of COPSAC₂₀₁₀ participants conducted in the period from 22 June 2011 to 5 January 2012. 618 responders of 690 study families

Study convenience	Unsatisfied	Satisfied	Very satisfied	N answered
Access to unscheduled visits	3%	49%	48%	612
Regular examinations by doctor	1%	38%	61%	618
Information on planned investigations	1%	32%	67%	618
Burden of study visits and procedures	1%	54%	45%	614
Continuity of research personnel	6%	51%	43%	618
Atmosphere at the research clinic	0%	18%	82%	615
Waiting time in the research clinic	0%	34%	66%	613
Parking accessibility	27%	60%	13%	618

Study procedures	Unsatisfied	Satisfied	Very satisfied	N participated
Blood sampling in parents	1%	37%	62%	609
Blood sampling in child	9%	43%	48%	565
Mucosal lining fluid sampling in child	2%	45%	53%	605
Hypopharyngeal aspiration in child	2%	47%	51%	608
VOC sampling in exhaled air	2%	43%	55%	601
Accelerometer fixed for 2 weeks on the ankle of the child	2%	53%	45%	207

We are applying new objective methods of disease assessment to target and refine our understanding of the different endotypes. These include breathomics, metabolomics local airway immune profiling, and multiple-breath washout, all applicable to clinical practice as non-invasive methods.

We first applied this longitudinal clinical approach in the COPSAC₂₀₀₀ birth cohort. Records from the family practitioners were compared with our data from the COPSAC₂₀₀₀ cohort showing no missing information on atopic disorders and infections, although some under-reporting on trauma [40]. Our studies have often showed strong statistical power compared with larger study numbers presumable because of the highly specific end-points and exposure assessments and the accurate information on time of onset.

Study compliance

The intensive regime with several hour-long visits at the clinic every 6 months is very resource demanding for the research unit and certainly for the families often needing to take days off from work. And yet the adherence is good with approx. 80% follow-up after 7 years in our first birth cohort, COPSAC₂₀₀₀. This first birth cohort only included mothers with a history of asthma that is likely to have strengthened motivation and adherence. Still, the early indications from the COPSAC₂₀₁₀ novel unselected birth cohort are encouraging with a very high adherence rate of 98% at age 1 year and 95% at age 2 years.

Recruitment bias

All pregnant women attending general practitioners in our recruitment area covering both urban and rural areas were targeted for an invitation to participate in the study. Regular health check during pregnancy with the general practitioner is free and recommended for all women, and the invitations were therefore without bias. Still, with a defined aim to analyse risk of asthma, eczema, and allergy, we find the expected over-representation of these diseases compared with non-participants. This confirms that it is unlikely that any cohort study with a disease focus will be truly unselected. The participants differed from non-participants with respect to socioeconomic factors, including employment and income level, while there was no apparent bias with respect to other suspected risk factors such as the mode of delivery, living with pets, smoking, or alcohol intake during pregnancy.

Cohort maintenance

In an Internet-based survey, we found a generally very high level of satisfaction with the visits to the clinic including access to acute help, level of information, appreciation from the personal at the clinic, and the sampling taken from the child (Table 3). In particular, only 9% were displeased with the repeated blood samplings from the infant.

Ethics

We are cognizant of the need to ensure the highest ethical standards when involving pregnant women and young children in research, emphasizing the autonomy and respect for the decisions of participants. The study conforms to the Declaration of Helsinki in its latest version, and the Danish Ethical Committee and the Danish Data Protection Agency have approved the projects. The safety of the randomized control study of two supplements during pregnancy has been reviewed by the Danish

Medicines Agency and is monitored by the Copenhagen GCP unit. The persons involved in the study participate voluntarily and after written informed consent by both parents. The participating families are informed that the study is unlikely to benefit their child and that discoveries will only benefit future generations. The analyses of the biobank are anonymized, and the results are not provided to the families. The participants have the advantage of a consultation by a paediatrician when they have any symptoms from the airways or skin, which is expected to facilitate early diagnosis and treatment. Successful early diagnosis and effective treatment will reduce morbidity and therefore improve quality of life.

In the COPSAC₂₀₀₀ birth cohort study, we conducted semi-structured qualitative interviews with parents and children to assess ethical questions pertaining to such comprehensive and invasive clinical research. It was the consistent conclusion that it is possible to conduct invasive clinical research on infants and young children in a manner that parents find ethically sound. Altruism was their primary motivation and secondly the comfort of close and easy access to paediatricians and other specialists [41], while the children themselves after 7 years of participation emphasized the friendship they had built with the COPSAC study group [42]. In this novel COPSAC₂₀₁₀ cohort, we used Internet-based survey tools to ask the parents anonymously about the motivation for participation (Table 3). Again altruism was the main reason given by 65% of the cohort, a particular interest in asthma, eczema, and allergy was the second most common reason given by 21% of the cohort.

Conclusion

Preventive measures against asthma, eczema, and allergy have largely failed due to our lack of understanding

of the disease origins. The COPSAC studies apply a translational clinical research approach based on long-term studies of birth cohorts with deep phenotyping and exposure assessment, genotyping, and sequencing of the microbiome. Combined with randomized controlled trials, insight into the genetic regulation of basic biological processes, and state-of-the-art systems biology methods, this approach holds the promise to improve our understanding of the processes causing disease in individual patients and the interaction between heredity and environment. Identification of the environmental factors that promote or protect against future disease raises realistic hope of prevention. This may provide the basis for the development of novel diagnostic tests, identification of molecular drug targets, and the possibility of individualized treatment. This research programme will eventually have significant effect on public health, as these diseases affect approximately one-fifth to one-third of all families in westernized countries and may represent communality with other chronic inflammatory diseases developing later in life.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

- Figure S1. Diary page – translated to English.
- Figure S2. Obtaining breath samples.
- Figure S3. Obtaining mucosal lining fluid.
- Figure S4. Handling of blood samples obtained at 18 months of age.

- Figure S5. Hypopharyngeal aspiration.
- Figure S6. Nasopharyngeal aspiration.
- Figure S7. Asthma/persistent wheeze treatment algorithm.
- Figure S8. Rhinitis classification and treatment.
- Figure S9. Schematic presentation of the relationship between the randomized trials on nutritional intervention during pregnancy and the trial on azithromycin in the offspring.

On-Line Repository

Deep Phenotyping of the Unselected COPSAC₂₀₁₀ Birth Cohort Study

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Children – history and data storage

Diary

Daily diary cards were used from birth monitoring symptoms as detailed in the methods section.

Figure S1: Diary page – translated to English

November 2012			Date		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
yes=x, no=0			Weekday		thu	fri	sat	sun	mon	tue	wed	thu	fri	sat	sun	mon	tue	wed	thu	fri	sat	sun	mon	tue	wed	thu	fri	sat	sun	mon	tue	wed	thu	fri
No symptoms, no medication (If this field is checked - no need to fill out the rest of the vertical fields)																																		
Lung symptoms			Cough																															
			Breathlessness																															
			Wheeze																															
Lung symptoms treatment	Acute medication	Blue spray																																
	Preventive medication	Brown spray																																
		Asthma tablet																																
Cold																																		
Pneumonia																																		
Inflammation of the thorax																																		
Middle ear infection, otitis media																																		
Fever																																		
Gastric/intestine infection																																		
Child home from institution																																		
Eczema																																		
Treatment with cutan steroid																																		

Visit

Each visit was coded as either a scheduled or acute visit (unscheduled patient initiated visit due to worsening of symptoms), as described in the methods section. The scheduled visits were coded as planned visit at specific time points (24 week or 36 week of pregnancy, 1 week, 1 month, 3 months, 6 months of age and 6 monthly hereafter till 3 years of age and yearly until 7 years of age). For children diagnosed with asthma additional scheduled visits were planned every 3 months and coded as an asthma control visit. Healthcare utilization and costs were registered according to NIH guidelines (1), including absence from day care due to infectious or asthma-related symptoms (see diary above), and all asthma treatment (name, dose and duration) including treatment initiated outside the research unit (name, dose and duration), diagnoses (ICD10) and asthma related health care contacts (emergency

department, hospital admissions, general practitioners etc.). All symptoms related to asthma or other airway symptoms, eczema, and allergy were registered (see standard operating procedures below).

Database

All information on medical history was obtained through personal interview and based on predefined questions and closed answers and entered into a dedicated database, including information regarding any disease. All information from the diary cards, medical history and objective measurements was entered into the database and subsequently double checked against source data and locked for further editing. Quality assurance followed “Good Clinical Practice” guidelines.

The technical infrastructure for the cohort database system consisted of a 3-tier solution. An Oracle 10G database was used for storage. Business logic and business rules such as audit trail and consistency checks were implemented using Oracle database functionality. The end-user layer was implemented through a thick client developed in Microsoft Visual Basic. For easier access to data with regard to follow up and reporting, a large number of database views were created. These views were used when performing queries towards the database, using the tool *Hyperion Intelligence Explorer*.

Children – objective assessments

Fetal Ultrasound

Information regarding fetal anthropometrics at approximately 20 week of gestation was obtained from ultrasounds measurements performed at the out-patient clinics, which the pregnant women attended as part of the Danish prenatal screening program.

Data included head circumference (HC) calculated from the biparietal diameter and occipito-frontal diameter, abdominal circumference (AC) and the length of femur (FL) determined by an ultrasound nuchal translucency scan in week 13. For the weight estimation Hadlock

equation including HC, AC, and FL was used ($\text{Estimated weight(grams)} = 10^{(1.326 - (0.00326 * AC * FL / 100) + (0.0107 * HC / 10) + (0.0438 * AC / 10) + (0.158 * FL / 10))}$). (2)

All local pregnancy clinics used the same scanning software (Astraia Software, Astraia software GmbH, Munich, Germany) and guidelines for biometric measures. Data was double-checked using reference ranges issued by The Danish Society of Obstetrics and Gynaecology. (3)

Dual energy X-ray absorptiometry (DXA)

A whole body DXA- scan was performed in the children at the age of 3 years with “Lunar iDXA densitometer” (GE Healthcare, Fairfield, CT, USA). The scan was done from head to toes in one movement, lasting approximately 3 minutes. DXA measures body composition by three basic compartments: fat, bone mineral and fat free tissue. Deriving assessment includes fat mass, lean mass, bone mineral content and bone mineral density in pre-defined regions and for the whole body.

Physical Examination

The children were examined by one of the COPSAC research unit’s doctors at each scheduled visit and at acute visits, focusing on the lungs, ears, and skin.

- Lung auscultation was described as either normal or abnormal (tachypnea, wheeze, breathlessness, chest recessions, as well as the appearance of crackles, rhonchi and/or reduced respiration sounds).
- Ear examination was categorized as: normal; acute otitis media; otitis media with effusion or having tubes based on anamnestic information, otoscopy and tympanometric evaluation (see below).
- Eczema was described using 35 predefined localizations and 9 different characteristics (erythema, oedema, excoriations, lichenification, crusts, dryness, vesicles, squamization and cracks). Hanifin and Rajka criteria were assessed to diagnose eczema. (4) A SCORAD test was performed at each visit to evaluate severity.

- Body temperature was assessed.

CRP measurement

CRP was measured at all acute visits or when needed using QuickRead 101 (QuickRead Instrument, Orion Diagnostica, Espoo, Finland).

Tympanometry

Tympanometric evaluation of the middle ear pressure was performed at 1, 2 and 3 years of age (MT10, Interacoustics, Denmark) on both ears. Middle ear effusion was defined as a flat curve (B-curve) or a pressure below -200 dpa (C2-curve). (5,6)

Lung function tests

Multiple Breath Washout Technique - MBW

Lung function was as assessed by Multiple Breath Washout Technique (MBW) at every visit from three years of age. (7,8) During steady breathing of 100% O₂ through a face mask, the concentration of end-tidal N₂ was measured until the concentration reached 1/40th of the initial concentration. The technique gives important information on overall ventilation inhomogeneity through calculation of lung clearance index (9-11)

Whole- body plethysmography

Specific airway resistance (sRaw) was measured from 3 years of age using whole body plethysmography (Master Screen Body; Erich Jaeger GmbH; Würzburg, Germany).

Measurements of air flow at the mouth and pressure changes in a sealed bodybox during tidal breathing gives the opportunity to assess the lungs airway resistance independent of effort. (12)

Spirometry

Spirometry (Vitalograph: Spirotrac II) was obtained from 5 years of age before and after inhalation of a standard dose of β 2-agonist. FEV1, FVC and MMEF were assessed according to standard quality requirement. (13)

Exhaled Nitric Oxide

Fractioned Nitric Oxide (FeNO) was measured from 5 years of age using an Aerocrine NO system (CLG77AM chemiluminescence analyzer from Ecophysics AG, Duernten, Switzerland). (13)

Breath analysis of Volatile Organic Compounds

Breaths samples were collected at 4 weeks,

18 months of age and at all acute visits.

Breath samples were collected in a 750 ml sampling bag of chemically inert material connected to a single shutter patient valve (Ambu® Silicone Resuscitator, Ambu A/S, Ballerup, Denmark) and facial mask (Ambu® UltraSealPaediatric, Ambu A/S, Ballerup, Denmark) covering mouth and nose.

Figure S2: Obtaining breath samples



Electronic Nose

The “breath print” was measured by an electronic nose (eNose). A Cyranose 320 eNose (Smiths Detection Inc., Smiths Detection, Watford, United Kingdom) was used to analyze patterns of Volatile Organic Compounds (VOCs) in samples of exhaled air. The analytical system of the Cyranose 320 is based on a 32-carbon sensor array, which reacts with the organic volatiles by absorbing and expanding and the results are recorded as the change in electronic resistance through the sensors. The sampling bag was connected to the inlet of the eNose. The eNose operates by a cycle of events including a purge of ambient air for baseline values and a purge of the breath sample for analysis. The software (PC-Nose Version 6.9.1.2,

Cyrano Sciences Inc. ©2005, Smiths Detection, Watford, United Kingdom), accompanying the eNose, visualizes the outcome (the 32-sensor response) as a “smell print” that is unique for each mixture of VOCs. The ΔR values for each measurement were saved as a .met data-file.

Gas Chromatography – Mass Spectrometry

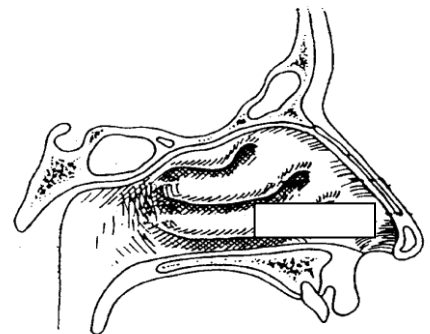
Following the eNose sampling the remainder of the breath sample was emptied under pressure over a stainless steel two-bed sorption tube, filled with carbograph 1TD/Carbopack X (Markes International, Llantrisant, Wales, United Kingdom). Before and after loading, the tubes were capped airtight. The carbon tubes were shipped to The Department of Health Risk Analysis and Toxicology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands for thermal desorption gas-chromatography *time-of-flight* mass-spectrometry (GC-TOF-MS) analysis. (14)

Immune function

Nasal imprint for cytokines and chemokines

Upper airway mucosal lining fluid was sampled from the upper airways at 1 and 24 months of age as well as at all acute airway visits. It was collected with a pair of 3x15mm strips of filter paper (Accuwik Ultra, fibrous hydroxylated polyester [FHPE] sheets, cat no. SPR0730, Pall Life Sciences, United Kingdom) inserted onto the anterior part of the inferior nasal turbinate of both nostrils. The strips were left for 2 minutes. After removal, the filter papers were frozen at -80°C and stored until analysis.

Figure S3: obtaining mucosal lining fluid



The two filter papers from each individual were thawed and immersed together in 300µl Milliplex Assay Buffer (Millipore, Cat no. L-AB, MesoScale Discovery®, Gaithersburg, MD, USA). Thereafter, the filter papers were placed in cellulose acetate tube filter (0.22 µm pore size, Spin-X Centrifuge Tube Filter, Sigma-Aldrich, Cat no. CLS8161, MesoScale Discovery®,

Gaithersburg, MD, USA) in an Eppendorf tube and centrifuged for 5 minutes in a cooled centrifuge (4°C) at 16000g.

The levels of Interleukin-12p70, CXCL10 (IP-10), Interferon-gamma (IFN- γ), Tumor necrosis factor-alpha (TNF- α), CCL4 (MIP-1 β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-17, IL-1 β , CXCL8 (IL-8), Transforming growth factor beta1 (TGF- β 1), IL-10, and IL-2 in the extracted upper airway mucosal lining fluid were analyzed in duplicates using the Ultrasensitive MesoScale Discovery Multi-spot Human TH1/TH2 11-Plex Cytokine assay and 9-plex Chemokine assay. Samples were read using the Sector Imager 6000 (MesoScale Discovery®, Gaithersburg, MD, USA). Assays were conducted according to standard manufacturer's protocols. The lower limit of detection was set as the mean signal from blanks +3 standard deviations (SD): IL-12p70: 1.44pg/ml, IP-10: 31pg/ml, IFN- γ : 4.09pg/ml, TNF- α : 0.61pg/ml, MIP-1 β : 4.3 pg/ml, MCP-1: 16pg/ml, MCP-4: 2.8pg/ml, IL-4: 1.05pg/ml, IL-5: 1.24pg/ml, IL-13: 2.71pg/ml, eotaxin-1: 22pg/ml, eotaxin-3: 45pg/ml, TARC: 7.2pg/ml, MDC: 25pg/ml, IL-17: 0.36 pg/ml, IL-1 β : 1.48pg/ml, IL-8: 3.0pg/ml, TGF- β 1 pg/ml: 5.2, IL-10: 1.00pg/ml and IL-2: 1.48pg/ml.

Skin Prick Test

Skin prick test was assessed twice: at 6 months and 18 months of age. Standard allergen extracts (Soluprick® SQ, ALK Abello, Gentofte, Denmark) including milk, egg, dog, cat, positive and negative controls were tested. A drop of each of the extracts was placed at the children's volar forearm, whereupon a lancet was used to prick through the droplet.

Reactions were read after 15 min (positive control 10 min). The reaction was defined as the diameter of the wheal.

Antihistamines were not allowed 72 hours before the testing. Cortisone creams (group 1+2) were not applied on the tested location 24 hours before the test was performed and potent steroids (group 3+4+systemic use) were not used on any location 14 days before testing.

Specific IgE

Specific IgE was determined from blood samples at 6 and 18 months (see below) by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) against 15 common inhalant and

food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen's egg, cow's milk, fish, wheat, peanut, soybean, and shrimp). (15)

Eosinophils

Blood eosinophil count (10^9 cells/l) was assessed from blood samples at 6 and 18 months.

Physical activity

Accelerometry

Physical activity was measured using the Actical accelerometer (Philips Respironics, Bend, OR 97701, USA) at 24 months of age. The Actical is an omnidirectional, waterproof accelerometer, which uses piezoelectricity derived from microscopic crystalline structures to measure activity counts. (16) The subject wore the accelerometer for 10 days on either ankle. The activity counts were summed and written to the memory at every 15 seconds (an epoch length of 15 seconds).

The mechanical reliability of the accelerometers was investigated prior to the clinical study by testing all accelerometers on a motor driven arm, where reliability was defined as the degree to which repeated measurements under unchanged conditions showed the same results. Accelerometers were tested four at a time to test inter-device reliability and three times to test intra-device reliability. (17)

Motor and cognitive development

Language test

The language development was assessed at the age of 12 and 24 months using a web-based parent-report instrument, MacArthur Communicative Development Inventory (CDI) forms for infants (at 12 months of age) and toddlers (at 24 months of age). The CDI-forms have been translated to Danish(18) and have previously been used to assess the effect of maternal fish oil intake during lactation on linguistic development. (19)

Cognitive test

Cognitive development was assessed at 30 months of age, using the cognitive part of Bayley Scales of Infant and Toddler Development (BSID), Third Edition. (20) The test is given on an individual basis lasting 20–60 minutes. It is administered by examiners specifically trained in BSID test procedures. The examiner presents a series of test materials to the child and observes the child's responses and behaviors. The child is then given a raw score from which scaled score and developmental age is calculated. The sessions are video-recorded and stored allowing subsequent review.

Milestones/motor development

At the child's 1 week visit the parents received a registration form, with thorough instructions, based on The Denver Development Index (21) and WHO milestones registration (22). Dates of achievement of predefined motor milestones were registered prospectively by the parents and reviewed at each visit to the research clinic.

Blood Samples

Cord blood Sample

The mother's midwives received written instructions on how to collect cord blood by needle puncture from the umbilical cord vein. Cord blood collection bags (Pall Corporation, Port Washington, NY), containing 25 ml anticoagulant Citrate Phosphate Dextrose, could hold a maximum of 150 ml and midwives were instructed to collect as much blood as possible.

The father contacted the courier immediately after collection and the cord blood was transported to the laboratory within hours of collection. If the courier reached the laboratory after hours, or during weekends, the cord blood was refrigerated until time of analysis.

Preparation included dilution 10x (100 µl CB in 900 µl PBS) for counting with the NucleoCounter YC-100 (ChemoMetec A/S, Allerød, Denmark). A volume of cord blood containing 30×10^6 cells were resuspended in 1:1 volume of Synth-a-Freeze (Invitrogen, Grand Island, NY) and stored in two 5 ml cryo tubes. In 5 ml cord blood the supernatant were removed and the pellet were resuspended in 10 ml RNAProtect (Qiagen, Hilden, Germany)

and stored in cryotubes. The last portion of cord blood (5-12 ml) was spun (4300 rpm in 5 min.) and the supernatant (plasma) aliquoted in cryo tubes. All tubes were immediately frozen at -80°C. Whole blood cells in Synth-a-Freeze were stored at -140°C.

6 months blood sampling

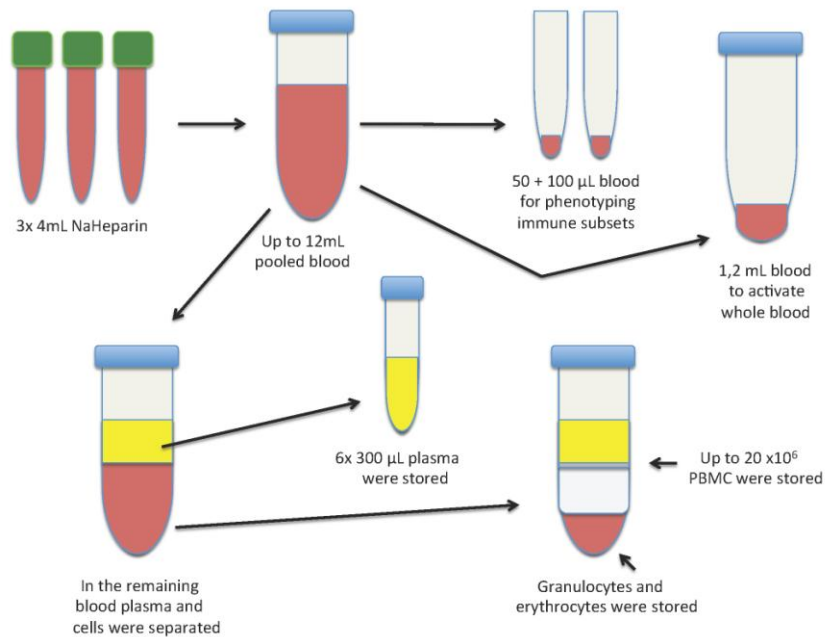
Blood was sampled from the study participants from the anterior cubital vein after anesthetizing the skin with a Lidocain and Prilocain mixture (Emla®, AstraZeneca AB, Södertälje, Sweden).

At 6 months, blood was sampled in 4 ml whole blood and 6 ml EDTA glasses. The blood was placed at room temperature for 30 minutes following sampling or refrigerated overnight before preparation. Blood tubes were centrifuged at 4300 rpm for 10 minutes after which the EDTA blood was separated into plasma and plasma cells and whole blood was separated into serum and serum cells. The material was stored in cryo tubes at -80°C.

18 months blood sampling

At 18 months, blood was sampled in up to three 4 ml Sodium Heparin glasses (12ml) and one 3 ml RNA glass (Tempus blood RNA tube with RNA stabilization solution) according to manufacturer's instructions. The RNA glass was stored untouched at -80°C within 30 minutes of sampling, while the Sodium Heparin glasses were sent to the laboratory, for further immunological analysis and storage, within 6 hours of sampling.

Figure S4: Handling blood samples obtained at 18 months of age



For immunophenotypic analysis, a volume of 50 µl and 100 µl blood was stained with titrated antihuman antibody cocktails specific for FITC-CD1c (eBiosciences, San Diego, CA), APC-eFlour®780-CD3 (eBiosciences), V500-CD4 (BD Biosciences, Franklin Lakes, NJ), PE-Cy7-CD14 (eBiosciences), PerCP-eFlour®710-CD16 (eBiosciences), eFlour®450-CD19 (eBiosciences), PE-CD141 (BD Biosciences), APC-CD303 (Miltenyi Biotec, Auburn, CA) and eFlour®450-CD3 (eBiosciences), V500-CD4, FITC-CD8a (eBiosciences), PC5.5-CD25 (Beckman Coulter, BREA, CA), PC7-CD56 (Beckman Coulter), APC-eFlour®780-CD127 (eBiosciences), PE-pan $\gamma\delta$ TCR (Beckman Coulter), eFlour®660-V α 24J α 18 TCR (eBioscience), respectively. The tubes were incubated 30 min at 4°C and the red blood cells were then lysed in Versalyse (Beckman Coulter), for 10 min at room temperature. Finally, Flow Count beads (Beckman Coulter) were added and the stained cells analyzed immediately on a BD FACSCantoII flow cytometer (BD Biosciences)(Figure S4).

For whole blood activation, 1.2 ml blood was diluted with 1.8 ml RPMI-1640 (Lonza, Walkersville, MD) supplemented with 1% (v/v) l-glutamine (Lonza) and 1% (v/v) Penicillin-Streptomycin (Lonza). Diluted blood was activated in duplicates with equal volumes of 10

ng/ml LPS, 1 µg/ml LPS (Invivogen, San Diego, CA), 50 µg/ml Peptidoglycan (Invivogen), 200 µg/ml Poly(I:C)-LMW (Invivogen), 4 µg/ml R848 (Invivogen), 400 ng/ml staphylococcal enterotoxin B (Sigma, St. Louis, MO), 200 µg/ml Alum crystals (Invivogen) with 10 ng/ml LPS, or RPMI-1640 alone. Following a 24-hour incubation period at 5% CO₂ and 37°C, supernatants were harvested and stored at -80°C for later analysis.

For biobanking, the remaining blood was centrifuged 15 min, 2000 *xg* and divided in a 2 ml top layer (plasma) and a remaining bottom layer (blood cells). Plasma was centrifuged at 3700 rpm, 15 min, and stored at -80°C for later analysis. The blood cells were diluted with an equal volume of RPMI-1640 supplemented with 1% (v/v) foetal bovine serum (FBS, Lonza) and layered on 10 ml Ficoll-Paque™ PLUS (GE Healthcare (Piscataway, NJ)). Following centrifugation at 2500 rpm, room temperature, 30 min without break, , the bottom layer (granulocytes and erythrocytes) were stored directly at -80°C for later analysis. Peripheral blood mononuclear cells (PBMC) were harvested and washed twice in ice-cold RPMI-1640 with 1% (v/v) FBS and spun down at 1200 rpm, 4°C. Depending on cell numbers, 4-5x10⁶ PBMC in icecold RPMI-1640, 20% (v/v) FBS and 10% (v/v) DMSO (Sigma) in one to four cryotubes were slowly frozen in a MrFrosty 1°C freezing container (Nalgene, Rochester, NY) at -80°C. The following day, the cryotubes were moved and stored at -140°C for later analysis.

6 years blood sampling

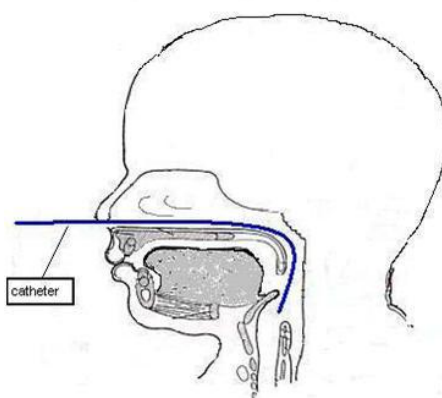
Blood will be sampled at age 6 years.

Microbiome

Hypopharyngeal aspiration

Hypopharyngeal sampling was performed in asymptomatic infants at the scheduled visits at 1 week, 1 month and 3 months. Furthermore, sampling was performed when the child attended the clinic for acute airway symptoms during the first 3 years of life. A soft suction catheter was passed through the nose under aseptic conditions by the physician. Aspiration was

Figure S5: Hypopharyngeal aspiration



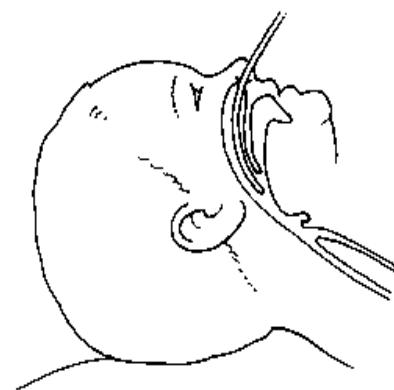
done intermittently assuring no suction was applied during retraction through the oropharynx and nasopharynx. The catheter was flushed with 1 ml of saline into a vessel for remaining secretions from the tube. Samples were transported at room temperature to the microbiology laboratories within 24 hours at the Department of Microbiology and Infection Control, Statens Serum Institute in Copenhagen, Denmark.

Each hypopharyngeal aspirate was mixed thoroughly. Samples were then plated on non-selective and selective media (SSI Diagnostica, Hillerød, Denmark). One set of plates, consisting of a blood agar plate (5% horse blood) and a chocolate agar plate (including lysed blood cells), was used for general culturing, a sabouraud agar plate was used for selective growth of yeast/fungi; the plates were incubated aerobically at 37°C for 18-20 hours. A similar set of blood and chocolate agar plates were incubated in microaerophilic conditions (5%CO₂, 3%H₂, 5%O₂ and 87%N₂ at 37°C) for 48 hours. Subsequently, microbial identification was performed according to their growth on selective media, characteristics of colonies, and cellular morphology. All bacterial identifications were confirmed biochemically by automated identification system VITEK 2 (Bio Mérieux, Craponne, France). Isolates were preserved at -80°C for future identification (i.e. sequencing). No quantification was performed.

Nasopharyngeal aspiration

Nasopharyngeal sampling was performed at the same time as the hypopharyngeal aspiration, i.e. in asymptomatic infants at the scheduled visits at 1 week, 1 month and 3 months as well as when the child attended the clinic for acute airway symptoms during the first 3 years of life. A soft suction catheter was inserted approx. 2-3 cm into the nasal cavity under aseptic conditions by the physician and aspiration from the nasopharynx was done. The catheter was flushed with 1 ml of UTM-medium (standard medium for viral analysis, 72813 UTM, SSI Diagnostica, Hillerød, Denmark). Samples were transported at room temperature to the Department of Microbiology and Infection Control, Statens Serum Institute in Copenhagen, Denmark within 24 hours, where it was stored at -20°C.

Figure S6: Nasopharyngeal aspiration



Fecal sample

Fecal microbiology was sampled at 1 week, 1 month and 12 months of age. A stool sample was collected in a clean container (40080, SSI Diagnostica, Denmark). The sample was collected at the clinic visit or the parents were instructed how to collect the sample at home. Samples were handled at room temperature until received at the Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark (SSI) within 24 hours. The samples were then plated on non-selective and selective media (SSI Diagnostica, Hillerød, Denmark). Set of plates used for general culturing, consisting of a blood agar plate (5% horse blood) and a chocolate agar plate (including lysed blood cells), and blue agar plate (selective for G- negative species, especially *Enterobacteriaceae*). A sabouraud agar plate was used for selective growth of yeast/fungi; the plates were incubated aerobically at 37°C for 18-20 hours. A similar set of blood and chocolate agar plates were incubated in microaerophilic conditions (5%CO₂, 3%H₂, 5%O₂ and 87%N₂ at 37°C) for 48 hours. All bacterial identifications were confirmed biochemically by automated identification system VITEK 2 (Bio Mérieux, Craponne, France). Isolates were preserved at -80°C for future identification. No quantification was performed. The rest of the fecal material was stored in glycerol and media at -80°C in cryovials.

Additionally a stool sample was obtained on day 14 after beginning of treatment for children participating in the antibiotics RCT. Samples were handled as described above.

Skin swab

Skin swabs were sampled once within at 12 months of age and again at the 36 months from the volar forearm and the axilla. Skin flora was sampled using a saline damped Eswab (72795 ESWAB + flocced swab (regular), 480CE, Copan Innovation, Brescia, Italy) for each of the two skin regions. The head of each swab was stored in the accompanying medium at -80°C.

The parents were instructed not to apply any cream or skin lotion 24 hours before the visit. Use of antibiotics as well as time since last bath was registered.

Exposures and biomarkers

Urine Sample

Urine was collected at 1 month and 12 months of age into a urine collection bag. The urine samples were immediately transferred to a 3.6mL Nunc Cryo tube™ (Thermo Fisher Scientific Inc., Roskilde, Denmark), and the aliquots were stored without addition of any preservatives at -80°C.

Hair cotinine and nicotine

Hair samples were collected at age 12, 24 and 36 months. A small lock of hair was cut close to the scalp, transferred to a Nunc Cryo tube™ (Thermo Fisher Scientific Inc., Roskilde, Denmark) and stored without addition of any preservatives at -80°C until analyses.

Environmental tobacco smoke exposure will be evaluated by measuring cotinine and nicotine levels in samples of the child's hair. (23)

Housedust Sample from Bed Linen

At the age of 6 months parents were supplied with a filter device to be used with the family's vacuum cleaner. They were instructed to vacuum the child's bed linen and mattress for 5 minutes. The sample was stored at home below 5°C for 3 days to assure that dust mites were killed whereupon the sample was sent to the clinical research unit by regular mail, where it was stored at -20 °C.

Parents

Parental history and exposures

Parents' asthma, rhinitis and eczema:

Parents' atopic status was determined by a structured clinical interview performed by the research doctors at the parents' first visit to the COPSAC research clinic during gestational week 24. Each parent was asked about history of symptoms, doctors' diagnoses of disease and/or daily treatment (> 1 year for asthma) with respects to asthma, rhinitis and eczema.

Furthermore the parents were asked about 'history of inhaled allergy' (yes/no), 'history of food allergy' (yes/no) and 'history of type IV allergy' (yes/no).

Dietary Questionnaire

During the initial visit to the research clinic, the mothers were presented with a food frequency questionnaire (FFQ) with details concerning their dietary habits 1 month prior to recruitment. The questionnaire was specially developed to address dietary habits among pregnant women in Denmark. (24)

Environmental Exposures

At the child's first visit at 1 week and at the 12, 24, and 36 months visits parents were interviewed from standardized questions about a wide range of exposures including environmental exposures (siblings, size and type of home, smoking at home, pets, fireplace, day care facilities, swimming in chlorinated water, etc.), socioeconomic exposures (household income, education and employment of parents, etc.) and details on nutrition (breastfeeding, introduction of solid foods etc.).

Parental biobanking

Vaginal Swab, Mother

Vaginal swabs were collected from asymptomatic pregnant women at gestational week 24 and 36 from fornix posterior of the vagina using flocked swabs (72795 ESWAB flocked regular, SSI Diagnostica, Hillerød, Denmark). 2 samples were obtained at each visit: one was smeared across a glass microscope slide, air dried and stored at room temperature for later microscopy; the second was transported in transport medium at room temperature to the Department of Microbiological and Infection Control, Statens Serum Institute in Copenhagen, Denmark. Here, the samples were characterized by conventional culture on selective and non-selective media within 24 hours after collection. One set of plates consisting of a blood agar plate (5% horse blood) and a chocolate agar plate (including lysed blood cells) was used for general culturing. A Sabouraud agar plate was used for selective growth of yeast/fungi; the plates were incubated aerobically at 37°C for 18-20 hours. A similar set of blood and chocolate agar plates were incubated in microaerophilic conditions (5%CO₂, 3%H₂, 5%O₂ and

87%N₂ at 37°C) for 48 hours. Additionally, one HBT (Human Blood Tween) plate was incubated in microaerophilic conditions for 48 hours for selecting *Gardnerella vaginalis*. Subsequently, microbial identification was performed according to growth on selective media, characteristics of colonies, and cellular morphology. All bacterial identifications were confirmed biochemically by automated identification system VITEK 2 (Bio Mérieux, Craponne, France). Isolates were preserved at -80°C for future identification. No quantification was performed.

Breast milk Sample

The mothers extracted a volume of 9 mL of breast milk 1 month post partum. Time of sampling during the feed was left to the mothers' choice, since the fatty acid composition does not change during a feeding. (25) 900 µL Antioxidant BHT (Butylatedhydroxytoluene) was added immediately after sample collection for preservation of fatty acids. The samples were frozen and stored at -80°C.

Blood Samples, Parents

Blood samples were obtained from both parents at week 24 of pregnancy and from the mother at 1 week post partum. The samples were centrifuged after 30 minutes at room temperature and separated in serum, plasma and cells and stored at -80°C. A maternal sample was stored using the antioxidant BHT (Butylatedhydroxytoluene) and blown with nitrogen for preservation of fatty acids.

Asthma – standard operating procedure

All COPSAC₂₀₁₀-children were examined in the clinic at episodes of troublesome lower airway symptoms, if symptoms were significant and the parents found the child needed medical attention.

Children diagnosed with persistent wheeze/asthma were seen in the clinic for routine controls every three months.

Definitions

The parents were informed of the following definitions, for registration in the COPSAC₂₀₁₀-diary cards:

Troublesome lower airway symptoms (TROLS): Symptoms from the *lower* airways such as wheeze or whistling sounds when exhaling, breathlessness or persistent troublesome cough severely affecting the wellbeing of the child, e.g. at night, during meals or when the child was laughing or crying.

Episode

- Three or more consecutive days of significant troublesome lower airway symptoms

Persistent wheeze and asthma

Persistent wheeze/asthma was diagnosed based on (1) recurrent episodes of TROLS recorded in the daily dairy cards as 5 episodes within 6 months, each episode lasting at least 3 consecutive days; (2) symptoms typical of asthma (recently termed "multi-trigger wheeze"(26)) including wheezing, breathlessness, and recurrent cough outside common cold; (3) need for intermittent rescue use of inhaled β_2 -agonist and (4) response to a 3-month course of inhaled corticosteroids and relapse of symptoms when stopping treatment.(27) Due to uncertainty of an asthma diagnosis early in life and diagnostic tradition, the term 'persistent wheeze' was used in the first 3 years while 'asthma was used for children fulfilling these criteria later in life.

Exacerbation (at least one of the following)

- Worsening of asthma requiring use of systemic corticosteroids or high-dose inhaled steroids

- Asthma- specific emergency room admissions
- Asthma-specific hospital admissions

Therapy

1.-2. Episode

- β 2-agonist 0,10 mg/dose as needed.

3. -4. Episode or $\geq$ two weeks of daily troublesome lung symptoms

- β 2-agonist 0,10 mg/dose as needed.
- Montelukast 4 mg/day for 7-14 days.

All subsequent episodes

- β 2-agonist 0,10 mg/dose as needed.
- Montelukast 4 mg/day for 7-14 days.

5. Episode

- Fluticasonpropionat 50 μ g 2 x 2 daily for three months.
- STOP treatment after 3 months (unless severe persistent symptoms) and evaluation of improvement during treatment.

Relapse

Defined as 2 or more episodes, or daily symptoms for 14 consecutive days within 12 months after ended 3 months daily ICS treatment and only if improvement during treatment

- Fluticasonpropionat 50 μ g 2 x 2 daily for 6 months.
- STOP treatment after 6 months (unless severe symptoms), and evaluation of improvement during treatment.

2nd and subsequent relapses

- Fluticasonpropionat 50 μ g 2 x 2 daily for 12 months.
- STOP treatment after 12 months (unless severe symptoms), and evaluation of improvement during treatment.

Exacerbation

- Prednisolon 1-2 mg/kg/day for 3 days.

or

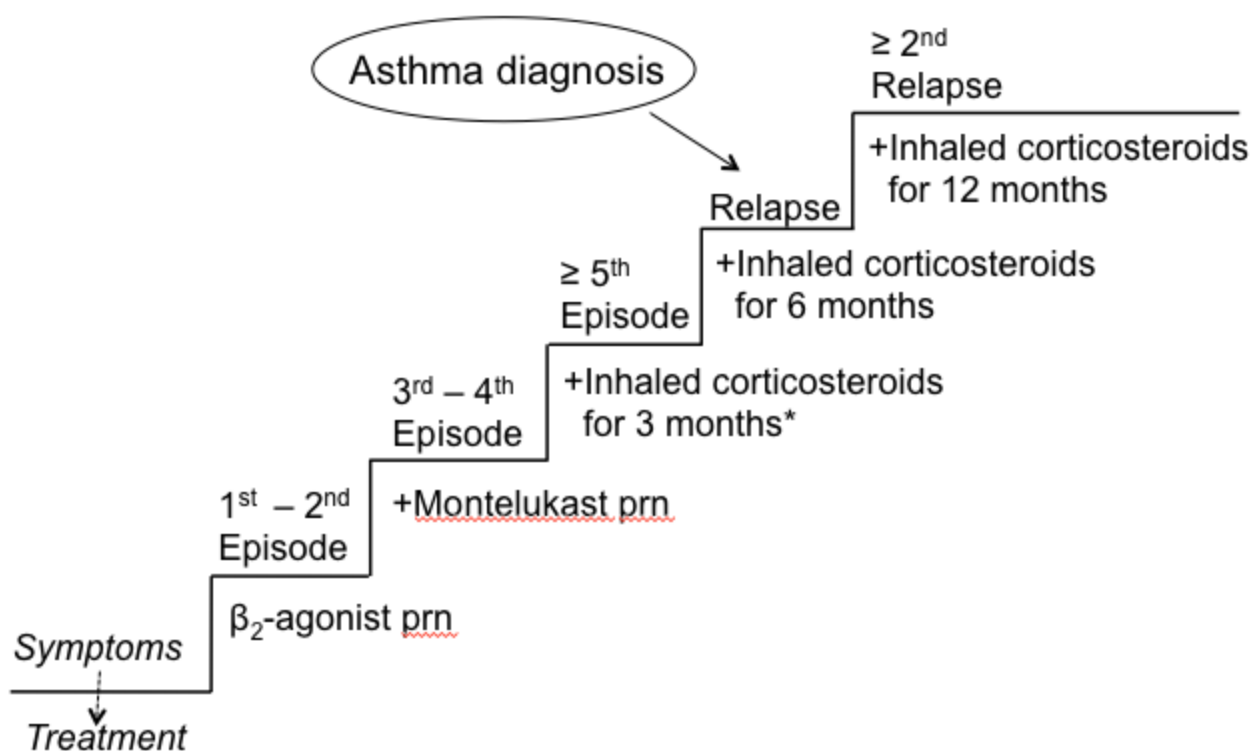
- Fluticasonpropionat 50 μ g 8 x 2 daily (~ 125 μ g 4 x 2 daily) for 2 weeks.

Control visit every three months

- Physical examination
- Growth
- Adherence to asthma medication verified from the national register on medications filled at Danish pharmacies.
- Revision of treatment or relapse. Children with no clear response to treatment were offered:
 - Chest X-ray
 - Genetic screening for all known mutations causing Cystic Fibrosis
 - If needed further investigations were carried out to rule out other underlying disease

The persistent wheeze/asthma diagnosis is dismissed after 1 year without need of inhaled corticosteroid according to the above algorithm (Figure S7).

Figure S7: Asthma / persistent wheeze treatment algorithm



* Failure to respond to treatment results in further pulmonary assessment

Eczema – standard operating procedure

Eczema was defined based on the criteria of Hanifin and Rajka, which requires the presence of 3 of 4 major criteria and at least 3 of 23 minor signs. (4) A SCORAD test was performed to evaluate disease severity.

Online recordings of skin lesions

Skin lesions with typical dermatitis morphologic features were described online according to predefined morphologic categories and localization. The skin morphologic categories of lesions were registered as erythema, edema, excoriations, lichenification, crusts, dryness, vesicles, squamatization and cracks on a scale from 0 to 3 (none, mild, moderate, and severe). The localization of the lesions was characterized in 35 predefined skin areas covering the whole body. Skin lesions caused by chicken pox, human parvovirus B19, Gianotti-Crosti syndrome, herpes, seborrheic dermatitis capitis, and virus exanthema were excluded from this recording. Seborrheic dermatitis capitis was defined as greasy scaling solely in the scalp with no other skin symptoms.

Therapy

General advice

- Use of moisturizing cream twice daily. Cream with a high content of lipids was preferable.
- Showers should be brief.
- The temperature in the bedroom should be cool.
- Avoid woolen clothes directly on the skin.
- Keep the nails short.
- Wash skin with lesions daily.

Medical treatment

The primary medical treatment was topical steroid cream.

The steroid cream group was chosen based on localization, age and occlusion of the area. In general the treatment was a group 1 or 2 topical steroid twice daily for 8-14 days until the eczema was in remission, followed by a one week treatment once daily and a week with

treatment every second or third day. A group 1 steroid was preferred when the eczema was located in the face or genital-areas and in infants. Precaution was taken when the area was occluded because of the risk of enhanced effect and side effects.

If topical steroids did not have a sufficient efficacy, had side effects or were not tolerated; pimecrolimus 1% or tacrolimus 0.03% was used according to current guidelines.

Skin infections were treated according to standard guidelines: Culture was performed, topical antibiotic cream was started initially and when necessary oral antibiotic treatment was added according to type of microbial growth.

Allergic rhinitis – standard operating procedure

Children with signs of rhinitis were evaluated when having acute symptoms and at scheduled visits in the COPSAC-clinic. The treatment was decided from the predominant symptoms independent of sensitization. Evaluation of the treatment effect was assessed at a new visit at the clinic three months after treatment start. Skin prick test was used to categorize symptoms but not for treatment decision.

Definitions

Rhinitis

Non-infectious rhinitis was defined from significant troublesome nasal symptoms including anterior or posterior rhinorrhea, sneezing, nasal blockage and/or itching of the nose or palate. These symptoms had to occur for two or more consecutive days, lasting minimum 1 hour a day without accompanying cold or flu. Time of year, signs of conjunctivitis (non-infectious conjunctivitis, itching or conjunctival injection), general symptoms of fatigue and irritability were registered.

Allergic rhinitis was defined as rhinitis with relevant allergic sensitization against ≥ 1 inhaled allergens. Sensitization was only considered relevant when the symptoms were predominant during the appropriate exposure: birch (April-May); grass (May-August); mugwort (July-August); moulds (May-October); house dust mites (October-February); animals (when exposed).

Non-allergic rhinitis was rhinitis symptoms with no or irrelevant sensitization.

If the symptoms had not been present for 12 months the rhinitis diagnoses was dismissed.

Sensitization

All children presenting rhinitis symptoms had skin prick test performed with the methodology described above.

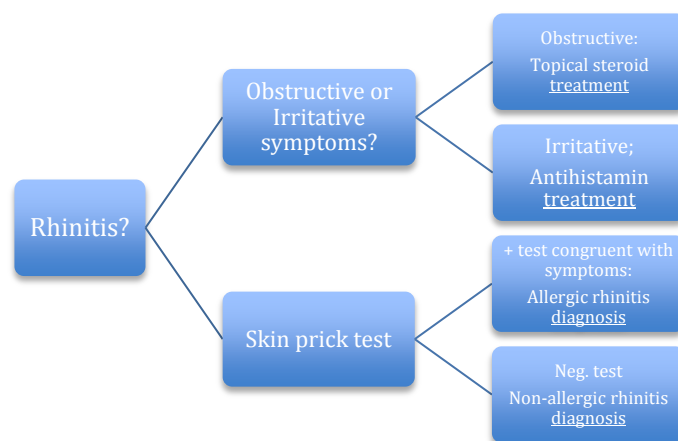
Specific IgE in blood was not assessed routinely for clinical purpose.

Treatment algorithm

Categories

The treatment was decided from the predominant symptoms independent of sensitization and classified as obstructive or irritative rhinitis. The classification in allergic or non-allergic rhinitis had no influence on treatment (Figure S8).

Figure S8: Rhinitis classification and treatment



Irritative rhinitis

If troublesome itching, rhinorrhea and fatigue were dominating symptoms, the rhinitis was treated with systemic antihistamines. If the antihistamine had effect the rhinitis was classified as irritative rhinitis.

Antihistamine, systemic treatment:

- Children 1-5 years of age: Desloratadin (mixture, 0.5mg/ml) 1.25 mg 1 times daily.
- Children 6-11 of age: Desloratadin (tablet, 5 mg) 2.5 mg 1 times daily.

Add-on: Continuous symptoms. Topical treatment:

- Children below 2 years of age: Levocabastin nasal spray 100 mcg 1 times daily.
- Children above 2 years of age: Levocabastin nasal spray 100 mcg 2 times daily

Obstructive rhinitis

If the obstructive symptoms dominated, the rhinitis was treated with topical steroids. If the steroid had effect on the symptoms the rhinitis was classified as obstructive rhinitis.

Local topical steroid treatment:

- Children below 12 years of age: Mometasonfuroat nasal spray (50 mcg) 1 puff in each nostril 1 time daily
- Children over 12 years of age: Mometasonfuroat nasal spray (50 mcg) 2 puffs (100 mcg) in each nostril 1 time daily

Children with nasal obstruction and no effect from topical steroids were offered nasal scope at ENT specialist to look for adenoid vegetations.

Allergic conjunctivitis

Troublesome eye symptoms such as non-infectious conjunctivitis, itching and conjunctival injection, was treated with topical antihistamine eye-drops:

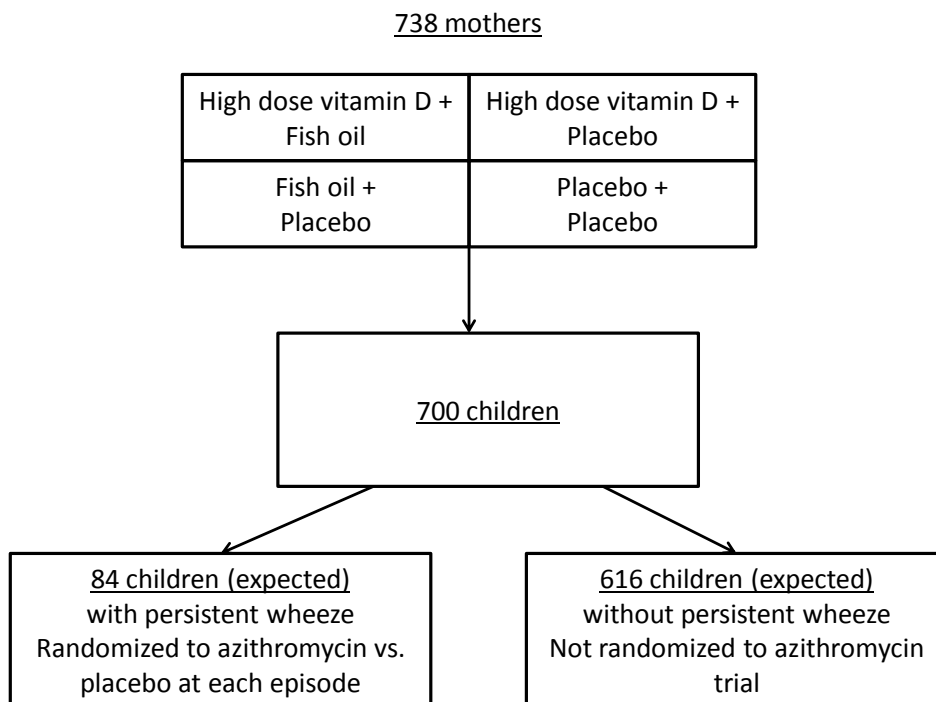
- Children below 2 years of age: Levocabastin eye drops (0.5mg/ml) 1 drop in each eye 2 times daily.
- Children above 2 years of age: Levocabastin eye drops (0.5mg/ml) 1 drop in each eye 3-4 times daily.

Add-on: When insufficient effect of Levocabastin:

- Natriumnedrocromil (20mg/ml) 1 drop in each eye up to 4 times daily

Randomized interventions

Figure S9: Schematical presentation of the relationship between the randomized trials on nutritional intervention during pregnancy and the trial on azithromycin in the offspring. The number of children participating in the trial on azithromycin are estimated since recruitment is still ongoing at the date of publication.



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