

Original article

The multinational birth cohort of EuroPrevall: background, aims and methods

Background/aim: The true prevalence and risk factors of food allergies in children are not known because estimates were based predominantly on subjective assessments and skin or serum tests of allergic sensitization to food. The diagnostic gold standard, a double-blind placebo-controlled food provocation test, was not performed consistently to confirm suspected allergic reactions in previous population studies in children. This protocol describes the specific aims and diagnostic protocol of a birth cohort study examining prevalence patterns and influential factors of confirmed food allergies in European children from different regions.

Methods: Within the collaborative translational research project EuroPrevall, we started a multi-center birth cohort study, recruiting a total of over 12 000 newborns in nine countries across Europe in 2005–2009. In addition to three telephone interviews during the first 30 months, parents were asked to immediately inform the centers about possible allergic reactions to food at any time during the follow-up period.

Results: All children with suspected food allergy symptoms were clinically evaluated including double-blind placebo-controlled food challenge tests. We assessed sensitization to different food allergens by measurements of specific serum immunoglobulin E and skin prick tests, collect blood, saliva or buccal swabs for genetic tests, breast milk for measurement of food proteins/cytokines, and evaluate quality-of-life and economic burden of families with food allergic children.

Conclusions: This birth cohort provides unique data on prevalence, risk factors, quality-of-life, and costs of food allergies in Europe, leading to the development of more informed and integrated preventative and treatment strategies for children with food allergies.

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Key words: birth cohort; children; EuroPrevall; food allergy; methods; study design.

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Background

Recent meta-analyses of population-based studies show considerable heterogeneity in the prevalence of perceived and confirmed allergic reactions, as well as sensitization to food. However, it remains unclear whether the variation observed was related to differences in study design, methodology, or genuine differences between populations (1, 2).

Adverse reactions to food have different prevalence patterns in different age groups. In children between birth and 4 years of age, the prevalence of perceived reactions to any food in population-based studies varied markedly and ranged from 3–35% (1, 3). Regarding specific food items, parents often reported adverse reactions to tomato with a prevalence of up to 13.8% in Swedish children (4), to cow's milk (up to 11% in Iceland) (4), to apple and to hen's egg (up to 8.5% and 6.7% respectively, both in Russia) (5). In the same age group, the prevalence of allergic reactions confirmed by food provocation tests was considerably lower. Cow's milk allergy was found to be highest in infants at 2.3% (The Netherlands) and 2.2% (Denmark) (6, 7), whereas hen's egg allergy at 1.7% in 3-year-old Danish children (8). Although these estimates were based on food provocation tests, they were mostly performed in an open or single blinded fashion, not as double-blind placebo-controlled food challenge tests (DBPCFC), which is widely considered the diagnostic gold standard for food allergies (9–11).

There is increasing public concern about the true prevalence and a possible increase of food allergies over time, particularly among parents, child care and school staff, health care providers, regulators and food producers (12, 13). Recent studies of children in mid-childhood have reported a rising prevalence of perceived peanut allergy, allergic sensitization to peanut and food challenge

test-proven peanut allergy in children from the USA and UK (14–16). In order to make valid estimates of the true prevalence of food allergies, we are still lacking high quality community studies based on objectively confirmed allergic reactions by DBPCFC.

To close the gap in our knowledge of food allergies, the European Commission is funding the translational integrated project EuroPrevall, officially launched in 2005. Over 60 partners including patient organizations, the food industry and research institutions from across Europe, Russia, Ghana, India and China are collaborating in basic and clinical research projects, as well as large epidemiological studies in both children and adults (<http://www.euoprevall.org>) (17).

Rationale of the birth cohort of EuroPrevall

Allergic reactions to food items, such as cow's milk, hen's egg, peanut, tree nuts, wheat, soy and fish often develop within the first few years of life. Whereas cow's milk and hen's egg allergy can resolve by school age (18, 19), peanut and fish allergy tend to persist throughout childhood and adulthood (20, 21). To detect factors associated with the persistence or resolution of food allergies over time, prospective longitudinal studies are necessary. Events during pregnancy and early childhood may be important in the development of food allergies. The time point of introduction of foods (lactation, weaning) or contact with creams or ointments containing food proteins could influence sensitization and clinical presentation of food allergy (22–27). To accurately assess the occurrence of possible risk factors for a disease that can start already in infancy and may resolve by school age, a prospective birth cohort with regular follow-up of participating children and families provides the best possible study design. A multi-center approach with

standardized assessment methods across all study centers will allow the evaluation of potential differences between populations.

Objectives of the birth cohort of EuroPrevall

Our primary aim is to establish and compare the prevalence of confirmed allergic reactions to food in young children from nine countries across major climatic and cultural regions in Europe. Food challenges are started at microgram (μg) levels to identify 'no observed adverse effect levels' (NOAELs) and 'lowest observed adverse effect levels' (LOAELs) (28). Among children with food allergies and control children, with this work we will further assess the level and pattern of allergic sensitization to food.

Secondary aims are to examine the role of possible determinants for the development of food allergies such as genetic background (using samples from both children and parents), maternal diet during pregnancy and breast feeding, postnatal food consumption, infections, psychosocial and environmental factors. We aim, furthermore, to evaluate the B-cell epitope recognition of milk, egg and peanut allergens and its association with the clinical course of patients' food allergy. We will also assess the potential of component-resolved diagnostics using microarrays of peptides derived from allergen sequences to improve the diagnosis of food allergies in infants and small children in whom only small blood volumes are obtainable (29, 30). To reduce the requirement for oral food provocation tests in the future, we will examine the possibility of developing population-based diagnostic decision points for allergen-specific serum immunoglobulin E (IgE) concentration for several foods. The figures now used widely are derived from investigation of highly selected and generally older groups of children, referred to tertiary centers. They may not be applicable to unselected infants prospectively identified in a birth cohort. In addition to these specific aims, the formation of the EuroPrevall birth cohort will build an important platform for future studies into the development of food allergies in and outside of Europe.

Methods

Study design and setting

The birth cohort study is being conducted as a collaborative multi-center study in nine regions from across Europe. The countries were chosen to give a geographic spread across the European continent, reflecting different climatic and cultural regions, including several countries for which no population-based food allergy studies have been previously published. The Nordic climatic region is represented by Reykjavik (Iceland); the maritime region by Southampton (UK) and Amsterdam (The Netherlands); in central Europe are Berlin (Germany), Lodz (Poland) and Vilnius (Lithuania); and the centers from the Mediterranean region include Madrid (Spain), Milan (Italy) and Athens (Greece) (Fig. 1).

This study is a longitudinal prospective evaluation starting at birth and included three routine follow-up assessments of all participants at 12, 24 and 30 months, regardless of whether or not they become food allergic. Additional clinical assessments at the centers were performed whenever parents reported possible food allergy symptoms in their children. These clinical evaluations followed a standardized protocol (described in detail below) to which all centers agreed before starting to recruit children. Short reminders about possible signs and symptoms of food allergies were given to the parents at the three follow-up assessments and additionally at 6 and 18 months, either by telephone, e-mail or text message (Fig. 2A, B).

Recruitment procedure, inclusion and exclusion criteria

Each center obtained approval from their governing ethics committee or review board before commencing recruitment. Due to large variation in the time to receive ethical approval, the centers started recruitment at different times: Germany, Greece, Poland, Iceland (October 2005), UK (November 2005), Lithuania (January 2006), and Spain (March 2006). The two centers in The Netherlands and Italy joined the birth cohort of EuroPrevall later and started recruiting in October 2006 and March 2007 respectively. Six centers recruited the mothers only postnatally, two only antenatally (Iceland and UK), and one mostly ante- but also postnatally (The Netherlands). From mothers who declined consent (also fathers in Germany and The Netherlands), we attempted to anonymously document basic socio-demographic and family allergy status information as fully as possible, in order to establish the representativeness of our study population.

Inclusion criteria were a minimal gestational age of 34 completed weeks and an APGAR score (a qualitative measurement of a newborn's success in adapting to the environment outside the uterus with scores from 0 to 10) of at least seven at 5 min after birth. Families lacking sufficient language or communication skills to be able to give informed consent or with newborns participating in interventional studies that examine atopy and allergic diseases were excluded from the study.

Sample size

The study size was chosen in order to screen a large population-based sample. With an estimated overall food allergy prevalence of 5% and a precision of the estimate of $\pm 5\%$ in a 95% confidence interval, it was calculated that 7300 children must be available for evaluation at the end of the study. Assuming a dropout rate of 15% during the follow-up period we planned to recruit at least 8625 children. Each of the initial six centers (Iceland, UK, Germany, Poland, Spain, and Greece) was to recruit 1500 mothers and their newborns over an 18-month period, commencing in October 2005. Three additional centers (Lithuania, The Netherlands, and Italy) entered the birth cohort at a later time and adhered to the same exact recruitment procedure and study protocol.

Baseline and follow-up questionnaires

Standardized questionnaires were developed, where possible based on pre-existing questionnaires used in previous epidemiological studies, such as ISAAC, INFABIO, and MAS (31–33). Additional study-specific questions were generated in two meetings through collaborative efforts of all centers. The questionnaires were piloted for applicability in all centers for comprehensibility and ease of use, and the final versions were translated into the country language and



Figure 1. Countries participating in the birth cohort study of EuroPrevall. Locations of the 9 study centers are indicated by stars.

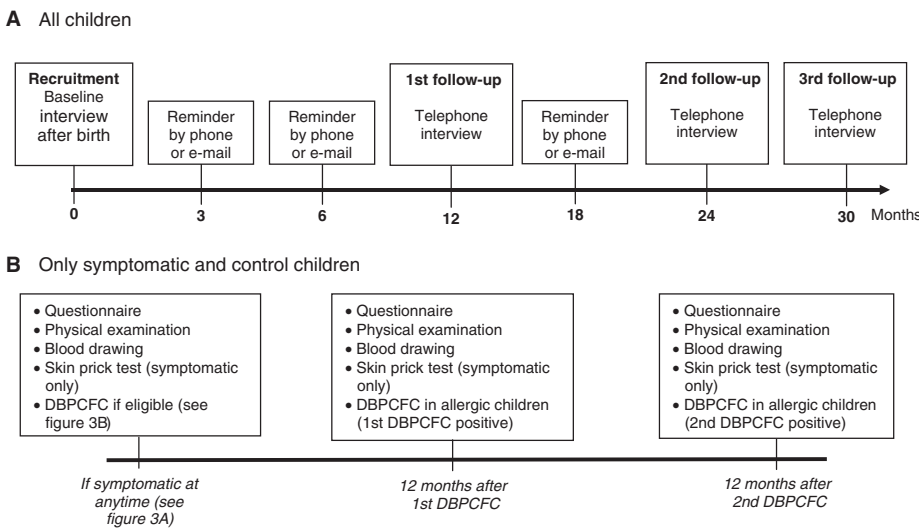


Figure 2. Time line of the birth cohort study of EuroPrevall. (A) Follow-up process for all children; (B) additional follow-up for symptomatic children eligible for double-blind placebo-controlled food challenge test (DBPCFC) and their controls.

back-translated into English by independent translators, according to the guidelines of the WHO (34). Trained interviewers administered the questionnaires either by telephone or face-to-face interview.

At recruitment, we collected information regarding the mother's pre-existing diseases, intake of foods, nutritional supplements, medications and exposure to cigarette smoke before and during pregnancy, as well as extensive socio-demographic and pet data. Data on allergic diseases of the mother, father and any blood-related siblings were assessed in detail.

At the three routine follow-up time points, we collected comprehensive data on the mother's intake of foods, nutritional supplements, medications and exposure to cigarette smoke during breastfeeding, weaning and food intake of the child, nutritional supplements, infections, medications, vaccinations, exposure to cigarette smoke, mould and pets, as well as any signs and symptoms of allergic diseases. Any changes in the allergy status and socio-demographics of the parents and siblings were also identified. The questionnaires of the birth cohort study of EuroPrevall will be accessible on the website of the coordinating institution after completion of the project.

Identification and evaluation of children with food allergy

Parents were advised to report to the study center if any signs or symptoms appear that could be caused by food (e.g. eczema,

gastrointestinal symptoms, wheezing). Using a standardized screening form, parents were invited to the study center if defined criteria are fulfilled (Fig. 3A). At this visit a physical examination was conducted including standard scoring for signs of eczema using the Scoring Atopic Dermatitis (SCORAD) assessment in children with atopic dermatitis (35), a symptomatic questionnaire was completed, 4–5 ml blood obtained for measurement of food-specific IgE and skin tests were performed (see below). Using this information the decision was made whether the child was eligible for a DBPCFC (Fig. 3B). If criteria for eligibility for a DBPCFC were met, two age-matched control children were to be recruited from the pool of noneligible children and followed with the same evaluation. Food allergies were classified as perceived (parents call with allergic symptoms related to food), probable (meets eligibility criteria for a DBPCFC, e.g. elevated allergen-specific serum IgE, but parents refused the food challenge) and confirmed (positive DBPCFC).

Double-blind placebo-controlled food challenge tests

To obtain a confirmed diagnosis for food allergy, DBPCFC tests were conducted in the study centers under the supervision of a trained pediatrician using a standardized protocol and recipes. Challenges were performed with skimmed milk powder (Dairy Crest Group, Esher, Surrey, UK), lyophilized hen's egg powder (F. R. Benson & Partners, Watford, UK), defatted peanut flour (Golden Peanut Company, Alpharetta, GA, USA), gluten powder (Kröner,

A 'Symptomatic children' after the telephone screening

- Every child with eczema despite emollients
- Child with the following signs or symptoms **definitely** associated with a specific food
 - Itchy tongue
 - Swelling or hives on the skin
 - Wheezing and/or stridor, asthma
 - Red, runny eyes or nose, sneezing, hay fever
 - Vomiting, diarrhoea, constipation, abdominal pain without fever, blood in stools
- Child with the following signs or symptoms **definitely** or **possibly** related to a specific food
 - Swollen lips
 - Recurrent itchy skin
 - Flushed skin
 - Anaphylaxis

B Eligibility for double-blind placebo-controlled food challenge tests (at least 1 criterion)

- Elevated allergen-specific serum IgE (>0.35 kU/l) unless child eats this food regularly without clinical signs or symptoms
- Positive skin prick test (≥ 3 mm mean wheal diameter) unless child eats this food regularly without clinical signs or symptoms
- Objective immediate type clinical signs or symptoms (≤ 2 hrs) after ingestion of a single food
- Repetitive subjective clinical signs or symptoms (on at least 2 occasions) after ingestion of a single food
- Clear improvement or absence of clinical signs or symptoms (e.g. eczema, diarrhoea, blood in stool) under an elimination diet

Figure 3. Criteria for (A) defining 'symptomatic children' after telephone screening; (B) eligibility for double-blind placebo-controlled food challenge tests.

Ibbenbüren, Germany), soy milk (Alpro, Ghent, Belgium) or fish (fresh cod). DBPCFC were performed titrated with increases of the allergen amount initially using 10-fold then 3-fold steps every 20 min. The amounts spanned 3 µg, 30 µg, 300 µg, 3 mg, 30 mg, 90 mg, 300 mg, 900 mg and 3 g food protein with the amount of food needed to give these protein amounts being calculated from the nutritional analysis provided by each manufacturer (or from standard food tables for cod) (Table 1). The allergenic foods were masked in extensively hydrolyzed cow's milk formula (Mead Johnson, Evansville, IN, USA) or amino acid formula (SHS, Heilbronn, Germany; Nutricia, Zoetermeer, The Netherlands) in bottle-fed infants or, for the older children, in vanilla-orange pudding made from amino acid, extensively hydrolyzed formula or rice milk and vanilla-flavoured starch with the addition of orange flavour (SHS International, Liverpool, UK) and beta carotene (beta carotene 10%; ID-number: E-160a; Synopharm, Barsbüttel, Germany) for additional blinding. Placebo meals consisted of the pure blinding matrix. Allergenic food content of all challenge materials prepared for use in each DBPCFC were tested in a central laboratory (Tepnel Biosystems, Deeside, UK).

Challenges were not performed in children with both a documented anaphylactic reaction with severe respiratory or cardiovascular symptoms after consumption of the allergenic food and elevated allergen-specific IgE. Children with sensitizations to multiple food allergens were challenged with all foods with at least a 1 : 2 ratio of placebo to active food in randomized order, e.g. cow's milk then placebo then hen's egg. Time intervals between each active or placebo provocation were at least 48 h. Due to national feeding recommendations of many of the birth cohort centers, the age at which food were introduced was staged, with cow's milk being challenged at any age, all other foods after 6 months of age with the exception of peanuts, tree nuts, seeds and fish, which are challenged only after 12 months of age. The parents were advised to avoid the specific food until the respective age. Active and placebo reactions are documented. Children were grouped according to their challenge outcome as either allergic or tolerant (Table 2). If both the active and placebo challenges were positive; the challenge was considered inconclusive and repeated. Following a negative challenge the food was reintroduced in increasing doses according to a standardized protocol under the supervision of the hospital staff.

Biological samples

At birth, cord blood was obtained in all children for future analyses of food specific IgE. Peripheral blood was obtained from children with perceived food allergy invited to the study centers for further analysis, as well as from the control children. From cord blood and

Table 2. Determination of allergic or tolerant children according to outcome of double-blind placebo-controlled food challenge tests

Active (verum)	Placebo	Classification
Positive	Negative	Allergic
Negative	Negative	Tolerant
Positive	Positive	Inconclusive*
Negative	Positive	Tolerant†

*Double-blind placebo-controlled food challenge must be repeated.

†Tolerant but placebo responder.

peripheral blood, serum was separated and frozen at -20°C until analysis. In a subgroup of 120 atopic and 120 nonatopic mothers, we collected placenta biopsies (Medical University of Vienna, Austria) and cord blood (Leiden University Medical Center, The Netherlands) for analyses of cytokine and Toll-like receptor expression. Breast milk samples are collected from mothers of symptomatic and control children still breast feeding and frozen at -20°C for future analyses of food proteins and cytokines (Charité, Berlin, Germany).

Measurement of food specific IgE

The serum of children with symptoms of a possible allergic reaction to food (Fig. 3A) and control children were tested for the six most common food allergens using the Phadia fx5 screening test (Phadia Diagnostics, Uppsala, Sweden). If the screening test was positive, the serum was analyzed for specific IgE antibodies to cow's milk, hen's egg, soy, wheat, fish and peanut. All measurements were performed centrally (allergy laboratory of the Department of Paediatric Pneumology and Immunology, Charité University Medical Center Berlin, Germany) using the Phadia ImmunoCAP 250 system (Phadia Diagnostics, Uppsala, Sweden). Additionally, component resolved diagnostics will be performed for individual allergens using ImmunoCAP-ISAC chip technology (originally developed by VBC Genomics, Vienna, Austria).

DNA and mRNA analyses

For children eligible for DBPCFC and their two age-matched controls, EDTA blood for DNA analysis and EDTA blood in RNA buffer (Applied Biosystems, now Life Technologies, Carlsbad, CA, USA) for mRNA analysis were frozen at -80°C for later analysis. Blood for DNA analysis was also obtained from parents. DNA

Table 1. Protein amounts and its equivalents used for double-blind placebo-controlled food challenge tests

Step	Protein amount	Skimmed milk powder	Equivalent cow's milk	Hen's egg powder	Equivalent hen's egg	Peanut flour	Gluten powder	Soy milk	Fish (Fresh Cod)
1	3 µg	8.3 µg	0.09 µl	6.4 µg	0.02 µl	6.0 µg	3.7 µg	0.09 µl	16.4 µg
2	30 µg	83.3 µg	0.9 µl	63.8 µg	0.24 µl	60 µg	37.4 µg	0.9 µl	164 µg
3	300 µg	833 µg	9.04 µl	638 µg	2.40 µl	600 µg	374 µg	9.04 µl	1.64 mg
4	3 mg	8.33 mg	90.4 µl	6.38 mg	24.0 µl	6.0 mg	3.74 mg	90.4 µl	16.38 mg
5	30 mg	83.3 mg	0.9 ml	63.8 mg	0.2 ml	60.0 mg	37.4 mg	0.9 ml	163.8 mg
6	90 mg	249.9 mg	2.7 ml	191.4 mg	0.7 ml	180.9 mg	112.2 mg	2.7 ml	491.4 mg
7	300 mg	833 mg	9.1 ml	638 mg	2.4 ml	603 mg	374 mg	9.1 ml	1.64 g
8	900 mg	2.50 g	27.3 ml	1.91 g	7.2 ml	1.81 g	1.12 g	27.3 ml	4.91 g
9	3 g	8.33 g	91 ml	6.38 g	24 ml	6.03 g	3.74 g	90.9 ml	16.38 g
Total dose	4.3 g	~12 g	~131 ml	~9.2 g	~1/2 egg	~8.7 g (about 4 large peanuts)	~5.4 g (about 35 g raw dried pasta)	~131 ml	~23.6 g (about medium filet)

analysis will be performed centrally (Institute of Inhalation Biology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany). Blood samples are being tracked upon arrival by a LIMS system with extracted DNA being aliquoted into tubes ready for storage in automatic sample stores. For DNA extraction Gentra kits were used (Puregene/Qiagen, Hilden, Germany); DNA amount is being quantified with a 8-channel spectrophotometer (Nanodrop, peqlab, Hilden, Germany) and quality tested by a PCR of AmelX/AmelY region with the product resolved on a microfluidics electrophoresis device (Bioanalyzer 2100, Agilent, Waldbronn, Germany). RNA analysis will be performed centrally (Department of Parasitology, Leiden University Medical Center, the Netherlands) for measurement of the expression of genes (mRNA) that are involved in pathogen recognition (Toll-like receptors) to indicate how microbial exposure might be related to the development of atopy in a child. Genes involved in the interaction of antigen presenting cells and T-cells will also be measured by quantitative PCR to determine how inhibitory pathways are related to expression of atopic disease. The expression of genes coding for the pro- and anti-inflammatory cytokines will be studied to assess the inflammatory status of the immune system of a child with allergies.

Skin prick test

Skin prick tests (SPT) were carried out in children with symptoms of a possible allergic reaction to food (Table 1) using ALK allergen solutions, histamine dihydrochloride as positive and saline solution as negative controls (ALK-Abelló, Hørsholm, Denmark). One drop of allergen solution was applied to the forearm and pricked with a 1-mm single-tine lancet (ALK-Abelló, Hørsholm, Denmark). SPTs were read after 15 min. Mean diameters of food allergens, positive and negative controls were calculated from the average of the largest wheal diameter plus largest wheal diameter perpendicular to this. Skin index (SI) was calculated as the ratio of allergen wheal size divided by the histamine wheal size (36, 37). The SPT result for each allergen was defined as positive if the mean wheal diameter was 3 mm or larger and the SI is greater than 0.6.

Study quality management

Before recruitment commenced, center pediatricians and other staff participated in a three-day training session, hosted by the coordinating center. Demonstrations of the standardized protocols for SPT, biological samples, standard scoring for signs of eczema (SCORAD, Kunz 1997) and DBPCFC were conducted and the investigators were observed practicing these protocols. All questionnaires, data entry and other documentation forms were demonstrated and standard operating procedures explained. In addition, further training sessions were held on a twice-yearly basis during the EuroPrevall congresses, as well as on-site training at the individual centers.

Site visits to each center were conducted by members of the coordinating center to ensure uniform implementation of the study and testing protocols, to document and correct any deviations, as well as to provide any specific training needs. Monthly reporting standards enabled close tracking of recruitment and evaluation of children with symptoms of food allergy and standardized forms.

Current status

By June 2009, recruitment for the birth cohort study has been completed in all centers except the Dutch center where inclusion of children will be continued until December 2009. So far, a total of

12 074 newborns and their families have been included in the study. For each individual country, as well as the entire cohort, we are currently analysing the prevalences of food allergies during the first 12, 24 and 30 months of life with respect to DBPCFC confirmed reactions, allergic sensitization to specific foods and parent-reported symptoms.

Conclusions

The EuroPrevall birth cohort study is the first worldwide that has been specifically designed to examine food allergies. It differs considerably from earlier birth cohorts that collected information on food allergies (38, 39), particularly by its large sample size, comprehensive questionnaires on prenatal maternal and postnatal nutrition, standardized objective methods including food provocation tests to confirm the diagnosis of food allergy, serological and genetic tests. The multinational birth cohort initiated by EuroPrevall will provide unique data on variations of the prevalence, risk factors, quality-of-life, and costs of perceived and confirmed food allergies in infants and children across major climatic and cultural regions of Europe. The results of this study are expected to provide the basis for the development of more informed and integrated strategies for preventing and treating food allergies.

Organizational structure

EuroPrevall overall co-ordinating center

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Birth cohort co-ordinating center

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8. Department of Paediatric Pulmonology, Emma Children's Hospital, Academic Medical Centre, Amsterdam, The Netherlands (A. Sprickelman);
9. Melloni Paediatric, University of Milan Medical School at the Macedonio Melloni Hospital, Milan, Italy (A. Fiocchi).

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