

Phase II of the International Study of Asthma and Allergies in Childhood (ISAAC II): rationale and methods

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ABSTRACT: International comparative studies, investigating whether disease incidence or prevalence rates differ between populations and, if so, which factors explain the observed differences, have made important contributions to the understanding of disease aetiology in many areas. In Phase I of the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence rates of symptoms of asthma, allergic rhinitis and atopic eczema in 13–14-yr-olds, assessed by standardised questionnaires, were found to differ >20-fold between the 155 study centres around the world. Phase II of ISAAC aims to identify determinants of these differences by studying informative populations.

A detailed study protocol was developed for use in community-based random samples of children aged 9–11 yrs. The study modules include standardised questionnaires with detailed questions on the occurrence and severity of symptoms of asthma, allergic rhinitis and atopic eczema, their clinical management, and a broad range of previous and current exposure conditions. In addition, standardised protocols were applied for examination of flexural dermatitis, skin-prick testing, bronchial challenge with hypertonic saline, blood sampling for immunoglobulin E analyses and genotyping, and dust sampling for assessment of indoor exposures to allergens and endotoxin.

To date, ISAAC II field work had been completed or started in 30 study centres in 22 countries.

The majority of centres are in countries that participated in International Study of Asthma and Allergies in Childhood Phase I and reflect almost the full range of the observed variability in Phase I prevalence rates.

Eur Respir J 2004; 24: 406–412.

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Keywords: Asthma, childhood, eczema, epidemiology, International Study of Asthma and Allergies in Childhood Phase II, rhinitis

Received: August 5 2003
Accepted after revision: March 22 2004

The coordination and central laboratory analyses of the European centres were supported by the Fifth Framework Programme of the European Commission, Brussels, Belgium (QLK4-CT-1999-01288). Support details for the local studies are given in the *Funding of local studies* section at the end of this article. Pharmacia Diagnostics (Uppsala, Sweden) generously made reagents available at a nominal cost. ALK (Horsholm, Denmark) generously provided reagents for fieldwork in several low-income countries without charge.

International comparative studies, conducted in a standardised way in different populations, have made important contributions to the understanding of disease aetiology in many areas, particularly cardiovascular diseases and cancer [1–5]. They investigate whether disease incidence or prevalence rates differ between populations and, if so, which factors explain the observed differences [1, 2].

In the 1990s, two international collaborative studies, one in children [6–10] and one in adults [11–14], chose the international comparative approach in the field of asthma and allergies. Both studies found large differences in prevalence rates between countries, which are likely to be determined to a large extent by environmental or lifestyle factors. Phase I of the International Study of Asthma and Allergies in Childhood (ISAAC I) used standardised written and video questionnaires on the occurrence and severity of symptoms of asthma, allergic rhinitis and atopic eczema, which were completed by children aged 13–14 yrs in 155 centres in 56

countries around the world [7]. The prevalence rates were found to differ >20-fold between study centres [7–10]. Phase II of ISAAC (ISAAC II) aims to identify determinants of the observed differences in prevalence rates. For example, the role of atopic sensitisation and bronchial responsiveness, as well as the influence of indoor exposures and lifestyle factors, are being investigated. The study methods are much more demanding than those of ISAAC I and are, therefore, being applied in a smaller, but informative, number of populations, *i.e.* centres with relatively high or low prevalence rates in ISAAC I or with unusual exposure and living conditions.

Objectives

The specific objectives of ISAAC II are to assess as follows:
1) the variation in the prevalence and severity of clinical

symptoms and objective markers of asthma, allergic rhinitis and atopic eczema in children living in different study centres worldwide; 2) the association between potential determinants and the occurrence and severity of asthma and allergies in children in different centres, and to make comparisons between and within study centres worldwide; and 3) the associations of genotypes known or suspected to play a role in childhood asthma and allergies with the measured phenotypes and to investigate gene-gene as well as gene-environment interactions in different study centres worldwide.

Methods

Study area and population

ISAAC II studies were conducted in children aged 9–11 yrs. This age group, although different from those studied in ISAAC I (13–14 yrs and 6–7 yrs), was chosen because children aged 10 yrs are known to participate and perform satisfactorily in all of the proposed tests, including spirometry and blood sampling (often problematic in younger children) and examination for flexural dermatitis (problematic in older children in some cultures). Valid measurements of these physiological parameters were considered more important than a direct comparison of age groups with ISAAC I. Study samples were drawn from populations living in defined geographical areas.

Study modules

The ISAAC II study protocol includes a series of modules that have been developed and agreed by the Steering Committee of the worldwide project [15, 16]. These modules are freely available for use in other studies, provided that they are strictly adhered to and properly referenced in any resulting publications.

Questionnaires. Standardised core questionnaires were developed on demographics, wheezing, allergic rhinitis and atopic eczema. These include detailed questions on the occurrence and severity of atopic symptoms, identical to those used for parents of children aged 6–7 yrs in ISAAC I [6]. Supplementary questionnaires dealt with additional respiratory symptoms, as well as the clinical management and medical treatment of asthma and wheezing, hay fever, rhinitis and flexural dermatitis. The risk factor questionnaire enquires about current and previous (first year of life) living conditions, and collects information about factors such as birthweight, gestational age, place of birth, socioeconomic status, breast feeding, family size, family history of atopic diseases, infections, vaccinations, crowding, pets, environmental tobacco smoke, cooking fuels, damp housing, type of windows, type of bedding, area of residence, physical activity and nutritional factors. All questionnaires were for completion by parents.

Examination for flexural dermatitis. Participating children were examined for visible flexural dermatitis according to a photographic protocol [17] and detailed instructions on its use in the field. The examination involved inspection of the skin: 1) around the eyes, 2) around the sides and front of the neck, 3) in front of the elbows, 4) behind the knees, and 5) in front of the ankles. The presence or absence of signs of visible flexural dermatitis was recorded for each of the five areas. A training package of high-quality clinical photographs and instructions for fieldworkers was developed, which also included a test set of pictures for central evaluation of fieldworker quality [18].

Skin-prick testing for atopy. Extracts of six common aeroallergens (*Dermatophagoides pteronyssinus*, *D. farinae*, cat hair, *Alternaria tenuis*, mixed tree pollen and mixed grass pollen) and control solutions produced by ALK (Hørsholm, Denmark) were used. Cockroach and other allergens of local relevance could be added. Briefly, a drop of each allergen extract and the positive (10 mg·mL⁻¹ histamine) and negative (diluent) control was placed on to the skin of the volar side of the left forearm and pierced vertically using 1-mm ALK lancets. After 15 min, the outer contour of the weal reaction was outlined using a fine felt-tip pen, and the result expressed as the mean of the lengths of the longest diameter and the perpendicular line through its centre. Detailed recommendations for training and monitoring of fieldworkers were provided in order to assess reproducibility and consistency over time.

Bronchial challenge with hypertonic saline. A minimum of two baseline spirometers was recorded, and the higher of two reproducible (within 5%) measurements of forced expiratory volume in one second (FEV₁) was recorded as baseline FEV₁. Bronchial reactivity was assessed by changes in FEV₁ after inhalation of nebulised saline using an ultrasound nebuliser [19]. The children inhaled hyperosmolar (4.5%) saline for periods of increasing duration: 0.5, 1, 2, 4, and 8 min. FEV₁ was measured 1 min after each inhalation period and the next challenge period followed after 3 min. If the FEV₁ fell by 10–15% from the baseline value, the exposure time was repeated. If, after two repetitions, the FEV₁ remained 10–15% below the baseline value, the duration of the inhalation period was doubled again according to the protocol. Either way, bronchial challenge was stopped if either the FEV₁ had fallen by ≥15% from baseline or the total inhalation period of 15.5 min had been reached. The saline canister and tubing were weighed before the first and after the last inhalation period in order to measure the aerosol dose delivered. Participating children were asked to withhold bronchodilator medication before the challenge. Regular use of inhaled steroids was recorded, but not withheld. In children with a baseline FEV₁ of <75% of the predicted value, no bronchial challenge was performed and an inhaled bronchodilator was administered.

Collaborative laboratory analyses

Serum samples for analyses of immunoglobulin E. Blood samples were collected by venipuncture into plain sample tubes and allowed to clot at room temperature. The serum was separated by centrifugation (2,500–3,000 revolutions per minute for 15 min) and pipetted off in 1-mL aliquots into plastic tubes with screw corks. Samples were frozen, stored at ≤-20°C and transported under dry ice.

Serum levels of total immunoglobulin (Ig)E and IgE directed against a panel of common inhalant allergens (Phadiatop™) were quantified using the Pharmacia CAP System™ (Pharmacia Diagnostics, Uppsala, Sweden). The analyses were performed in one central certified (SWEDAC) laboratory (Dept of Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden). In three centres, analyses had already been performed using the same methods but in another laboratory. The comparability of results obtained in different laboratories was tested in 400 samples from two centres [20], which were analysed in the central laboratory and another laboratory (two laboratories in the case of total IgE). There was good agreement between results: for total IgE levels, the concordance correlation coefficient was >0.99; and for specific IgE classes, the weighted κ was 0.88.

Dust samples for analyses of aeroallergens and endotoxins. Instructions for dust collection are in the ISAAC II manual and in the more detailed protocol [21], including photographic instructions, developed by Utrecht University (Utrecht, The Netherlands), which has been in use since 2001. Vacuum cleaners with a ≥ 800 -W engine were used in combination with ALK filter holders and 70-mm paper filters (ALK or Schleicher&Schuell, Dassel, Germany). Dust was sampled separately from two sites within homes: the mattress or sleeping place of the index child participating in the study, and the floor in the living room. The beds were vacuumed on the sheets or, if there were no sheets, directly on the mattress or sleeping surface. The original instructions were to vacuum soft surfaces for $2 \text{ min}\cdot\text{m}^{-2}$, covering an area of $\geq 2 \text{ m}^2$ ($\geq 4 \text{ m}^2$ if possible), and hard surfaces for $1 \text{ min}\cdot\text{m}^{-2}$, covering an area of $\geq 4 \text{ m}^2$ (ISAAC II manual). This was subsequently made more specific as follows: 1) mattress, whole area for 2 min; 2) carpeted floors, 2 m^2 for 4 min; 3) smooth floor with $\geq 4\text{-m}^2$ rug, 2-m^2 rug for 4 min; and 4) smooth floor with no rug or smaller rug(s), 4-m^2 smooth floor for 4 min (Utrecht University protocol). After collection of dust, the filter box with the filter was removed, the lid was attached and then the box was stored in a plastic bag at -20°C until analysis (ISAAC II manual). Alternatively, after dust collection, the filter with dust was transferred to a preweighed plastic tube directly after the vacuum cleaning, facilitating transport and analysis (Utrecht University protocol).

Laboratory analyses of the dust of European centres took place at Utrecht University, at the Institute for Risk Assessment Sciences. Dust of non-European centres was analysed at the laboratory of the Wellington Asthma Research Group (Wellington School of Medicine and Health Sciences, Wellington, New Zealand). After weighing, floor dust was extracted using Tween-20 in water [22] and analysed for endotoxin, using a kinetic chromogenic *Limulus* amoebocyte lysate (LAL) test, using only one batch of LAL reagents (LAL lot no. 1L676S and lipopolysaccharide standard lot no. 2L0090; BioWhittaker, Verviers, Belgium) for all ISAAC analyses. Endotoxin concentrations were expressed as units of endotoxin per gram of dust (1 endotoxin unit=0.09 ng). Mattress dust was extracted using phosphate-buffered saline containing 0.05% Tween-20, and analysed for the allergens *D. pteronyssinus* antigen 1 (Der p 1), *D. farinae* antigen 1 (Der f 1), dog antigen 1 (Can f 1) and cat antigen 1 (Fel d 1), using the enzyme immunoassay reagents and methods of Indoor Biotechnologies (Cardiff, UK), essentially as described previously [23]. The lower limits of detection are $8 \text{ ng}\cdot\text{mL}^{-1}$ for Der p 1, $6 \text{ ng}\cdot\text{mL}^{-1}$ for Der f 1, $20 \text{ ng}\cdot\text{mL}^{-1}$ for Can f 1 and $0.4 \text{ ng}\cdot\text{mL}^{-1}$ for Fel d 1 for five-fold diluted samples. Samples with undetectable amounts of allergen or endotoxin were assigned a value two-thirds that of the lowest observed amount per gram of dust for the specific component determined.

DNA preparation for genotyping. The alternative sources of DNA from study participants included frozen storage of whole blood or buffy coats and storage of whole blood as dried spots. Samples originating from populations with prevalence rates of category III infectious agents (such as hepatitis B and HIV) of $>1\%$ required extraction in a containment level 3 laboratory.

Whole blood and buffy coat samples were received frozen in tubes containing ethylenediamine tetra-acetic acid, with typical volumes ranging 4–10 mL. Extractions were performed using the Promega Wizard genomic DNA purification kit (Promega UK, Southampton, UK), using the standard protocol adjusted for sample volume. Extraction of blood spots was carried out using the Qiagen Midi Kit (Qiagen,

Crawley, UK). Blood spots were cut from filter paper and further fragmented using autoclaved scissors. Cell lysis and protein precipitation was carried out using volumes adjusted for sample volume.

Following extraction, all samples were quantified using a Molecular Probes Pico Green double-stranded DNA Quantification Kit (Invitrogen, Paisley, UK). Blood spots yielded 0.0–48.0, buffy coats 0.0–1,013.0 and whole blood 0.0–578.0 $\text{ng}\cdot\mu\text{L}^{-1}$ DNA. The corresponding total amount of DNA from each source was blood spots 0.9–2.1, buffy coats 3.5–69.5 and whole blood 47.0–69.0 μg . Primer extension preamplification was used to increase the quantity of DNA by a factor of 40. This involves using random primer sequences to amplify template DNA, resulting in whole genome amplification [24].

The aim of the genetic module is to genotype the samples with regard to all published genetic polymorphisms associated with childhood asthma and allergies. Several single nucleotide polymorphisms will be typed from each gene in order to create haplotypes that may be compared across populations. Single nucleotide polymorphisms will also be chosen according to their heterozygosity level, with minor allele frequencies of $\geq 20\%$. Genotyping will be carried out using the Sequenom System (Sequenom, Inc., San Diego, CA, USA), which is based on an assay of primer extension with detection by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF). Genotyping is performed at the University of Oxford (Oxford, UK).

Sampling options and sample size

Random samples of ≥ 10 schools had to be chosen from a complete sampling frame of all schools in a defined geographical area. Each centre was expected to provide data on $\geq 1,000$ children with completed parental questionnaires, examination for flexural dermatitis and skin-prick tests for atopy. Measurement of bronchial responsiveness and collection of blood and dust samples were performed in stratified subsamples. These consisted of ≥ 100 children chosen at random from those with a history of wheeze in the past year and ≥ 100 children selected at random from all those without wheeze in the past year (option B). This stratified sampling procedure substantially reduced the costs in comparison to testing the entire sample of children. It permits comparisons between centres of objective markers of asthma and atopy, such as bronchial responsiveness and total or specific serum IgE levels. At the discretion of local investigators, all tests could be performed on all participants (option A).

It was calculated that this sample size and sampling procedure (option B) would permit detection (with 95% confidence) of prevalence differences between two centres with 80% power at the following magnitude: wheezing 6 versus 10% (90% power); severe wheezing 1 versus 3%; atopic sensitisation 15 versus 20%; flexural dermatitis 2.5 versus 5%; and bronchial hyperresponsiveness 20 versus 40%.

Quality control

Major efforts were made to ensure comparable data quality in all study centres. A standardised study protocol was developed, which gave very detailed instructions for all study procedures. It provided detailed guidance for the training of fieldworkers, and some elements were centrally evaluated, e.g. skin examination for dermatitis. In addition, close collaboration and visits between study centres was encouraged and occurred throughout the study. There was a central

3-day workshop for training in ISAAC II methods at the University of Münster (Münster, Germany) in March 1998, at which almost all countries participating in ISAAC II were represented (exceptions were West Bank, Ghana and Ecuador). There were many visits of experienced collaborators to other study centres to train fieldworkers or monitor fieldwork. Relevant aspects of fieldwork were recorded and reported to the data centre. The analyses were carried out in central laboratories in Oxford, Stockholm, Wellington and Utrecht.

Data management and statistical analyses

All data from fieldwork and laboratory analyses were collated by the ISAAC II Coordinating and Data Centre at the University of Ulm (Ulm, Germany). The study centres submitted their data according to the coding and transfer manual, which provides detailed guidance. Prevalence rates will be calculated and associations of health outcomes with potential determinants will be investigated using bivariate and multivariate techniques. Data collected only in stratified subsamples (option B) will be analysed taking the sampling structure into account [25, 26].

Participating study centres

To date, ISAAC II fieldwork has been completed or started in 30 study centres in 22 countries (table 1). The data from 24 study centres have been accepted by the Coordinating and Data Centre in Ulm.

ISAAC II focused on "informative" populations, usually

identified on the basis of ISAAC I findings [7–10]. The countries included in ISAAC II reflect almost the full range of variability in asthma prevalence rates observed in ISAAC I (fig. 1). Thus, countries which were ranked at the top (e.g. UK and New Zealand) or bottom (e.g. Albania, Georgia and India) of the worldwide distribution of prevalence rates

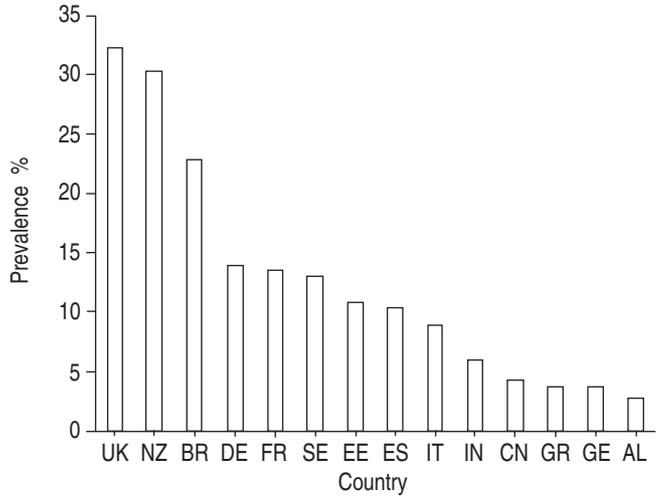


Fig. 1.—Mean 12-month wheeze prevalence obtained in the 13–14 yrs age group in the International Study of Asthma and Allergies in Childhood (ISAAC) Phase I in countries participating in ISAAC Phase II [9]. NZ: New Zealand; BR: Brazil; DE: Germany; FR: France; SE: Sweden; EE: Estonia; ES: Spain; IT: Italy; IN: India; CN: China; GR: Greece; GE: Georgia; AL: Albania.

Table 1.—Current status of International Study of Asthma and Allergies in Childhood Phase II (ISAAC II) data collection

Country	Centre	Sampling option	Fieldwork complete	Checking complete [#]
Albania	Tirana	B	Y	Y
Brazil	Pôrto Alegre	B	N	N
China	Beijing	B	Y	Y
	Guangzhou	B	Y	Y
	Hong Kong	B	Y	Y
Ecuador	Pichincha province	A	Y	N
Estonia	Tallinn	B	Y	Y
France	Créteil	B	N	N
Georgia	Tbilisi	B	Y	Y
Germany	Dresden	A	Y	Y
	Munich	A	Y	Y
Ghana	Kintampo	B	Y	Y
Greece	Athens	B	Y	Y
	Thessaloniki	B	Y	Y
Iceland	Reykjavik	B	N	N
India	Mumbai	B	Y	N
Italy	Rome	B	Y	Y
Latvia	Riga	A	Y	N
The Netherlands	Utrecht	A	Y	Y
New Zealand	Hastings	B	Y	Y
Norway	Tromsø	B	Y	Y
Spain	Almeria	B	Y	Y
	Cartagena	B	Y	Y
	Madrid	B	Y	Y
	Valencia	B	Y	Y
Sweden	Linköping	B	Y	Y
	Östersund	B	Y	Y
Turkey	Ankara	B	Y	Y
UK	West Sussex	B	Y	Y
West Bank	Ramallah	B	Y	Y

B: some tests performed in stratified subsample of wheezers and nonwheezers; A: tests performed in all children; Y: yes; N: no. [#]: data checking carried out by ISAAC II Coordinating and Data Centre, Dept of Epidemiology, University of Ulm, Ulm, Germany.



Fig. 2.—Location of study centres (■) at which International Study of Asthma and Allergies in Childhood Phase II fieldwork is ongoing or complete.

are represented in ISAAC II. The location of the study centres is shown in figure 2. Additional centres, which have been included due to interesting exposure and living conditions, are located in Ecuador, Ghana, Iceland, Norway, Turkey and West Bank.

Organisational structure

ISAAC II is managed by the ISAAC II Steering Group and by subcommittees who deal with specific aspects of the study. All members of the ISAAC II Steering Group are also members of the ISAAC II Steering Committee. ISAAC II study centres in countries with more than one centre are coordinated by National Coordinators. All studies have been approved by local ethics committees, and the international coordination and collaboration has been approved by the ethics committees of the universities of Münster and Ulm, Germany.

Outlook

The International Study of Asthma and Allergies in Childhood Phase II will be the most comprehensive study of the prevalence, risk factors, and genetics of asthma and allergies in children yet undertaken. The wide range of ethnicities, environments, lifestyles, and climatic and socio-economic conditions amongst the >30,000 children who have taken part in the International Study of Asthma and Allergies in Childhood Phase II will provide a unique cross-sectional view of the determinants of asthma and allergic disease and potentially the interactions between environmental and genetic factors.

Acknowledgements. The authors wish to thank all children, parents, teachers, fieldworkers and laboratory workers for their enormous contribution to this collaborative study.

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Laboratories: Blood samples were analysed for serum IgE levels at the Karolinska Institute, Stockholm, Sweden (B. Bj rkst n and M. van Hage-Hamsten). DNA analyses were performed at the Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK (W. Cookson, M. Moffatt and J. Cantelmo). Analyses of dust samples were carried out at the Institute for Risk Assessment Sciences, University of Utrecht, Utrecht, the Netherlands (B. Brunekreef, D. Schram, M. Boeve-Wietzes, G. Doekes, G. Terpstra, J. Spithoven and S. de Wind), and by the Wellington Asthma Research Group, Wellington School of Medicine and Health Sciences, Wellington, New Zealand (J. Crane and R. Siebers).

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Module coordinators: ISAAC II modules have been developed by subgroups of members of the Steering Committee. *Coordinators:* M. Burr (additional respiratory questions); C. Robertson (questions on disease management); S.K. Weiland (risk factor questionnaire); H.C. Williams (examination for flexural dermatitis); E. von Mutius (skin-prick test and serum IgE); C. Robertson (bronchial responsiveness to hypertonic saline); B. Björkstén (blood sampling and frozen storage).

Funding of local studies: *Almeria:* Fondo de Investigacion Sanitaria (grant code 00/1092E); *Ankara*:* Scientific and Technical Research Council of Turkey (grant code SPAG-2237), Treatment and Research Foundation of Turkey for Allergy, Asthma and Immunology, and the Research Foundation of Hacettepe University Faculty of Medicine; *Athens:* the Thorax Foundation Research Centre, Greece; *Cartagena:* Fondo de Investigacion Sanitaria (grant code 00/1092E). *Créteil:* French Institute of Health and Medical Research (Institut National de la Santé et de la Recherche Médicale (INSERM)) (grant codes IDS 337/4D001D and 737/69480), Ministère de l'Emploi et de la Solidarité (grant code 227/7HL02D), Mutuelle Générale de l'Education Nationale (grant code 257/8PL01F) and Agence de l'Environnement et de la Maitrise de l'Energie ADEME/PRIMEQUAL 96 (grant code FJ012B); *Dresden:* German Ministry of Education and Research (01 EE 9411-3); *Hastings:* Health Research Council of New Zealand, Asthma and Respiratory Foundation of New Zealand, and Hawkes Bay Medical Research Foundation; *Kintampo*:* *Linköping:* the Swedish Foundation for Health Care Sciences and Allergy Research; *Madrid:* Fondo de Investigacion Sanitaria (grant code 00/1092E); *Mumbai*:* Jaslok Hospital & Research Centre; *Munich:* German Ministry of Education and Research (01 EE 9411-3); *Östersund:* the Swedish Foundation for Health Care Sciences and Allergy Research; *Pichincha province:* Wellcome Trust; *Pôrto Alegre:* Rudolf und Clothilde Eberhardt Foundation, Ulm, Germany; *Ramallah:* Al-Quds University, Directorate General for International Cooperation and Belgian Technical Cooperation; *Riga*:* *Rome:* Lazio Regional Health Authority; *Tallinn*:* *Tbilisi*:* *Thessaloniki:* the Thorax Foundation Research Centre, Greece; *Tirana*:* *Utrecht:* Dutch Ministries of the Environment, of Health and of Transport, Rotterdam, the Netherlands; *Valencia:* Fondo de Investigacion Sanitaria (grant code 00/1092E); West Sussex: South Thames National Health Service Regional Research and Development project SPGS 573. *: centres supported, at least in part, by European members of the ISAAC Steering Committee.

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