DOCTORAL THESIS

HOUSEHOLD AEROALERGENS IN THE INCEPTION OF INFANT ATOPY AND ASTHMA. THE AMICS STUDY.

Maties Torrent Quetglas

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PREFACE

The present doctoral thesis consists of a series of ten articles (8 published and 2 in press) based on data collected from the Asthma Multicentre Infant Cohort Study (AMICS), carried out in Ashford (UK) and Barcelona and Menorca (Spain). In addition to the articles themselves, a general introduction and a general discussion are included to put together the findings of the articles in the light of present scientific evidence. A section on the implications for the etiologic research of asthma and for public health and a list of final conclusions are also included. I have been responsible of the AMICS cohort in Menorca since its origin in 1997, which has been funded by different grants from the Spanish Ministry of Health, the European Commission and a private foundation. The main initial objective of the European project was to measure the role of common domestic aeroallergens in the inception of atopy and asthma. The Menorca cohort extended his objectives including additional data on dietary habits and blood levels of organochlorine compounds. The project continues in Menorca and at the moment of presenting this thesis the 9 years follow-up work is being carried out.

ACRONYMS

AMICS: Asthma Multicentre Infant Cohorts Study

CI: Confidence Interval

DDE: Dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

Der f 1: Dermatophagoides Farinae

Der p 1: Dermatophagoides Pteronisinus

ELISA: Enzyme-linked Immunoabsorbent Assay

Fel d 1: Cat allergen

FFQ: Food Frequency Questionnaire

HDM: House Dust Mite

Ig E: Immunoglobulin E

INMA: INfancia y MedioAmbiente (Spanish research network on Childhood and Environment).

n3-n6: Omega 3-Omega 6

OR: Odds Ratio

PCB: Polychlorinated Biphenyls

POC: Persistent Organic Components

PUFA: Polyunsaturated fatty acids

p.p.b.: parts per billion

RR: Relative Risk

SD: Standard Deviation

SNP: Single Nucleotide Polymorphism

SPT: Skin Prick Test

SUMMARY

Background

Specific sensitisation and asthma have traditionally been linked to allergen exposure through inhalation. In more recent years, however, several studies have challenged the assumption that the initiation of asthma is related to allergen exposure, while the available epidemiological evidence is considered to be consistent with a role of allergen exposures in the development of specific sensitisation; that sensitisation to common aeroallergens is linked with an increased risk of wheeze and asthma is well established. Available evidences on the relationship between allergen exposure and atopy or asthma, however, is mostly derived from cross-sectional surveys, a few prospective studies of selected 'high-risk' populations, surveys of asthmatic children, and studies in areas where exposures to house dust mite are extremely low. Prospective studies conducted in different areas with enough variation to exposure, including several allergens and in non-selected representative populations were needed to simultaneously test different possible explanations to this apparent incongruity.

Objectives

The main objective was to assess, in a prospective manner and in a general population, the role of early life exposures to Der p1 and Fel d1 upon the inception of sensitisation and asthma.

Additional objectives are: (1) To determine the Der p1 and Fel d 1 allergen levels in the indoor home environments in three different European settings; (2) To ascertain the level of home exposure to such respiratory irritants as NO_2 and tobacco smoke; (3) To make an estimate of the influence to atopy and asthma of some pregnancy and early life dietary habits; (4) To assess the association of cord serum and at 4 years blood levels of DDE and other organochlorine compounds with atopy and asthma during early childhood; (5) To determine the role and interaction with other factors of breastfeeding.

Methods

Pregnant women and their children were recruited for the Asthma Multicentre Infant Cohort Study (AMICS). Three cohorts (Ashford in the United Kingdom and Menorca and Barcelona in Spain) followed the same research protocol. A total of 1611 new-born children were initially enrolled in the cohorts, from whose homes Der p1 and Fel d1 allergens were measured in household dust samples at three months of age for 1474 (91.5%) participants; blood extraction and specific IgE determinations were performed on 1019 (69.1%) of children at four years of age, and skin prick tests were performed at six years of age on 1182 (80.2%) of them. Wheeze and diagnosed asthma were reported in yearly questionnaires. On the Menorca cohort, prenatal exposure of organochlorine compounds was measured in cord serum in 405 (83%) children and additional information on dietary habits was collected. The role of the different exposures collected on the inception of atopy and asthma was analysed.

Results

Exposure to Der p1 early in life was not related with a positive specific prick test, nor with asthma or persistent wheeze at 6 years. Fel d1, however, showed an association with all these outcomes: the relationships suggest a steep increase in risk at low levels of exposure, and a flattening above 1 μg/g of dust (third vs. first tertile OR: 4.43 for positive specific prick test and 2.6 for diagnosed asthma at six years of age). Our results also indicate that prenatal exposure to DDE residues may contribute to development of asthma. With respect to diet, our data shows a protective effect of fish intake during pregnancy for the new-born on the risk of atopy and of atopic wheeze at six years. Fish intake at six years also showed a protective role on atopy and a high consumption of fruity vegetables at this age was found to have a beneficial effect on current wheeze and atopic wheeze.

Conclusions

Dose-response relationships between allergen exposure and sensitisation or asthma may be allergen specific and non-linear; a minimum threshold level would be needed to induce sensitisation, but no dose-response relationship would exist above this level. In addition, the effect of a particular allergen appears to be very similar upon atopy and asthma inception. According to our data, it seems improbable that interventions to reduce domestic allergen levels alone will have a major impact on the incidence of sensitization or asthma in childhood. Other exposures, as chemical contaminants or certain components of diet, may also play an important role in the inception of atopy and asthma and should be further investigated.

RESUM

Antecedents

L'atòpia i l'asma s'han associat tradicionalment a l'exposició als al.lèrgens. Recentment, diversos estudis han posat en dubte que el procés d'iniciació de l'asma tingui relació amb l'exposició als al.lèrgens, mentre l'evidència epidemiològica que estableix una associació entre exposició als al.lèrgens i la corresponent sensibilització específica es segueix considerant vàlida. Per altre part, l'associació entre sensibilització als al.lergens comuns i asma és ben establerta. Les evidències que relacionen l'exposició a al.lergens amb l'atòpia i l'asma, però, es basen sobretot en estudis transversals, algun estudi prospectiu en poblacions d'alt risc, estudis en nens asmàtics i estudis a zones on l'exposició als àcars de la pols es extremadament baixa. Per a poder avançar en aquest coneixement i poder entendre la incongruència que representa que l'exposició estigui associada a l'atòpia però no a l'asma, quan aquestes dos últimes estan fortament associades, cal fer estudis prospectius en poblacions representatives i no seleccionades en vàries zones amb suficient variabilitat en les exposicions, on es quantifiquin diferents al.lergens.

Objectius

El principal objectiu en el moment de dissenyar el projecte era estimar, de manera prospectiva i en població general, el paper de l'exposició durant les

primeres etapes de la vida del Der p 1 i el Fel d 1 en la iniciació de la sensibilització i l'asma.

Altres objectius addicionals son: (1) Determinar els nivells de Der p 1 i de Fel d 1 a l'interior dels habitatges en tres entorns europeus diferents; (2) Establir el nivell d'exposició domèstica a certs irritants respiratoris com el NO_2 i el fum del tabac; (3) Fer una estimació de la influència dels hàbits alimentaris durant l'embaràs i els primers anys de vida en l'atòpia i l'asma; (4) Avaluar la influència dels nivells de DDE i altres compostos organoclorats a la sang de cordó i als 4 anys en l'atòpia i l'asma a l'infància; (5) Determinar el paper de l'alletament matern i la seva interacció amb altres factors en el procés d'iniciació de l'atòpia i l'asma.

Mètodes

Per a l'estudi AMICS (Asthma Multicentre Infant Cohort Study) es van incloure dones embarassades i els seus fills d'una població del Regne Unit (Ashford), de la ciutat de Barcelona i de l'illa de Menorca seguint un mateix protocol. En total es van incloure 1611 nou nats; als domicilis de 1474 (91.5%) d'aquests nens es va recollir pols per a mesurar-hi els nivells de Der p 1 i de Fel d1 quan els nens tenien tres mesos d'edat; als quatre anys es va fer una extracció de sang per a determinar les IgE específiques a 1019 (69.1%) nens i als sis anys es van fer les proves d'al.lèrgia cutània a 1182 (80.2%). Cada any es va administrar un qüestionari sobre sibil·lants o xiulets i altres símptomes respiratoris i sobre diagnòstic d'asma. A la cohort de Menorca, a més, es va quantificar l'exposició prenatal a organoclorats a la sang de cordó de 405 (83%) nens. A Menorca

també es van administrar qüestionaris addicionals referents a la dieta durant l'embaràs, als quatre i als sis anys. S'ha analitzat el paper de les diferents exposicions recollides en el procés d'iniciació de l'atòpia i l'asma.

Resultats

L'exposició a Der p1 a l'inici de la vida no mostra relació amb els test específic d'al·lèrgia cutània ni amb el diagnòstic d'asma ni la presència de sibil·lants persistents als 6 anys. L'exposició al Fel d 1, per altre banda, mostra una associació amb tots ells: la relació observada suggereix un fort increment del risc a nivells baixos d'exposició i un aplanament de l'associació per sobre de 1 μg/g de pols (OR de 4.43 del tercer tercil d'exposició respecte al primer per una prova d'al·lèrgia cutània específica positiva i 2.6 per el diagnòstic d'asma als sis anys). Els nostres resultats també mostren que l'exposició prenatal al DDE pot contribuir al desenvolupament de l'asma. Pel que fa a la dieta, les nostres dades mostren un efecte protector del peix consumit per la mare durant l'embaràs per el risc d'atòpia i dels sibil·lants atòpics als 6 anys per al nou nat. El consum de peix als sis anys també mostra un efecte protector de l'atòpia i el consum de verdures de fruita en aquesta edat va mostrar un efecte beneficiós al disminuir les sibil·lants als sis anys.

Conclusions

Les associacions dosi-reposta entre exposició a al·lergens i la sensibilització o l'asma probablement son específiques per cada al·lergen i no lineals, essent

necessari un dintell minin d'exposició per a induir la sensibilització, però per sobre d'aquest dintell no hi hauria relació dosi-resposta. A més, les nostres dades mostren que l'efecte d'un al·lergen específic és molt similar per l'atòpia i per l'asma. A partir d'aquest resultats, sembla poc probable que les intervencions dirigides a reduir els nivells domèstics d' al·lergens puguin, per sí mateixes, tenir cap impacte important en la incidència d'atòpia o d'asma a la infància. Altres exposicions, com els contaminants químics ambientals o certs components de la dieta, poden tenir un paper important en el procés d'iniciació de l'atòpia i l'asma i s'haurien de seguir investigant en el futur.

1. INTRODUCTION

1.1.The asthma epidemic

Asthma has become one of the major infant health problems in industrialised countries, where its prevalence has been increasing during the last decades. Asthma currently affects to about one in seven children in Europe, with an important proportion having a history of atopy. In this sense, the ISAAC study (phase I), including some three quarters of a million children in more than a hundred centres distributed in more than fifty countries, represented a major effort in measuring the magnitude of the problem. Although this increase in prevalence is almost universal, affecting both industrialised and developing countries, marked variations exist between and within countries with up to fifteen fold differences between countries, which provides an epidemiological opportunity for investigating into the causes of asthma (1).

Given the speed and widespread of this increase in prevalence, it is widely accepted that is most likely to be due to environmental and lifestyle factors, more than to genetic variations.

1.2. The aetiology of Asthma

During the last decades, multiple clinical and epidemiological studies have identified factors which play a role in the aetiology of asthma. Among these factors, several of them are not modifiable, as the genetic background posed by the family history or the gender. Family size and birth order are also non modifiable factors by themselves, but they may well reflect different exposures, namely to infectious agents, though the role of previous pregnancies and breast-feeding practices of the mother may not be neglected. Anyhow, the 'natural' reduction in family size occurred in most industrialised countries may have contributed to the increase in asthma prevalence.

For what respects to clearly environmental and lifestyle, and thus potentially modifiable, risk factors, exposure to Environmental Tobacco Smoke and other respiratory irritants, smoking during pregnancy, breastfeeding, early dietary influences and a wide variety of socio-economic factors such as housing density, affluence and family occupation (farming for example) have shown in different studies to play a significant role. Lower respiratory tract infections during the first year of life have also been linked to asthma aetiology.

Based on the observation that a considerable proportion of incident cases of asthma occurred during the first years of life, it became clear in the nineties that the etiologic research clearly had to put his focus on exposures which occur early in life (2-5), as are those mentioned in the previous paragraph.

Atopy is strongly related with asthma, with which in addition shares a number of other risk factors. The kind of relationship between atopy and asthma, however, is of a complex nature and may not be seen as a classical cause-effect relationship; the possibility that in fact they are two manifestations of a common background pathological entity, probably sharing genetic components, may not be discarded. In this sense, some authors have postulated that the asthmatic condition itself may play a role in determining a specific pattern of atopic sensitisation and that the process of becoming sensitised to allergens would parallel, rather than cause, the development of asthma (6-9).

Last but not least, it seems axiomatic that allergen exposure is necessary for the development of specific sensitisation and subsequent atopic diseases, this opening a potential strategy for prevention. It has been held that the risk of childhood respiratory allergies is correlated with the intensity of allergen exposure in early life. If true, this would imply that lowering the levels of allergen, particularly in the home, would reduce the incidence of these diseases. Indeed, exposure to allergens had traditionally been linked to the aetiology of asthma (10), but this has been posed into questions in more recent years, as several studies have not found an association between early life allergen exposure and the incidence of wheezing or asthma (11).

According to the available evidences on the role of the known risk factors, different hypothesis have been proposed to explain the increase on asthma

prevalence. The hygiene hypothesis has been the most widely accepted and is still widely considered as the most plausible working hypothesis to explain both the temporal changes and the regional differences in asthma and atopy prevalence (12). From the first formulations of the hygiene hypothesis, focused on the Th1/Th2 paradigm, which equilibrium would be modulated by the exposure to common childhood infections (13-15) its focus has turned on the effect of parasitic infections, exposure to microbes, in particular to endotoxins, and pets or other not identified substances present in farms which would determine the production of regulatory T cells (16-19). This theories are nowadays under much discussion on the light of new immunological findings (20-21), and there is increasing evidence suggesting that patterns of T-cell immunity to inhalant allergens in genetically diverse human populations are more heterogeneous than previously assumed. Asthma susceptibility might also be associated with variation in genes encoding components of innate immunity (22-24), particularly those known to be expressed in lung tissue such as *TLR10* and *TLR6*, making them biologically plausible candidate genes for asthma.

The other major hypothesis behind the increase in asthma prevalence, at the time the AMICS study was planned, referred to an increased level of allergens in the households because of a better isolation and less ventilation of the homes (25-29). In this line, a number of groups are testing, by randomised trial and observational means, the associations between early house dust mite exposure and the development of specific sensitisation and asthma.

Intervention studies to reduce allergen levels in the homes have shown the ability to significantly reduce allergen exposure at home (30), but results on its impact on reducing incidence of atopy and asthma are not very optimistic for the moment (31-32). Several birth cohort studies have reported effects of allergen reduction measures on the development of atopic phenotypes. In the Manchester Asthma and Allergy Study (MAAS), in which high-risk children were randomly allocated to a stringent environmental intervention or a normal regime, at the age of 1 year this intervention resulted in a significant decrease of attacks of severe wheeze with shortness of breath (33), but the benefits in terms of reduction of respiratory symptoms were transient and atopic sensitization was not prevented (34). Similarly, in the Study on the Prevention of Allergy in Europe (SPACE), HDM reduction programs showed an association between HDM allergen exposure and specific sensitization in the first years of life (35) but this effect was only transient (36-37). In the Isle of Wight study, a reduction in current wheeze, night cough, bronchial hyper responsiveness and atopy at the age of 8 years was found after strict food-allergen avoidance and reduction of HDM exposure by the use of an acaricide and mattress covers in the first 9 months of life (38). In the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study, the use of mite allergen impermeable mattress covers significantly reduced exposure to Der p 1 and Der f 1 allergens at 1 year of age, and slightly reduced upper airway symptoms and nocturnal cough without a cold in the first 2 year of life, but no evidence of protection against other respiratory symptoms and atopic dermatitis was found and, at age 1, sensitization to mite allergens was similar in the active and the placebo groups (31, 39). In the Australian CAPS study, a significant effect of allergen avoidance

on the prevalence of sensitization was found, but no differences in the prevalence of wheeze at the age of three (40). If allergen levels in the household were one of the major risk factors in the incidence of asthma, allergen reduction intervention studies should find a clear effect on the reduction of asthma incidence in children but, as mentioned, the role of allergen exposure on the inception of asthma, traditionally accepted, has been posed into question in recent years (11)

1.3. Difficulties in the research on the aetiology of asthma

Despite intensive research during the last three decades into the field of asthma aetiology, no major advances have been made in the prevention of this pathology which prevalence (and incidence?) has increased dramatically during the same period.

Different reasons may explain the difficulties found in the research of asthma aetiology and which may have avoided a major advance in proposing prevention strategies. Several are the major methodological difficulties inherent to the etiological research of asthma and which may be grouped in several areas:

The natural history of asthma, with a variable and intermittent course including remissions and relapses, makes it difficult to establish the cause-effect time sequence.

The same definition of infant asthma, which may present with different phenotypes, posses important problems and may lead to important misclassification between asthmatics and non-asthmatics. Until asthma is not well established (issue not always easy to be determined), the diagnosis is usually based on wheezing, but not all wheezing corresponds to asthma. In fact, different wheezing phenotypes have been described: early wheezers, late wheezers and persistent wheezers, each one showing a different degree of overlap with asthma.

Asthma at any age is an ill-defined disease, and definition in epidemiological studies mostly relies on reporting symptoms to questionnaires originally devised and tested in English speaking populations. The difficulty increases in the case of children, whose appraisal relies on parental reporting. These limitations in the measurement of asthma, specially at a very young age, may have also played a role in the reliability of results presented referring to very young children. The use of repeated measures, as cohort studies allow, may at least partially overcome the problem as persistent reporting of wheezing increases specificity in the definition.

The prevalence/incidence confusion is one of the major problems faced in the research done in this field. The factors associated with the incidence of asthma may well not be the same than those responsible for maintaining the illness, that is the prevalent cases. Similarly, factors responsible for the initiation of asthma and factors acting as a triggers for asthma attacks may not be the

same. In a similar way, conclusions drawn from studies directed to evaluate the effect of different strategies intended to the remission or alleviation of symptoms, may not be transferable to primary prevention strategies.

Allergen exposure is a necessary component for the development of specific sensitisation and subsequent atopic asthma. The relationship between allergen exposure, atopic sensitization and asthma, however, is of a complex nature and has recently been a subject of controversy on the light of new findings (12, 32, 41-42). In addition, as shown with occupational asthma (43), the relevant exposures for the inception of asthma are the first contacts with the allergen, probably within a time window of about a year (44). Only prospective studies covering the first year of life may adequately collect the relevant exposures at the appropriate time.

An additional difficulty when investigating into the causes of asthma is the important genetic component and gene-environment interactions involved. A first major implication of this for the aetiologic research of asthma is that conclusions drawn from high risk groups, as atopic families, may not apply to the general population.

1.4. The inception of atopy

In parallel to asthma, the prevalence of atopy has also increased in the last decades and is said to affect to about a quarter of all children in Europe with

important variations between and within countries. Atopy and asthma in fact share some common risk factors and present similar difficulties for the implementation of effective preventive measures.

Unlike what has happened with the scientific discussion about allergen exposure and the inception of asthma, the controversy concerning the role of allergen exposures in the development of atopy and specific sensitization has not openly been raised being still widely assumed that a higher exposure to allergens early in life would increase the risk of atopy (11, 41, 45).

Most of the evidence on the relationship between allergen exposure and atopy, however, is derived from cross-sectional surveys, a few prospective studies of selected 'high-risk' populations (10, 46), surveys of asthmatic children (47), and studies in areas where exposures to house dust mite are extremely low (11, 44, 48-49).

It is probable that any direct associations with allergen exposure are strongly influenced by both genetic (32, 44, 48, 50-51) and other environmental factors, such as exposure to pets or other substances present on farms (51-59). Moreover, the shape of the association seems to vary depending on the particular allergen considered and is unlikely to be linear (45, 58-61). Comparisons between studies, several with conflicting findings, are always difficult because of their different methodologies and because of their different aeroallergen environments and varying genetic backgrounds.

Results from intervention studies looking at the impact of reducing household allergen exposure on atopy are not conclusive for the moment (31) and recent publications have failed to find any effect of early reduction of house dust mite allergen exposure by means of mattress covers on sensitization and atopy at 4 years in children with allergic mothers (34, 62).

1.5. The allergen exposure / atopy / asthma pathway

Specific sensitisation and asthma had traditionally been linked to allergen exposure through inhalation (10, 63). In more recent years, as mentioned earlier, several studies have challenged the assumption that the initiation of asthma is related to allergen exposure (11, 64-65), while the available epidemiological evidence seemed to be consistent with a role of allergen exposures in the development of specific sensitisation (41, 44-45).

Since there is relatively well established evidence that sensitisation to common aeroallergens is linked with an increased risk of wheeze and asthma (58), an intriguing and relevant question arises: if aeroallergen exposure is linked to atopy and atopy is linked to wheeze and asthma, how to explain that aeroallergen exposure does not show an association with wheeze and asthma? (42).

Several reasons could explain the incongruities found in this respect, including the influence of factors that could counterbalance atopic sensitisation like contact with pets, farm environments or endotoxins (51-53), geographical differences in relation to different levels of exposure (66) or the influence of susceptibility factors (32, 50) in studies in which the population has been selected from high risk families (46). Or maybe, in fact, the inception of atopy, similarly to asthma, is not related to the level of allergen exposure at home, or not in a linear way. Some of these explanations can only simultaneously be tested in prospective studies conducted in different areas with enough variation to exposure, including several allergens and in non-selected representative populations (64, 67). The difficulties in relation to the measurement of asthma stated above, specially at very young age, may have also played a role in the conflicting results published referring to infants.

The Asthma Multicentre Infant Cohort Study (AMICS), including non-selected populations of children followed since before birth in different international settings presenting with very different exposure experiences, represents a unique opportunity to give a broader picture of the complex allergen exposure, atopy and asthma relationship.

1.6. The AMICS study

Our main objective when setting up the Asthma Multicentre Infant Cohort Study (AMICS) was to assess, in a prospective manner and in a non-selected population, the role of early life exposures to two major household

aeroallergens (Der p1 and Fel d1) upon the subsequent development of sensitisation and asthma.

The origin of the AMICS study was a cohort study set up in Ashford (UK) in 1993, and which represented the first large scale population to be recruited specifically for detailed etiologic study of childhood allergy and asthma and which purposively included unselected and general population representative children. In 1993, an observational cohort study rather than an intervention study was devised in Ashford on the basis that the knowledge on the role of exposure to allergens on the inception of atopy and asthma was not sound enough to devise an intervention strategy.

Two other centres in Spain (Barcelona and Menorca) started in 1996 and 1997 the constitution of other cohorts following the same research protocol. Some time later, Munich and Kuopio also set up cohorts with a common core protocol with the other centres. For the purpose of the present thesis, and given the delay in the constitution of these later cohorts, only data for the first three centres (Ashford, Barcelona and Menorca) will be included.

The main methodological features of the AMICS study are:

1.6.1. Recruitment and questionnaire data collection

In each of the centres, pregnant women in their second trimesters were invited to participate. In Ashford and Menorca, population based cohorts were recruited

while the Barcelona cohort was hospital based. Recruitment took place over two to three year periods between 1993 and 1998. Children born to these mothers formed the several birth cohorts.

Questionnaire information was collected during pregnancy, in the two months visit at home, and by means o a yearly telephonic questionnaire up to the age of six year. Standardised questionnaires were used for the measurement of household characteristics, family history, smoking habits of parents, pets at home, breastfeeding practices and dietary questionnaires .

1.6.2. Parents tests and blood samples

Blood sampling and skin prick testing of the mothers (and of a smaller proportion of fathers) was performed and cord blood was collected at birth. Serum aliquots of the parents and cord blood were frozen at –60ºC and stored for later analysis.

1.6.3. Aeroallergen exposures

When their children were aged two months, the mothers were invited to complete a questionnaire enquiring into certain structural and behavioural characteristics of their homes. Coincident with the administration of this questionnaire, dust samples from the living room floor (if carpeted) and child's mattress were collected using vacuum cleaners and a standard dust trap (ALK) containing 7µm filter paper. Where there were bare floors (many of the homes

in Barcelona and Menorca), and consequently insufficient dust, a $1m^2$ area of the sofa was vacuum cleaned. Der p 1 and Fel d 1 concentrations were determined using an enzyme-linked immunoabsorbent assay (ELISA).

1.6.4. Irritant exposures

At the two month visit, the mean concentration of NO_2 in the living room of each home was measured over a two week period using commercially available diffusion tubes. Passive exposure to cigarette smoke within the home was assessed by detailed questionnaire administered to mothers in each of the cohorts at the same visit.

1.6.5. Symptoms

Early respiratory symptoms were collected by an annual and standardised questionnaire administered to the mothers of all children in the cohorts. The same instrument was used to collect information about eczema, early infections and potentially confounding variables. At the age of 5½ years, among children in the Ashford and Barcelona cohorts, an estimate of bronchial responsiveness was made using peak expiratory flow responses to a six-minute exercise test. After appropriate tuition and under supervision, each child recorded a set of three baseline readings which were required to have a variability of less than 20%. Readings, together with recordings of apical heart rate, are repeated five and ten minutes after exercise; and subsequently before and ten minutes after inhalation of 200µg salbutamol administered via a spacer device. For the

Menorca cohort, bronchial responsiveness was measured at 6½ years following the same protocol but without including the exercise test.

1.6.6. Specific sensitisation

Venous blood samples, collected at age four years, were assayed for specific IgE antibodies to Der p 1, Fel d 1 and grass pollens using a paediatric kit (Pharmacia CAP). This is an automated system which uses fluorescence to detect IgE. Results greater than or equal to 0.35 kU/l were considered positive.

Atopy at around 6 years was assessed by skin prick tests with commercial extracts including in the three centres at least:

- pollen mixture
- *Dermataphagoides pternyssinus*
- cat fur

Positive (histamine) and negative (saline) control solutions were included in the test series. Tests were carried out on the volar surface of the forearm using ALK lancets and read after ten minutes.

1.6.7. Follow-up

The chief difficulties and the main threat to validity in cohort studies lie in losses to follow-up or missing data. In our study, we had performed prick tests at age 6 years for 80% of the children for whom early life household allergen measurements were available; 89% had information on diagnosis of asthma at

this age. Loss of follow-up was marked in one of the three centres, but we obtained very similar results when restricting the analysis to the other two, whose combined follow-up rates were 87% for skin prick test and 95% for a diagnosis of asthma, making it improbable that any important selection bias was introduced in this manner

1.7. Extension of the AMICS study in Menorca. The INMA network

In parallel with the Menorca AMICS study, a cohort of about 100 new-born children had been constituted in Flix (Tarragona, Spain), an area with a high level of exposure to Persistent Organic Components (POCs) due to the presence of an important chemical industry, with the objective to study the impact of the exposure to organochlorine compounds on the neurodevelopment of children.

In order to corroborate and to extend the findings of the Flix cohort at 1 year of age, and given that the Menorca cohort had cord-blood samples frozen which could still be analyzed for POC's and a very low level of losses of follow-up, a common protocol was designed for a new follow-up at 4 years including the McCarthy test on neurodevelopment, the California Test on Social Competence and blood sampling.

The findings on neurodevelopment are not on the aims of the present thesis, but the chemical exposures measured, namely DDT and DDE, were also analysed for its impact on the aetiology of asthma.

The Flix and Menorca cohorts later became one of the origins of an Spanish national multidisciplinary research network on Childhood and Environment (INMA, Infancia y Medioambiente).

Additionally, since the implementation of the AMICS cohort in Menorca we had an added interest on the role of diet on atopy and asthma aetiology, and included for the Menorca cohort a Food Frequency Questionnaire (FFQ) to estimate diet during pregnancy and a FFQ referred to the diet of the children at the ages of 4 and 6 years. Changing dietary patterns has been one of the hypothesised factors playing a role in the increase on asthma incidence, but published data to support associations with specific dietary components is very limited.

2. RATIONALE

When the AMICS cohort was first planned about 15 years ago, exposure to household allergens was believed to be one of the major causes to the increase in atopy and asthma prevalence, and the relevant exposures were claimed to be those occurring early in life, probably within the time window of the first year of life.

In fact, based on this assumption, several intervention studies have started since then to evaluate the impact of allergen reduction measures early in life in reducing the incidence of atopy and allergic diseases.

However, the evidence that childhood exposures to domestic or other allergens had increased in parallel with the rising incidence of allergic diseases was scarce. Similarly, the evidence that the risk of childhood respiratory allergies is correlated with the intensity of allergen exposure in early life is mostly derived from cross-sectional surveys, a few prospective studies of selected ‘‘high-risk’’ populations (10, 46), surveys of asthmatic children (47), and studies in areas where exposures to house dust mite are extremely low (11, 44, 48-49).

Recently, data has been published challenging the assumption that there is any such relationship for the initiation of asthma (21, 41-42, 64), but no major controversy has openly been raised concerning the role of allergen exposures in the development of specific IgE sensitization (41, 45).

In order to overcome most of the limitations of previous studies, the AMICS study was planned to assess in a prospective way the relationship between exposure to aeroallergens in early life and the development of specific sensitization, wheeze and asthma up to the age of 6 years in non selected populations exposed to a wide range of allergen levels.

3. OBJECTIVES

Two are the main objectives of the present thesis:

- To measure the role of the early exposure level to two major household aeroallergens (Der p 1 and Fel d 1) in the inception of atopy.

- To measure the role of the household levels of Der p 1 and Fel d 1 early in life in the aetiology of asthma and wheeze.

Additional objectives of the AMICS study relevant to this thesis are:

- To determine the Der p1 and Fel d 1 allergen levels in the indoor home environments in three different European settings.

- To ascertain the level of home exposure to such respiratory irritants as NO_2 and tobacco smoke.

- To make an estimate of the influence to atopy and asthma of some pregnancy and early life dietary habits.

- To assess the association of cord serum and at 4 years blood levels of DDE and other organochlorine compounds with atopy and asthma during early childhood.

- To determine the role and interaction with other factors of breastfeeding.

4. PAPERS

4.1. Exposures in the AMICS study:

4.1.1. PAPER # 1: X Basagaña, M. Torrent, W Atkinson, C Puig, M Barnes, O Vall, J Sunyer, P Cullinan. Domestic aeroallergen levels in Barcelona and Menorca (Spain). Pediatric Allergy and Immunology 2002; 13:412-7.

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4.2. The role of Allergen exposure in the inception of atopy and asthma:

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4.2.2. PAPER # 5: Maties Torrent, Jordi Sunyer, Laura Muñoz, Maria Victoria Iturriaga , Paul Cullinan, Anthony Newman Taylor, Cecilia Figueroa, Oriol Vall, Josep M Anto. Early life domestic aeroallergen exposure and IgE sensitization at age four. J Allergy Clin Immunol 2006;118:742-8.

4.2.3. PAPER # 6: Matias Torrent, Jordi Sunyer, Raquel Garcia, Jessica Harris, Maria V Iturriaga, Carme Puig, Oriol Vall, Josep M Anto, Anthony J Newman Taylor and Paul Cullinan. Early life allergen exposure and atopy, asthma and wheeze up to 6 years of age. Am J Respir Crit Care Med (in press)

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PAPER # 6: Matias Torrent, Jordi Sunyer, Raquel Garcia, Jessica Harris, Maria V Iturriaga, Carme Puig, Oriol Vall, Josep M Anto, Anthony J Newman Taylor and Paul Cullinan. Early life allergen exposure and atopy, asthma and wheeze up to 6 years of age. Am J Respir Crit Care Med (in press)

EARLY LIFE ALLERGEN EXPOSURE AND ATOPY, ASTHMA AND WHEEZE UP TO 6 YEARS OF AGE

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Short running head: ALLERGEN EXPOSURE IN ATOPY-ASTHMA INCEPTION

ABSTRACT

Rationale: although widely assumed that incidence of childhood respiratory allergies to common aeroallergens is directly related to allergen exposure in early life, few longitudinal studies have investigated this issue and available data are scarce and mainly limited to high risk groups.

Objectives: to assess, in a prospective manner and in a general population, the role of early life exposures to Der p1 and Fel d1 upon the inception of sensitization and asthma.

Methods: pregnant women and their children were recruited for the AMICS study. Overall, 1611 newborns were initially enrolled in three cohorts in the UK and Spain. Der p1 and Fel d1 allergens were measured in household dust samples at three months of age for 1474 (91.5%) participants and skin prick tests were performed at six years of age on 1182 (80.2%) of them. Wheeze and diagnosed asthma were reported in yearly questionnaires.

Results: exposure to Der p1 early in life was not related with a positive specific prick test, nor with asthma or persistent wheeze at 6 years. Fel d1, however, showed an association with all these outcomes (third vs. first tertile OR: 4.43 for positive specific prick test and 2.6 for diagnosed asthma).

Conclusions: dose-response relationships between allergen exposure and sensitization or asthma may be allergen specific and non-linear; a minimum threshold level would be needed to induce sensitization, but no dose-response relationship would exist above this level. In addition, the effect of a particular allergen appears to be very similar upon atopy and asthma inception.

Key words: wheeze, sensitization, skin prick test, Der p1, Fel d1.

INTRODUCTION

Specific sensitisation and asthma have traditionally been linked to allergen exposure through inhalation (1-2). In more recent years, however, several studies have challenged the assumption that the initiation of asthma is related to allergen exposure (3-5), while the available epidemiological evidence is consistent with a role of allergen exposures in the development of specific sensitisation (6-8). Since there is relatively well established evidence that sensitization to common aeroallergens is linked with an increased risk of wheeze and asthma (9), it is an intriguing and relevant question whether allergen exposure is or is not a risk factor for asthma (10). Several reasons could explain the incongruities found in this respect, including the influence of factors that could inhibit atopic sensitisation like contact with pets, farm environments or endotoxins (11-12), geographical differences in relation to different levels of exposure (13) or the influence of susceptibility factors (14-15) in studies in which the population has been selected from high risk families (16). Some of these explanations can only be simultaneously tested in prospective studies conducted in different areas with enough variation to exposure, including several allergens and in non-selected representative populations (4, 17).

Our objective when setting up the Asthma Multicentre Infant Cohort Study (AMICS) was to assess, in a prospective manner and in a general population, the role of early life exposures to two major household aeroallergens (Der p1 and Fel d1) upon the subsequent development of sensitization and asthma.

Here our findings from three independent European populations, one in the UK and two in Spain, with quite different aeroallergen exposure profiles (18-19), are presented. At age four, we found an effect on sensitization (20) and wheeze (21) to cat allergen but not to house dust mite, though both phenotypes are not considered to be fully expressed at this age (22). Now we have the opportunity to assess the prospective relationship between exposure to aeroallergens in early life and the development of specific sensitization, wheeze and asthma up to the age of 6 years in non selected populations.

SUBJECTS AND METHODS

Pregnant women and their children were recruited in three concurrent cohorts (Ashford (UK), Menorca Island and Barcelona city (Spain)) following the same research protocol. Cohorts were population-based in Ashford and Menorca and hospital-based in Barcelona. In all centres women agreed to participate after they were told the objectives of the study; each provided written consent. The study was approved by the ethics committee of the participating centres.

Information collected prospectively included: details of the pregnancy, cord blood, a household visit at the age of three months for collection of dust and ambient air NO_2 samples, blood sampling and skin prick testing of the mothers (and of a smaller proportion of fathers), a yearly questionnaire up to the age of six years enquiring into respiratory symptoms and diagnosis, household characteristics and exposures (pets, passive smoking, cooking and heating

appliances), venipuncture of the children at the age four years and a skin prick test including Dermatophagoides pteronyssinus and cat epithelia allergens at the age of 6. All data collection methods, household sampling and prick test technique were standardized between centres (21). A common training was followed by the fieldworkers of the three centres and the same dust collection appliances and the same prick test technique, including use of the same equipotent extracts (prepared by Leti Laboratories for the Spanish centres and by ALK-Abello for the UK centre) and the same 1 mm. prick test lancets, was used.

Dust samples were collected from living room and from children's mattresses during the first three months of life. Results obtained using living room or bed levels were very similar. In this report, living room levels were used for the main analysis because, apart from showing lower levels, bed levels were more affected by non-differential misclassification (risk estimates tended more to null, data not shown) . House dust mite allergen (Der p1) and cat allergen (Fel d1) concentrations were estimated following a standard protocol (18-19).

Out of 1611 newborn children initially enrolled, household dust samples were collected for 1474 (91.5%) and 1182 (80.2%) completed skin prick tests at age six. Dust samples and prick tests were available for about 87% of children in Ashford and Menorca and 59% of those in Barcelona. For information on asthma at age six, these percentages were 95% and 72%. Due to differences in loss of follow-up in the three centres we repeated all analysis after exclusion of children from Barcelona and tested for heterogeneity between centres.

House dust mite and cat allergen concentrations were log transformed given its skewed distribution. Five outcome variables were defined: a positive (wheal >= 3 mm) prick test to Der p1 and to Fel d1 at age 5-6, a medical diagnosis of asthma up to age six as reported by the parents, persistent wheeze (any wheeze between 5 and 6 years of age having had wheezing between 3 and 4) and current wheeze at 6 years (any wheeze during the sixth year of life). To examine the independent effects of allergen concentrations on the risk of the different outcomes defined, we first used generalized additive models (GAM) which allow the fitting of a non-linear exposure-response relationship and give an illustrative graphic representation of any association. Second, we fitted multiple regression models for binary data for each of the dependent variables defined. Allergen concentrations were introduced into the models as tertiles of the pooled levels of the three centres. Interactions with allergen exposure, including factors such as gender and parental atopy or asthma, were tested using interaction terms in the regression model; only maternal atopy showed significant interaction and stratified models by maternal atopic status were constructed. Stratified analysis were also performed for atopic and non-atopic asthma and wheeze. Statistical analyses were conducted using Stata (Stata Corporation, TX, USA).

RESULTS

The concentrations of Der p 1 and Fel d 1 antigens in living room dust are shown in Table 1 separately by centre, along with the proportions of children

with positive prick tests to these aeroallergens, a diagnosis of asthma by the age of 6 years or having wheezed in their fifth or sixth year of life and those considered to be persistent wheezers. Exposure profiles were very different between the centres with high levels of Der p1 in Menorca and high levels of Fel d1 in Ashford.

Non adjusted comparisons of sensitisation, diagnosed asthma, persistent wheeze or wheeze at 6 years by tertile of dust allergen exposure (table 2) showed no associations for house dust mite exposure with any one of the outcomes. On the other hand, for cat allergen exposure a statistically significant association was observed with wheeze at age six, and the associations with diagnosed asthma, persistent wheeze and positive specific prick test to Fel d 1 were at the limit of statistical significance. None of these relationships was modified by centre as shown by the heterogeneity tests; nor were there any important changes after exclusion of children from Barcelona. Table 2 also shows the positive associations between sensitisation and asthma or wheeze, all these associations were highly significant; the sense of the association was the same for the three centres, although the association between positive specific prick test to Der p 1 and wheezing was lower in Barcelona (p for heterogeneity between centres < 0.05).

All variables that showed an association with any of the outcomes on bivariate analysis are shown in table 3. Male gender, high home crowding, maternal or paternal atopy and maternal or paternal asthma significantly increased the risk of specific sensitisation to at least one of the two aeroallergens studied. Male

gender, a lower gestational age at birth, lower NO_2 levels, smoking during pregnancy, maternal atopy, maternal and paternal asthma, lower respiratory tract infection during the first year of life and a shorter breastfeeding period were risk factors for diagnosed asthma or wheeze. Other variables analysed that did not reach statistical significance for any of the outcomes included: birth order, birth weight, cat or dog ownership (or number of them) during the first year of life, household damp, season in which the dust was collected and maternal education.

Figure 1 depicts, on a continuous scale, the relationship of Der p1 and Fel d1 exposures with each one of the outcomes studied based on a GAM model constructed for each exposure-outcome pair. There was no association between house dust mite exposure and specific sensitisation, asthma or wheeze. In contrast, Fel d1 exposure showed a positive association for all outcomes; in each case the risk appeared to increase at low levels of exposures and to flatten above 1 μg/g of dust. Wide confidence intervals at the extremes highlight the low precision of the risk estimates of the GAM models at this points.

Multiple regression models for Der p 1 exposure (table 4) confirmed that the level of exposure early in life was not related with subsequent specific sensitisation. Male gender, with an Odds Ratio estimate of 1.85 (95% CI, 1.24-2.76) and an atopic mother (OR 2.07, 95% CI 1.38-3.10) were the main risk factors for this outcome. Of the three sites studied, Ashford had the lowest risk of a positive prick test to Der p1 and Barcelona, the highest. Fel d1 exposure,

on the other hand, showed a strong association with the sensitisation (table 4), although no difference in risk was observed between the second and third tertiles of exposure. Other risk factors associated with a positive prick test to cat were high home crowding, maternal atopy and maternal asthma. By centre, the major risk of sensitisation to cat was in Barcelona and the lowest in Menorca.

A diagnosis of asthma and persistent wheeze were each positively related to Fel d1 exposure, although only reaching statistical significance for the former. There were no relationships between Der p1 exposure and any of the respiratory outcomes (table 4). The observed risk factors, included in the multivariate models because of a 'p' value lower than 0.10, for a diagnosis of asthma and wheeze, with very similar estimates for the Fel d1 and the Der p1 models, were: having a positive specific prick test, maternal and paternal asthma, smoking during pregnancy, a lower respiratory tract infection during the first year of life and being exposed to higher levels of NO_2 early in life - while being breastfed for more than two weeks appeared to have a protective effect. Children in Ashford had the highest risk of an asthma diagnosis and of wheeze, even after adjusting for the relevant variables, but again no interaction with centre was observed. Very similar results were obtained when taking wheeze at six years instead of persistent wheeze as the dependent variable.

We looked extensively for possible interactions with exposure to the two aeroallergens of interest, with only maternal atopy suggestive of any such effect. Table 5 shows the models stratified by maternal atopy as well as the p values for interactions for each allergen and outcome model. In children of

atopic mothers, exposure to Der p1 seemed to protect against a positive prick test to house dust mite. For asthma diagnosis a higher risk was associated with aeroallergen exposure if the mother was atopic, this was especially the case for Fel d1 exposure.

Finally, we analysed different possible associations with aeroallergen exposure depending on whether the child's asthma or wheeze was accompanied by specific sensitisation. Very similar results were obtained for sensitised and non-sensitised children with no evidence of any interaction by sensitisation status.

DISCUSSION

AMICS is the first international birth cohort study designed to investigate the relationship between exposures very early in life and childhood sensitization and asthma in a general non selected population. The main exposures of interest were household aeroallergens, namely house dust mite and cat, of which a wide range of exposures were apparent in the three settings. Our results strongly suggest that the relationship between exposure to inhaled allergens in early life and the development of sensitization and asthma is allergen dependent, Der p 1 and Fel d1 showing different dose-response patterns. In addition, within the range of exposures observed, for the same allergen, the role of aeroallergen exposure is similar upon sensitization and upon asthma.

With respect to cat allergen exposures, we found a non-linear relationship between home exposure to Fel d 1 in early life and corresponding sensitisation, by skin prick test, a diagnosis of asthma or wheeze up to the age of six years. The relationships suggest a steep increase in risk at low levels of exposure, and a flattening above 1 μg/g of dust. This result is in agreement with previously published literature, although we did not observe a reduction of risk, as others have, at high exposure levels (9). In our population, we did not find any effect of cat ownership, nor of the number of cats owned, either on the risk of sensitization or on asthma. Such effects have been reported by some authors (12) but not by others (23), or to be limited to sensitization but not to asthma (5). These inconsistencies may reflect particular behaviors in relation to cat keeping.

In contrast to cat, we did not find any significant associations between early life domestic Der p1 allergen exposure and any one of the outcomes studied. The lack of any apparent association of house dust mite with sensitization or with asthma up to the age of 6 years would confirm our preliminary findings concerning wheeze up to the age of 4 years (21) and Ig E sensitization at age four (20), and is in agreement with the results observed in another highly exposed population in Michigan (24). These findings are in apparent contradiction to other published literature from observational studies which suggest that sensitization to house dust mite is directly related to levels of early allergen exposure. These surveys, however, are generally derived from small studies of selected populations from atopic or asthmatic families (16), from surveys based on asthmatic children alone (25) or from studies in settings where exposures to house dust mite were extremely low (3, 26). In this sense,

our finding of an interaction effect of maternal atopy on the relationship between allergen exposure and sensitization or asthma is specially relevant, indicating that results from studies in descendants of high risk families may not apply to the general population. Other studies have also pointed out the importance of parental history as a modifying independent variable in the relationship between early dust mite exposure and atopic outcomes (24), though the effect modification has not always been shown to work in the same direction.

Our findings suggest that, for a given allergen, the effects on both the inception of specific sensitization and of asthma are very similar, a consistency that remained after stratification by maternal atopy. This consistency is in contrast with previous studies (3-5) and would resolve an intriguing incongruity in the understanding of the exposure-atopy-asthma relationship. The difference between our results and those from previous studies may be partially due to the fact that we have covered a wide range of exposure levels, but only a few subjects were exposed to very low levels of allergens, especially for Der p1, and the different effect upon sensitization and upon asthma may only occur at this very low levels. In fact, in the Ashford cohort, the only one of our three cohorts with a sufficient number of children exposed to low levels of house dust mite allergen, a weak, non significant, risk at very low levels of exposure with attenuation thereafter was found (27).

We have extensively analyzed possible confounding variables, none of them showed any significant effect on the relationship between exposure and sensitization or asthma. The main variables found to be associated with

sensitization to both allergens were maternal atopy and maternal asthma, while high home crowding increased the risk of sensitization to Fel d1 and males showed a higher risk of Der p1 sensitization. For asthma, the main predictor variables were having a positive prick test, maternal and paternal asthma and having lower respiratory tract infections during the first year of life. Although not reaching statistical significance, a mother smoking during pregnancy or a child being exposed to high levels of NO_2 during early life showed a 50% increase in the risk of asthma or wheeze up to the age of six years. The only protective factor observed was breastfeeding for at least two weeks. An important residual effect by center still remained after adjusting for all relevant variables collected.

We have also examined many potential interactions within our data, only maternal atopy showing any effect. Other authors have reported that any effect of allergen exposure on atopy and asthma is restricted to children of atopic or asthmatic parents, and especially children of atopic or asthmatic mothers (28-29). For diagnosed asthma, we obtained some indication of a restricted effect of allergen exposure, specifically of Fel d1, in children of atopic mothers, an interaction not seen with maternal asthma. For specific skin prick test positivity we observed a protective effect of Der p1, especially at very high levels of exposure, among children of atopic mothers, as if a tolerance effect appeared. This effect has not previously been described for Der p1, maybe because lower levels of exposure had been observed. Importantly, among those with no maternal atopy the association of allergen exposure with each one of the outcomes studied is quite similar for Der p1 and Fel d1, the different role of these two allergens appearing to be restricted to children with an atopic mother.

The results we are presenting constitute, to our knowledge, the first prospective report on the role of domestic allergen exposures in representative community samples covering a wide range of exposures in different international settings. Although the three environments had very different household exposure profiles and incidence rates of both atopy and asthma, the role of the allergens studied was similar in the three settings, lending important strength to the generalisability of the results obtained.

After accounting for allergen levels and other relevant variables, the risk of developing sensitization varied greatly between locations, from an odds ratio of 0.25 for Menorca to 2.12 for Barcelona, taking Ashford as reference. The risk of asthma also remained quite different between locales after multivariate adjustment, though differences were not of the same magnitude nor parallel to those of sensitization. These differences, however, are especially important when looking at diagnosis of asthma, and less so if looking at wheeze, probably indicating different diagnostic criteria in the three settings. Nonetheless, in the analysis of risk factors, the results are virtually the same when considering asthma or wheeze, giving higher consistency to our findings.

Prospective cohort study designs are the most robust epidemiological method for assessing the risk of exposures that occur a long time before the onset of disease, allowing the collection of relevant data at the appropriate time. The main threat to validity is losses to follow up or missing data. In our study, we had performed prick tests at age 6 years for 80% of the children for whom early

life household allergen measurements were available; 89% had information on diagnosis of asthma at this age. Loss of follow-up was marked in one of the three centres, but we obtained very similar results when restricting the analysis to the other two, whose combined follow-up rates were 87% for skin prick test and 95% for a diagnosis of asthma, making it improbable that any important selection bias was introduced in this manner. We recognize the limitations of using a single measure of exposure which may not accurately reflect the total effective relevant exposure; there are however data to suggest that allergen levels are consistent over time within households (30). A further limitation is our measurement in dust of only Der p1 (and not Der f1); however, given the high levels of Der p1 found in our study, any effect of mite exposure should have become apparent taking into account Der p1 alone, at least in the UK centre, where Der f1 levels are known to be very low. In any case, very low correlations have been found between Der p1 and Der f1 levels in the European Community Respiratory Health Survey II (31), which allows us to conclude that the relationship observed for Der p1 alone is not confounded by the unmeasured levels of Der f1; strictly speaking, our conclusions in relation to mite exposure refer to Der p1, but, as far as we know, there is no evidence of any differential causal relationship between the different types of mite allergens with atopy or respiratory outcomes. Negative confounding by some unmeasured and potentially protective factor - perhaps endotoxin exposure - is one possibility that we can not explore with our data.

In summary, we did not find any association between house dust mite exposure and specific sensitization, nor with asthma or wheeze; for Fel d1 exposure there

was a non-linear risk curve for all these outcomes. We suggest that the effects of allergen exposure on the inception of atopy or asthma may approach an 'all-or-nothing' event; the different exposure-effect curve observed for the two allergens studied could be explained by the different exposure ranges observed: above the threshold for house dust mite and including the threshold for cat allergen exposure. In addition, we found that the effects of a particular allergen on the inception of atopy and on asthma are very similar, not appearing in our data the reported incongruity in the exposure-atopy-asthma relationship, namely that exposure would be related to atopy but not to asthma, while atopy and asthma are clearly associated. The different effect observed in other studies may be explained by the range of exposures covered, which might include the threshold level for one of the outcomes but not for the other, or could be due to a dominant inclusion of high risk families. If we are correct then any exposure thresholds for sensitization or asthma appear to be exceedingly low making it improbable that intervention to reduce domestic allergen levels alone will have a major impact on the incidence of sensitization or asthma in childhood.

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Table 1. Domestic allergen concentrations in early life (geometric mean (95% CI)) and specific prick test positivity, asthma diagnosis, persistent wheeze and wheeze at 6 years by centre.

	ASHFORD			BARCELONA			MENORCA		
At 3 months of age	N	geometric mean (95%CI)		N	geometric mean (95%CI)		N	geometric mean (95%CI)	
House dust mite allergen concentrations (μg/g)	624	1.36 (1.17 - 1.59)		369	0.77 (0.65 - 0.92)		481	9.04 (7.93 - 10.31)	
Cat allergen concentrations (μg/g)	623	3.12 (2.55 - 3.81)		368	0.38 (0.31 - 0.45)		481	0.43 (0.36 - 0.51)	
At age one	N	n	%	N	n	%	N	n	%
Cat ownership	627	208	33.17	393	34	8.65	478	8	1.67
At age six	N	n	%	N	n	%	N	n	%
Specific prick to									
House dust mite*	552	39	7.07	256	63	24.61	419	52	12.41
Cat *	551	35	6.35	256	13	5.08	419	3	0.72
Asthma diagnosis	604	118	19.54	312	29	9.29	459	13	2.83
Persistent wheeze†	602	87	14.45	214	23	10.75	478	31	6.49
Wheeze at 6 years	604	108	17.88	311	43	13.83	481	41	8.52

* Positive specific Prick Test: >= 3 mm.
† Defined as wheeze at 5-6 years and at 3 or 4 years.

Table 2. Prevalence of positive specific prick test, diagnosed asthma, persistent wheeze and wheeze at age 6 by category of aeroallergen exposure and prevalence of respiratory outputs by specific prick test result

	Positive specific prick test†		Diagnosed Asthma		Persistent Wheeze		Wheeze at age 6	
Allergen in dust	n	%	n	%	n	%	n	%
Der p1 concentration								
< 0.83 μg/g	48	13.4	66	16.1	55	15.2	74	18.0
0.83-6.46 μg/g	53	12.6	56	12.5	44	10.5	59	12.9
≥ 6.46 μg/g	52	10.6	30	6.5	37	8.3	49	10.4
p-value‡	*0.617*		*0.219*		*0.240*		*0.193*	
p- heterogeneity	*0.623*		*0.854*		*0.279*		*0.524*	
Fel d1 concentration								
< 0.25 μg/g	5	1.3	27	6.3	34	8.5	48	11.0
0.25-1.39 μg/g	18	4.8	44	10.3	35	8.9	49	11.3
≥ 1.39 μg/g	27	6.3	82	17.9	67	15.5	86	18.6
p-value‡	*0.060*		*0.069*		*0.051*		*0.036*	
p-heterogeneity	*0.477*		*0.587*		*0.212*		*0.247*	
Specific Skin Prick Test								
Der p 1								
< 3 mm			114	10.7	87	8.8	118	11.0
≥ 3 mm			35	22.7	40	32.8	57	37.0
p-value‡			< 0.001		< 0.001		< 0.001	
p-heterogeneity			0.259		0.031		0.032	
Fel d 1								
< 3 mm			127	10.9	109	10.2	150	12.8
≥ 3 mm			21	41.2	17	41.5	24	47.1
p-value‡			< 0.001		< 0.001		< 0.001	
p-heterogeneity			0.479		0.252		0.258	

† Positive (≥ 3 mm) specific prick test to Der p 1 or Fel d 1 according to the specific allergen in dust
‡ Adjusted for site
minimum of p-values for heterogeneity between centres

Table 3. Unadjusted associations between specific prick test, diagnosed asthma, persistent wheeze and wheeze at 6 years with variables of interest

Variables	N	Positive Specific Prick Test† to Der p 1 n (%)	Positive Specific Prick Test† to Fel d 1 n (%)	Diagnosed asthma n (%)	Persistent wheeze n (%)	Wheeze at age 6 n (%)
Sex						
Female	762	54 (9.3)	19 (3.3)	66 (10.3)	50 (8.5)	73 (11.2)
Male	847	100 (15.5)*	32 (5.0)	94 (12.8)	91 (13.6)*	119 (16.0)*
Birth order						
No firstborn	854	72 (10.8)	25 (3.7)	90 (12.0)	81 (11.7)	104 (13.8)
Firstborn	741	78 (14.2)	26 (4.7)	69 (11.1)	59 (10.4)	86 (13.6)
Gestational age						
≥ 37 weeks	1495	140 (12.3)	46 (4.0)	144 (11.3)	126 (10.7)	175 (13.5)
< 37 weeks	103	13 (15.9)	5 (6.1)	15 (16.9)	15 (19.0)*	17 (19.1)
Home crowding						
"Low" (≤ 2 persons per room)	1363	136 (12.6)	37 (3.4)	135 (11.2)	123 (11.0)	162 (13.2)
"High" (> 2)	127	8 (7.5)	13 (12.3)*	20 (16.4)	15 (13.0)	22 (18.0)
NO_2						
<10 ug/m^3	702	52 (8.4)	29 (4.7)	91 (13.6)	81 (12.3)	102 (15.0)
≥ 10 ug/m^3	730	80 (15.0)	21 (3.9)	61 (10.0)*	53 (9.9)	78 (12.5)
Maternal Social Class						
I-II Professional / technician	316	35 (13.9)	19 (7.6)	39 (13.7)	33 (12.6)	46 (15.9)
III Skilled non-manual	253	15 (7.7)*	8 (4.1)	14 (6.1)	9 (4.2)*	11 (4.8)*
III Skilled manual	609	64 (13.6)	11 (2.3)*	48 (9.3)	44 (9.2)	71 (13.4)
IV Unskilled	259	26 (12.3)	9 (4.3)	30 (13.2)	33 (15.6)	38 (16.7)
Maternal smoking at pregnancy						
Never	1157	114 (12.6)	39 (4.3)	107 (10.6)	94 (10.0)	132 (12.9)
Ever	382	34 (12.1)	12 (4.3)	47 (14.6)*	44 (15.3)*	56 (17.3)*
Maternal atopy†						
No	1094	85 (10.4)	26 (3.2)	101 (10.9)	81 (9.7)	118 (12.6)
Yes	478	62 (16.3)*	24 (6.3)*	56 (13.5)	57 (14.4)*	71 (16.6)*
Paternal atopy†						
No	576	43 (9.1)	17 (3.6)	63 (12.3)	61 (13.0)	82 (16.0)
Yes	390	48 (14.9)*	22 (6.8)*	60 (16.7)	40 (11.8)	53 (14.5)
Maternal asthma						
No	1446	133 (12.1)	39 (3.5)	124 (10.1)	108 (9.6)	155 (12.4)
Yes	163	21 (16.9)*	12 (9.7)*	36 (25.2)*	33 (24.4)*	37 (25.5)*
Paternal asthma						
No	1442	132 (12.0)	43 (3.9)	128 (10.4)	114 (10.1)	163 (13.0)
Yes	143	19 (16.5)*	8 (7.0)	32 (25.2)*	26 (21.8)*	28 (21.7)*
LRTI at year 1						
No	890	97 (13.9)	38 (5.4)	73 (9.3)	65 (9.0)	89 (11.3)
Yes	540	43 (9.7)	13 (2.9)	82 (16.5)*	67 (14.5)*	91 (18.2)*
Birth weight						
≤ 2500 grs.	118	13 (15.7)	5 (6.0)	13 (13.3)	15 (17.4)	17 (17.0)
> 2500 grs.	1493	141 (12.3)	46 (4.0)	147 (11.5)	126 (10.7)	175 (13.5)
Breast feeding						
< 2 weeks	479	40 (10.7)	20 (5.3)	76 (18.1)	59 (14.8)	71 (16.6)
2 – 12 weeks	417	43 (13.4)	11 (3.4)	39 (10.9)	31 (9.7)	48 (13.3)
> 12 weeks	563	61 (13.3)	19 (4.2)	37 (7.3)*	42 (8.9)	61 (11.8)

Variables included are those with a p-value < 0.10 for anyone of the outcomes.
† Positive specific prick test ≥ 3 mm
* p-values < 0.05

TABLE 4. MULTIVARIATE MODELS FOR POSITIVE PRICK TEST, DIAGNOSED ASTHMA AND PERSISTENT WHEEZE IN RELATION TO DER P1 AND FEL D1 EXPOSURE

	Positive Specific Prick Test† (N=1059)		Diagnosed Asthma (N=911)		Persistent Wheeze (N=859)	
Allergen in dust	OR	(95% C.I.)	OR	(95% C.I.)	OR	(95% C.I.)
Der p 1 concentration[1]						
0.83-6.46 μg/g	0.88	(0.53 -1.46)	0.67	(0.40 -1.12)	0.59	(0.32 -1.08)
≥ 6.46 μg/g	0.74	(0.42 -1.32)	0.68	(0.37 -1.25)	0.74	(0.38 -1.46)
	(N=1058)		(N=911)		(N=858)	
Allergen in dust	OR	(95% C.I.)	OR	(95% C.I.)	OR	(95% C.I.)
Fel d 1 concentration						
0.25-1.39 μg/g	4.09	(1.14 -14.67)	1.59	(0.75 -3.36)	0.73	(0.34 -1.54)
≥ 1.39 μg/g	4.43	(1.22 -16.07)	2.6	(1.27 -5.34)	1.56	(0.79 -3.08)
Atopy at 6 years[2]			3.32	(1.94 -5.67)	5.61	(3.24 -9.70)
Male	-		-		1.14	(0.70 -1.87)
Firstborn	0.98	(0.51 -1.89)	-		-	
High home crowding	2.65	(1.18 -5.97)	-		-	
Maternal Social Class						
III Skilled non-manual	0.56	(0.22 -1.42)	0.56	(0.26 -1.21)	0.49	(0.19 -1.26)
III Skilled manual	0.45	(0.20 -1.03)	0.74	(0.42 -1.30)	0.94	(0.49 -1.79)
IV Unskilled	0.59	(0.25 -1.42)	0.75	(0.40 -1.43)	1.52	(0.76 -3.03)
Maternal atopy	1.84	(0.95 -3.58)	-		1.21	(0.72 -2.04)
Maternal asthma	2.08	(0.94 -4.58)	2.81	(1.60 -4.96)	3.28	(1.74 -6.18)
Paternal asthma	-		2.66	(1.51 -4.70)	2.38	(1.25 -4.53)
LRTI during first year	-		1.82	(1.15 -2.88)	1.47	(0.89 -2.43)
NO2 ≥ 10 ug/m^3	-		1.53	(0.87 -2.70)	-	
Smoking during pregnancy	-		1.46	(0.85 -2.49)	1.59	(0.89 -2.82)
Breast feeding						
2-12 weeks	-		0.52	(0.29 -0.92)	0.52	(0.28 -1.00)
> 12 weeks	-		0.56	(0.32 -0.98)	0.74	(0.41 -1.33)
Barcelona	2.12	(0.90 -4.96)	0.45	(0.19 -1.06)	0.49	(0.18 -1.38)
Menorca	0.25	(0.07 -0.89)	0.22	(0.10 -0.47)	0.79	(0.42 -1.49)

† Prick test to Der p 1 or Fel d 1 according to the specific allergen in dust

[1] Each column presents 2 multivariate regression models, one for Der p1 and the other for Fel d1, both adjusted for confounding variables. For Der p1, the confounding variables are not shown since odds ratios were very similar to those of the Fel d1 model.

[2] Positive skin prick test to any allergen

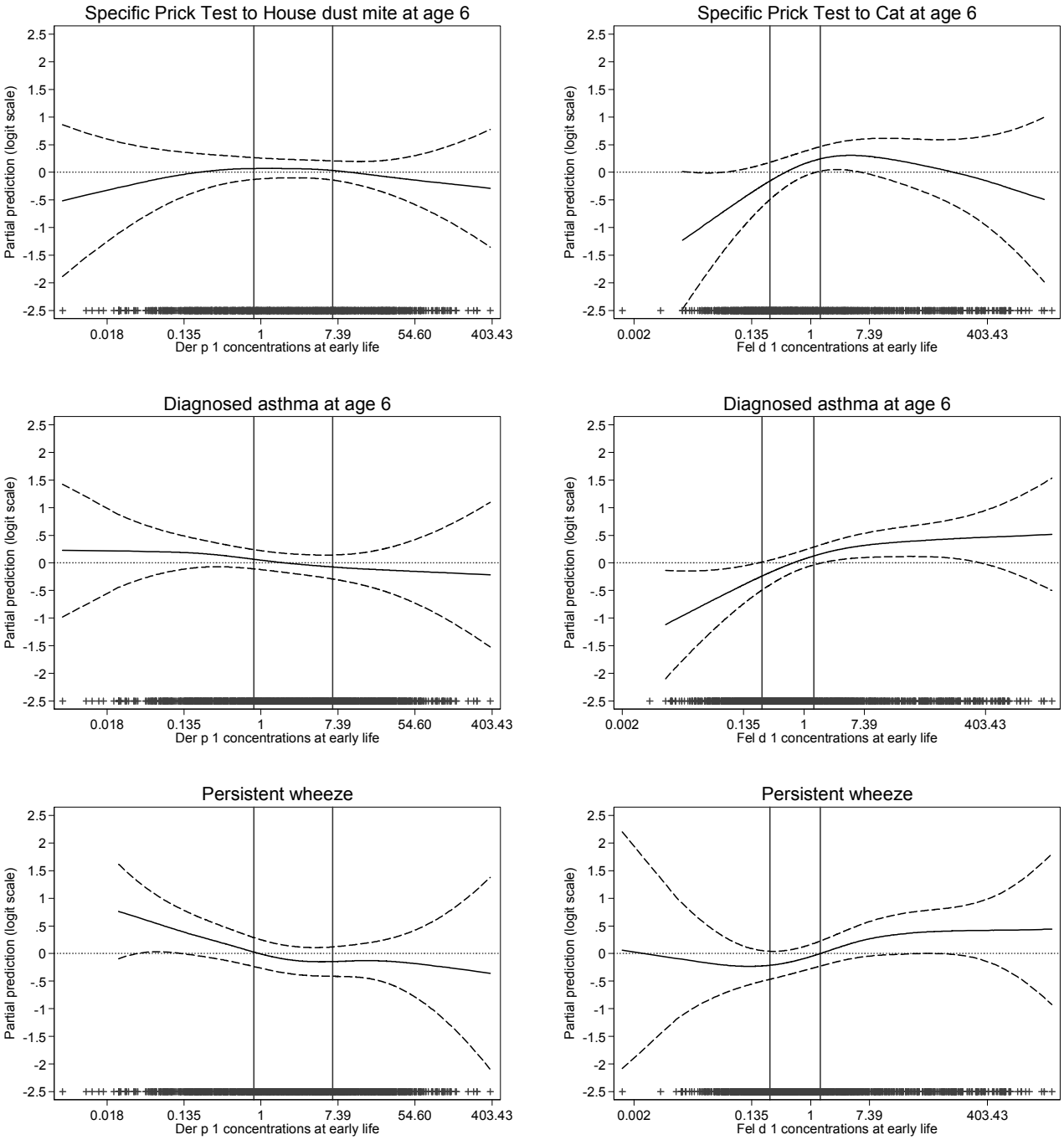
- Not included due to p> 0.10.

Table 5. Adjusted association between Der p 1 and Fel d 1 allergen exposure and specific prick text, diagnosed asthma and persistent wheeze stratified by maternal atopy

	Maternal atopy		No maternal atopy		p-value for interaction
	OR†	(95% C.I.)	OR†	(95% C.I.)	
Der p 1 concentration					
Positive specific prick test					
0.83-6.46 μg/g	0.45	(0.20 -1.02)	1.26	(0.64 -2.48)	0.061
≥ 6.46 μg/g	0.28	(0.11 -0.70)	1.44	(0.67 -3.09)	0.003
Diagnosed asthma					
0.83-6.46 μg/g	1.48	(0.63 -3.48)	0.48	(0.25 -0.91)	0.044
≥ 6.46 μg/g	0.71	(0.25 -2.02)	0.55	(0.26 -1.17)	0.606
Persistent wheeze					
0.83-6.46 μg/g	0.59	(0.25 -1.41)	0.54	(0.25 -1.15)	0.798
≥ 6.46 μg/g	0.39	(0.15 -1.03)	0.88	(0.38 -2.05)	0.321
Fel d1 concentration					
Positive specific prick test					
0.25-1.39 μg/g	2.61	(0.49 -13.99)	1.23	(0.35 -4.38)	0.852
≥ 1.39 μg/g	2.34	(0.44 -12.37)	1.82	(0.53 -6.30)	0.852
Diagnosed asthma					
0.25-1.39 μg/g	5.76	(0.66 -50.68)	1.32	(0.60 -2.88)	0.224
≥ 1.39 μg/g	18.43	(2.20 -154.0)	1.55	(0.71 -3.41)	0.037
Persistent wheeze					
0.25-1.39 μg/g	1.47	(0.49 -4.38)	0.55	(0.23 -1.34)	0.144
≥ 1.39 μg/g	2.49	(0.88 -7.07)	1.37	(0.61 -3.08)	0.269

† Odds ratio adjusted for the same variables than in table 4,

Figure 1. Probability (logit scale) of skin prick test positivity, diagnosis of asthma and wheeze up to the age of six years according to exposure to house dust mite (Der p1) and cat (Fel d1) allergens in early life.



| Vertical lines indicate tertile cut points (0.83 and 6.46 μg/g of dust for Der p1 and 0.25 and 1.39 μg/g of dust for Fel d1)

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.3. The role of other exposures in the AMICS-Menorca study

4.3.1. DDE exposure and asthma:

4.3.1.1. PAPER # 7: Jordi Sunyer, Maties Torrent, Laura Muñoz-Ortiz, Núria Ribas-Fitó, Daniel Carrizo, Joan Grimalt, Josep M Antó, Paul Cullinan P. Prenatal dichlorodiphenyldichloroethylene (DDE) and asthma in children. Environ Health Perspect 2005;113:1787-90.

4.3.1.2 PAPER # 8: Jordi Sunyer, Maties Torrent, Raquel García-Esteban, Nuria Ribas-Fitó, Daniel Carrizo, Isabelle Romieu, Josep M Antó, Joan O. Grimalt. Early exposure to dichlorodiphenyldichloroethylene, breastfeeding and asthma at age six. Clin Exp Allergy. 2006;36:1236-41.

4.3.2. Diet on atopy and asthma

4.3.2.1. PAPER # 9: Romieu I, Torrent M, Garcia-Esteban R, Ferrer C, Ribas-Fitó N, Antó JM, Sunyer J. Maternal fish intake during pregnancy and atopy and asthma in infancy. Clin Exp Allergy. 2007;37:518-25.

4.3.2.2. PAPER # 10: Leda Chatzi, Matias Torrent, Isabelle Romieu, Raquel Garcia-Esteban, Carlos Ferrer, Jesus Vioque, Manolis Kogevinas, Jordi Sunyer. Diet, wheeze, and atopy in school children in Menorca, Spain. Pediatric Allergy and Immunology (in press).

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5. GENERAL DISCUSSION

AMICS is the first international birth cohort study designed to investigate the relationship between exposures very early in life and childhood sensitization and asthma in a general non selected population. The main exposures of interest were household aeroallergens, namely house dust mite and cat, of which a wide range of exposures were apparent in the three settings. Our results strongly suggest that the relationship between exposure to inhaled allergens in early life and the development of sensitization and asthma is allergen dependent, Der p 1 and Fel d1 showing different dose-response patterns. In addition, within the range of exposures observed, for the same allergen, the role of aeroallergen exposure is similar upon sensitization and upon asthma.

5.1. On methodology

Prospective cohort study designs are the most robust epidemiological method for assessing the risk of exposures that occur a long time before the onset of disease, allowing the collection of relevant data at the appropriate time. The main threat to validity is losses to follow up or missing data. In our study, we had performed prick tests at age 6 years for 80% of the children for whom early life household allergen measurements were available; 89% had information on

diagnosis of asthma at this age. Loss of follow-up was marked in one of the three centers, but we obtained very similar results when restricting the analysis to the other two, whose combined follow-up rates were 87% for skin prick test and 95% for a diagnosis of asthma. Moreover, loss of follow-up, venesection at four years and prick test at 6 years were unrelated to any of the outputs or exposures of interest, making it improbable that any important selection bias was introduced in this manner. In addition, two of the three cohorts included in the study were representative of the general population: all medical professionals from both the public and the private health system asked the woman to participate in the study. In this way, 94% of eligible mothers in Ashford and almost 70% in Menorca were included in the cohorts.

We included all relevant variables hypothesized to play a role on the inception of atopy and asthma at the time the cohort was planned. We have extensively analyzed possible confounding variables, none of them showed any significant effect on the relationship between aeroallergen exposure and sensitization or asthma. The main variables found to be associated with sensitization to both allergens were maternal atopy (importantly measured by prick test to both parents) and maternal asthma, while high home crowding increased the risk of sensitization to Fel d1 and males showed a higher risk of Der p1 sensitization. For asthma, the main predictor variables were having a positive prick test, maternal and paternal asthma and having lower respiratory tract infections during the first year of life. Although not reaching statistical significance, a mother smoking during pregnancy or a child being exposed to high levels of NO_2 during early life showed a 50% increase in the risk of asthma or wheeze up

to the age of six years. The only protective factor observed was breastfeeding for at least two weeks. An important residual effect by center still remained after adjusting for all relevant variables collected.

Some limitations have to be recognized. Using a single measure of exposure may not accurately reflect the total effective relevant exposure; there are however data to suggest that allergen levels are consistent over time within households (68-69). A further limitation is our measurement in dust of only Der p1 (and not Der f1); however, given the high levels of Der p1 found in our study, any effect of mite exposure should have become apparent taking into account Der p1 alone, at least in the UK center, where Der f1 levels are known to be very low. In any case, very low correlations have been found between Der p1 and Der f1 levels in the European Community Respiratory Health Survey II (70), which allows us to conclude that the relationship observed for Der p1 alone is not confounded by the unmeasured levels of Der f1; strictly speaking, our conclusions in relation to mite exposure refer to Der p1, but, as far as we know, there is no evidence of any differential causal relationship between the different types of mite allergens with atopy or respiratory outcomes. Negative confounding by some unmeasured and potentially protective factor - perhaps exposure to endotoxins or other bacterial products - is one possibility that we can not explore with our data; the role of these factors had not clearly been hypothesized at the time the AMICS cohort was planned.

An additional advantage of cohort studies is the possibility of including new outputs of interest and, if biological samples have been stored, even to analyze

exposures which were not thought at the beginning of the project. These research opportunities have been exploded in the AMICS study in Menorca including neurodevelopment measurements at four years of age and making additional analysis of the stored biological samples, namely DDE in cord blood.

5.2. On the range and variability of exposures and outcomes

The results we are presenting constitute, to our knowledge, the first prospective report on the role of domestic allergen exposures in representative community samples covering a wide range of exposures in different international settings. However, although the three environments had very different household exposure profiles and incidence rates of both atopy and asthma, the role of the allergens studied was similar in the three settings, lending important strength to the generalisability of the results obtained.

Levels of Der p 1 were much higher in Menorca, showing levels which are in the higher range of those published at the international level (67, 71-73). In Menorca, the proportion of homes with living room levels >2 μg/g was 85%, and 51% had levels >10 μg/g. These proportions are 42% and 15% in Ashford and 27% and 9% Barcelona.

Levels of Fel d 1 in Barcelona and Menorca were much lower than those in Ashford (74), which presents levels similar to Germany (11) and New Zealand (67). This difference may be explained by a lower rate of cat ownership: 10% in

Barcelona, 14% in Menorca and 32% in Ashford. Maternal atopy was not related to having lower levels of allergens, which suggests that mothers with atopy do not take effective measures to reduce allergen exposures. Furthermore, there were no differences in cat ownership by maternal sensitization to Fel d 1.

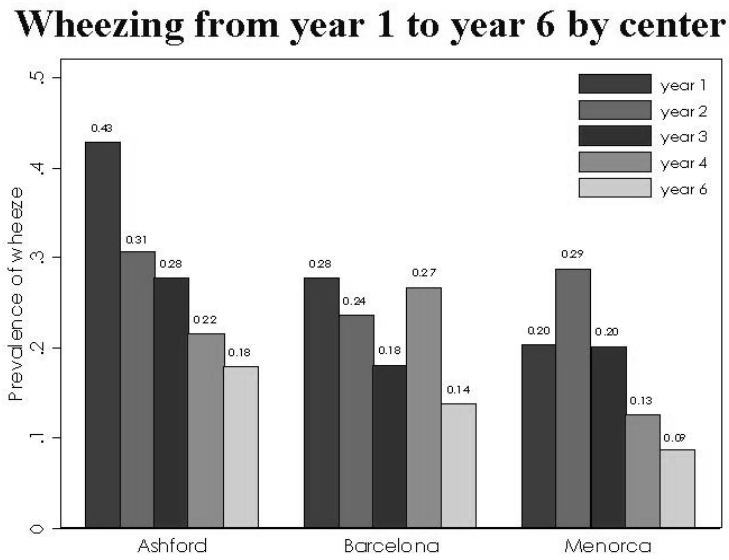
Homes in the three centers had significantly different concentrations of indoor NO2, with those in Barcelona showing the highest levels (median NO2 level of 23.87 p.p.b., while Ashford and Menorca had 5.79 and 6.06 respectively). In comparison with other European cities, the measured concentrations in Barcelona were high but similar to those measured in London, Southampton and Genoa (75-77). Conversely, indoor NO2 concentrations from Ashford and Menorca were similar to the very low average concentrations measured in less polluted cities in different european countries (77-79), the most important difference between the first and the second group of European cities being their values of outdoor NO2.

Baseline smoking habits and changes in smoking habits during pregnancy significantly differed between the three communities studied. Smoking prior to pregnancy was more common in Barcelona (46.2%) than in Minorca (39.8%) or Ashford (31.6%). Cessation rates during pregnancy also differed and 18% of women in Ashford, 20.4% in Minorca and 31.9% in Barcelona were still smoking during the first trimester. The UK center also had the highest proportion of women who had never smoked and Barcelona had the lowest rate of ex-smokers, that is women who had stopped before pregnancy.

Overall, the three centers included show very different exposure profiles to the main exposures of interest: Barcelona presented a much higher exposure to indoor NO_2 and to smoking of the mothers, while Menorca showed a very high exposure to Der p 1 and Ashford to Fel d 1. Nonetheless, in the analysis of risk factors for asthma and wheeze the results are virtually the same for the three centers (no interaction by center is seen), giving high consistency to our findings.

The three centers also greatly differed in the incidence of the outputs of interest. The proportion of children with positive prick tests to house dust mite was much greater in Barcelona, and Menorca had a much lower proportion of children with a positive skin prick test to cat than the other two centers, this not paralleling the exposure levels observed in the households of the three centers. Diagnosis of asthma, wheeze at six years and persistent wheeze by the age of 6 years also presented a wide range of rates in the different centers.

With respect to wheeze, from the fourth year of life Menorca has a clear lower rate than the other two centers. For the first three years of life the pattern by center is not that clear, probably reflecting the mixture of 'wheezings' included at these ages, including both infectious and allergic wheeze.



After accounting for allergen levels and other relevant variables, the risk of developing sensitization varied greatly between locations, from an odds ratio of 0.25 for Menorca to 2.12 for Barcelona, taking Ashford as reference. The risk of asthma also remained quite different between locales after multivariate adjustment, though differences were not of the same magnitude nor parallel to those of sensitization. The incidence of asthma in Menorca is very low in comparison to the other two centers and the known etiologic factors do not explain why; this would indicate that some important factors are missing in the etiologic research of atopy and asthma. Genetics most probably plays an important role, but also other environmental factors as diet, contamination, exposure to chemicals or contact with animals or other farm (or nature) products, among others, should be more deeply investigated.

5.3. Allergen exposure on the inception of atopy and asthma

With respect to cat allergen exposures, we found a non-linear relationship between home exposure to Fel d 1 in early life and corresponding sensitisation, a diagnosis of asthma or wheeze up to the age of six years. The relationships suggest a steep increase in risk at low levels of exposure, and a flattening above 1 μg/g of dust. This result is in agreement with previously published literature, although we did not observe a reduction of risk, as others have, at high exposure levels (58). In our population, we did not find any effect of cat ownership, nor of the number of cats owned, either on the risk of sensitization or on asthma. Such effects have been reported by some authors (53) but not by others (59), or to be limited to sensitization but not to asthma (65). These inconsistencies may reflect particular behaviors in relation to cat keeping.

In contrast to cat, we did not find any significant associations between early life domestic Der p1 allergen exposure and any one of the outcomes studied. The lack of any apparent association of house dust mite with sensitization or with asthma up to the age of 6 years would confirm our preliminary findings concerning wheeze up to the age of 4 years (80) and Ig E sensitization at age four (81), and is in agreement with the results observed in another highly exposed population in Michigan (82). These findings are in apparent contradiction to other published literature from observational studies which suggest that sensitization to house dust mite is directly related to levels of early allergen exposure. These surveys, however, are generally derived from small studies of selected populations from atopic or asthmatic families (46), from

surveys based on asthmatic children alone (47) or from studies in settings where exposures to house dust mite were extremely low (11,83). In this sense, our finding of an interaction effect of maternal atopy on the relationship between allergen exposure and sensitization or asthma is specially relevant, indicating that results from studies in descendants of high risk families may not apply to the general population. Other studies have also pointed out the importance of parental history as a modifying independent variable in the relationship between early dust mite exposure and atopic outcomes (82), though the effect modification has not always been shown to work in the same direction.

We have examined many potential interactions within our data, only maternal atopy showing any effect. Other authors have reported that any effect of allergen exposure on atopy and asthma is restricted to children of atopic or asthmatic parents, and especially children of atopic or asthmatic mothers (49, 84). For diagnosed asthma, we obtained some indication of a restricted effect of allergen exposure, specifically of Fel d1, in children of atopic mothers, an interaction not seen with maternal asthma. For specific skin prick test positivity we observed a protective effect of Der p1, especially at very high levels of exposure, among children of atopic mothers, as if a tolerance effect appeared. This effect has not previously been described for Der p1, maybe because lower levels of exposure had been observed. Importantly, among those with no maternal atopy the association of allergen exposure with each one of the outcomes studied is quite similar for Der p1 and Fel d1, the different role of these two allergens appearing to be restricted to children with an atopic mother.

Our findings suggest that, for a given allergen, the effects on both the inception of specific sensitization and of asthma are very similar, a consistency that remained after stratification by maternal atopy. This consistency is in contrast with previous studies (11, 64-65) and would resolve an intriguing incongruity in the understanding of the exposure-atopy-asthma relationship. The difference between our results and those from previous studies may be partially due to the fact that we have covered a wide range of exposure levels, but only a few subjects were exposed to very low levels of allergens, especially for Der p1, and the different effect upon sensitization and upon asthma may only occur at this very low levels. In fact, in the Ashford cohort, the only one of our three cohorts with a sufficient number of children exposed to low levels of house dust mite allergen, a weak, non significant, risk of sensitization at very low levels of exposure with attenuation thereafter was found (85).

5.4. The role of DDE

The levels of DDT and DDE found in Menorca are high in comparison to other regions of Spain, and an utilization in agriculture until more recently than expected (its utilization was forbidden in 1978) may not be ruled out. Maybe we have a quite unique population for the study of the association of these exposures and asthma.

DDE at birth increased the risk of diagnosed asthma and of persistent wheeze at age 6, but this was not the case for DDE measured at 4 years of age. DDE

levels did not modify the protective effect of breastfeeding on childhood asthma. These results suggest an effect of prenatal DDE exposure on childhood asthma probably via its hormone-like effect (86).

Our results suggest that in a community without known current DDT releases, the major source of DDE in children comes from maternal exposures since children with artificial feeding had a decrease in their body burden. The lack of an effect with DDE measured at 4 years of age suggests that the critical time window occurred early in life. The time window of exposure is becoming a key aspect in the study of diseases involving systems with a long developmental length such as the immunological and respiratory system.

Moreover, we found no modification according to DDE levels on the protective effect of breastfeeding in asthma. Breastfeeding is an important way of ingesting organochlorine compounds during infancy. The lack of effect after stratification of breastfeeding duration by pre-natal levels of DDE suggested that the post-natal effects of DDE (incorporated through breastfeeding) on asthma are probably less relevant than pre-natal exposure, as some authors have also suggested for neurodevelopment (87).

Why DDT did not show any association could in part be due to the opposite effects of DDE and DDT, but also to the lower levels of DDT than DDE in our population. We also found higher DDE levels at birth among mothers who breastfed for a shorter time among those mothers who breastfed, though differences were not significant. When we selected only firstborn children, DDE

levels at birth among children with shorter breastfeed duration were much higher than among mothers with longer duration, although we did not find any modification by smoking as other studies had found (88-89). This adds support to the estrogenic effect of DDE.

We did not find any association of DDE levels with atopy measured by Ig E at age 4, which we later confirmed by prick test at age 6, in contrast to a study in school children measuring total IgE (90- 91). This finding might indicate that the association between DDE and asthma does not involve the immunological cells related with specific IgE production. The unmodified association between DDE and wheezing found among non-atopic children (and if any stronger association) strengthen this possibility, although we had a small power to test interaction given the low prevalence of asthma. Similarly, two studies on other organochlorine compounds, such as PCBs and dioxins, found a negative association with allergic reactions in children (92) and IgE sensitization in rats (93).

Overall, this study strengthens the evidence for an effect of DDE on asthma by measuring the disease at age 6 and does not support a DDE effect modification of the protective effect of breastfeeding on asthma. Moreover, it suggests the early exposure as a more relevant time-window than exposure at age four. Furthermore, it reinforces the lack of effect of DDE on atopy. The hormone-like activity of DDE appears as the best explanation which deserves further research at mechanistic level.

5.5. The role of diet

In the nineties, diet was recognized as a potential risk factor for asthma and specially four types of dietary constituents were considered: breastfeeding and food avoidance in infancy; antioxidant vitamins, specifically vitamin C; dietary cations as sodium and magnesium and n3-n6 fatty acids. In order to assess diet as a risk factor for early childhood asthma and its interrelationship with other risk factors, it was claimed that prospective cohort studies of the effects of early childhood diet on the development of asthma in children, specially from birth to age six, were needed (94-102).

Diet (except breastfeeding) was not one of the original exposures of interest in the international AMICS project but given the growing interest on this subject and previous experience of the Menorca research team in nutritional epidemiology, the Menorca cohort included diet as an additional objective. Food Frequency Questionnaires during pregnancy and at the ages of four and six years were included. Advances in nutritional epidemiology in relation to the etiology of asthma since the AMICS cohort was constituted have been modest and our data may contribute to build evidence in this field.

Our results suggest that fish intake during pregnancy may provide a protective effect on the risk of eczema and atopy in the offspring. Previous longitudinal studies have evaluated the association of the content of n-3 PUFAs in colostrum and breast milk on the risk of atopic disease in the offspring (103-

106), but not a consistent pattern has been observed. Recent publications suggest that consumption of fish during pregnancy decreased the risk of SPT positivity (107) or asthma diagnosis (108) among children, but both surveys had assessed diet during pregnancy retrospectively. To our knowledge, our data represent the first longitudinal study to assess prospectively in a general population of pregnant women the impact of maternal diet during pregnancy on the incidence of atopic diseases up to age 6 years.

n-3 PUFAs, important nutrients of both fish and breast milk, are likely to play a protective role in the incidence of atopic-related outcomes, though its role in the incidence of atopy is not well understood. Two reports of randomized-controlled trials suggest that dietary n-3 PUFAs supplements during pregnancy (109) or in early postnatal period (110) could have immunomodulatory properties and/or associated clinical effects on atopy and asthma in offspring. A recent review on nutrition and allergic disease concluded that the beneficial effect of n-3 PUFAs on asthma and allergy is biologically plausible and that recent supplementation studies during pregnancy and early life are promising (111). Our data on fish intake during pregnancy collected in a prospective manner suggest that a component of fish, most likely n-3 PUFAs might modulate the risk of atopy in children.

In our study, the protective effect of fish consumption on atopic diseases was observed in offsprings of both atopic and non-atopic mothers and breastfeeding did not modify the association of fish intake with atopy in children, but the impact of fish intake on persistent wheeze and atopic wheeze was only present

in children without breastfeeding. Several compounds of breastfeeding may act to protect the infant from developing asthma and therefore, the impact of n-3 PUFAs might be more easily detected among non-breastfed infants.

Children in Menorca had a considerable intake of fish, fruits and fruity vegetables. Fruity vegetables (tomatoes, eggplants, cucumber, green beans, zucchini) contain many potentially important antioxidants that cannot easily be quantified.

Our data showed a potential protective effect of fruity vegetables and fish intake at age six on childhood wheeze and atopy respectively. When we simultaneously included fruity vegetables and fish intake in the multivariate models, results remained very similar, showing an independent beneficial effect on the prevalence of atopy and wheeze. Although other studies on nutrition and asthma in children have observed a beneficial effect of fruit intake on asthma symptoms (98, 112) we did not observe any significant associations in the present study.

We tested for interaction between maternal fish intake during pregnancy and children's fish intake at age 6.5 years and we found no significant interactions. The results from both analyses are consistent and indicate that a high fish intake during pregnancy and childhood may provide protection on the risk of atopic sensitization. Having assessed both maternal dietary habits during pregnancy and children dietary habits is one of the strengths of our study with

respect to previous reports on the role of diet on asthma and atopy outcomes in childhood, allowing to better evaluate the relevant time-window of exposure.

6. IMPLICATIONS

6.1. Implications for the etiologic research of asthma

Some important factors must be missing in the etiologic research of atopy and asthma given the enormous differences in risk of different populations after taking into account most of the accepted risk factors. Genetics most probably plays an important role, but also other environmental factors as diet, contamination, exposure to chemicals or contact with animals or other farm (or nature) products, among others, should be more deeply investigated.

Although the importance of diet on the etiology of asthma was recognized in the nineties, advances in nutritional epidemiology in relation to the etiology of asthma have been modest, this representing an important potential for future research.

External validity of epidemiological research is also important if we want to have an overall impact when proposing preventive measures. Results obtained from high risk groups or in environments with a narrow range of exposures may not be applied to other populations.

The time window of exposure is a key aspect in the study of diseases involving systems with a long developmental length such as the immunological and respiratory system. Only cohort studies may accurately collect relevant exposures at different time points.

The association between some chemical contaminants, as DDE, and asthma may not involve the immunological cells related with specific IgE production, thus having a different effect on atopy and on asthma.

Breastfeeding is a combined exposure including some intrinsic components of breast milk (as immunoglobulins or n-3 PUFAs), but also some contaminants or different levels of nutrients depending on the diet of the mother. From the research point of view is important to distinguish the role of this different components.

The genetic determinants of atopy or asthma are of a complex nature and family history may not adequately capture the risk involved; this could in part explain the incongruities found when studying the modifying effect of family history.

6.2 Implications for public health

Despite being a vehicle of potential toxic components, breastfeeding has a clear overall positive effect on the development of children, including a protective effect on asthma.

Prior to implementing intervention measures, a sound body of evidence should exist, and this evidence should come from different settings. Poor results of the interventions to reduce house dust mite allergen levels on the incidence of atopy and wheeze may in part be due to the fact that evidence linking atopic illnesses to household levels of allergens were mostly based on studies done in selected populations or in populations presenting a quite narrow range of exposures.

7. CONCLUSIONS

1. ***The effects of allergen exposure on the inception of atopy or asthma may approach an 'all-or-nothing' event.***

2. ***The effects of a particular allergen on the inception of atopy and on asthma are very similar.***

Focusing on the role of allergen exposures, we did not find any association between house dust mite exposure and specific sensitization, nor with asthma or wheeze; for Fel d1 exposure there was a non-linear risk curve for all these outcomes. We suggest that the effects of allergen exposure on the inception of atopy or asthma may approach an 'all-or-nothing' event; the different exposure-effect curve observed for the two allergens studied could be explained by the different exposure ranges observed: above the threshold for house dust mite and including the threshold for cat allergen exposure. In addition, we found that the effects of a particular allergen on the inception of atopy and on asthma are very similar, not appearing in our data the reported incongruity in the exposure-atopy-asthma relationship, namely that exposure would be related to atopy but not to asthma, while atopy and asthma are clearly associated. The different effect observed in other studies may be explained by the range of exposures covered, which might include the threshold level for one of the outcomes but not for the other, or could be due to a dominant inclusion of high risk families.

3. ***Any exposure thresholds (specially for Der p 1) for sensitization or asthma appear to be exceedingly low.***

4. ***It seems improbable that interventions to reduce domestic allergen levels alone will have a major impact on the incidence of sensitization or asthma in childhood.***

If we are correct then any exposure thresholds for sensitization or asthma appear to be exceedingly low making it improbable that intervention to reduce domestic allergen levels alone will have a major impact on the incidence of sensitization or asthma in childhood. Even in the case this dramatic reduction in domestic aeroallergen levels was possible, exposure in other places would still occur given the universal presence of allergens in many other places frequented by children: even if allergen exposure reduction had a role, it would not probably be enough to avoid it at home. Additionally, any undesired effect of the interventions, specially when using acaricides or any chemicals, should be evaluated.

5. ***We may have been looking in the wrong direction and other factors, as diet, should be more deeply investigated.***

Despite intensive research in the field of atopy and asthma etiology in the last decades, no major advances in terms of prevention have been made. For what respects aeroallergens we may have been looking in the wrong direction and other factors, as diet, should be more deeply investigated.

6. ***DDE at birth may increase the risk of diagnosed asthma and of persistent wheeze at age 6.***

Persistent Organic Compounds, DDT and DDE among others, may play a role in the inception of diseases involving systems with a long developmental length such as the immunological and respiratory systems. For this substances, very early life exposures appear to be the most relevant time-window for a significant effect.

7. ***Diet may play an important role on the inception of atopy and asthma; consumption of fish and some vegetables appear as possible beneficial components.***

Dietary habits have changed in an important way during the last decades almost universally and this may have played an important role in the rising incidence and prevalence of asthma and atopy. To disentangle the specific dietary factors involved, however, is a complicated task, given the methodological difficulties of nutritional epidemiology. Diet includes different specific components and we do not know if they are specific components or their equilibrium which play a role.

8. ***Advances in the knowledge of the genetic components involved will most probably be one of the major contributions to this field in the near future.***

Genetics are, no doubt, a major determinant of the probability of becoming asthmatic or atopic, the interaction of genetics with environment playing most probably a major role. Advances in the knowledge of the specific genetic components involved will be one of the major contributions to this field in the near future. Family history of atopy or asthma are too rough indicators of genetic risk and identifying the specific markers involved will allow to better understand the gene-environment interactions involved.

8. FUTURE RESEARCH PLANS

Genetic analysis of collected samples

Part of the blood collected at age four was preserved with EDTA and was frozen at –60ºC until DNA was extracted. At present, genotyping of some asthma genes has been performed. Genotyping has been carried out in the Centre de Recerca Genòmica de Barcelona (CRG) which is part of the multinode Spanish Genotyping Centre-CeGen (WWW.cegen.org.es). They have developed several genotyping assays covering candidate genes related to asthma/allergy, neurological processes, gene-environment interaction as well as endocrine disruptors, using different platforms depending on the category of markers (High throughput, medium-high throughput and lowthroughtput technologies). They have implemented assays on candidate asthma genes, GST null alleles, GSTP val104 functional variant, and are now developing new assays for candidate genes involved in innate immunity (TLR family genes) and hormonal disruption (in relation to TH metabolism and foetal growth). SNP selection is based on indirect strategy (LD, tagSNPs) in order to capture as much variability as possible from the genomic region from candidate genes. When appropriate, functional reported markers have been included.

Follow-up at 9 to 12 years of age period:

We have obtained a new grant for a new follow-up visit during the period 2007-2009, when the children will have between 9 and 12 years. This follow-up will include:

- Questionnaire referring to respiratory symptoms and atopy outputs and to exposures with special emphasis on diet and physical activity.
- Antropometry and body composition.
- Pulmonary function (espirometry)
- Exhaled air NO.

New dust samples collection

An application has been submitted to the Framework Program Seven of the European Community on “Health Effects of Indoor Pollutants – Toxicological, Microbiological and Epidemiological Approach’ combining several on-going cohort studies including the Menorca cohort. The objectives of this project, among others, are:

- To complete allergen characterisation of household dust, quantifying different subtypes of house dust mite allergens (Der p1, Der f1, Der f2).

- To characterise microbial exposure in the household, including: traditional markers, as endotoxins, and other microbial biomass components (ergosterol, 3OH fatty acids, muramic acid). Other components with an inflammatory potential, as gram positives or fungal glucans will also be analyzed.

- To assess repeatability of household dust analysis.

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10. ANNEXES:

Annex I:

List of other publications including Menorca AMICS cohort data

Annex II:

Funding grants of the Menorca AMICS study

Annex I

List of other publications including Menorca AMICS cohort data

1: Carrizo D, Grimalt JO, Ribas-Fito N, Torrent M, Sunyer J. In utero and post-natal accumulation of organochlorine compounds in children under different environmental conditions. J Environ Monit. 2007;9:523-9. Epub 2007 May 14.

2: Julvez J, Ribas-Fito N, Torrent M, Forns M, Garcia-Esteban R, Sunyer J. Maternal smoking habits and cognitive development of children at age 4 years in a population-based birth cohort. Int J Epidemiol. 2007 Jun 5; [Epub ahead of print]

3: Julvez J, Ribas-Fito N, Forns M, Garcia-Esteban R, Torrent M, Sunyer J. Attention behaviour and hyperactivity at age 4 and duration of breast-feeding. Acta Paediatr. 2007;96:842-7.

4: Alvarez-Pedrerol M, Ribas-Fito N, Torrent M, Julvez J, Ferrer C, Sunyer J. TSH concentration within the normal range is associated with cognitive function and ADHD symptoms in healthy preschoolers. Clin Endocrinol (Oxf). 2007;66:890-8.

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Annex II

Funding grants of the Menorca AMICS study

- Spanish Ministry of Health: Grant FIS 97/0588. Years 1997-1999.
- Spanish Ministry of Health: Grant FIS 00/0021-02. Years 2000-2002.
- European Commission. Contract Number QLK4-2000-00263 (concerted action). Years 2001-2003.
- Grant of '4ª convocatoria de ayudas a la investigación en enfermedades neurodegenerativas de La Caixa' Years 2001-2003.
- Spanish Ministry of Health: Thematic Research Network G03/176. Years 2003-2005
- Spanish Ministry of Health: Grant FIS PI041705. Years 2005-2007.
- Spanish Ministry of Health: Grant FIS PI061756. Years 2007-2009.