Microbial exposures, cleaning products and child health

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PREFACE

Early life exposure to indoor biocontaminants may have an effect on the development of the immune system and the neuropsychological development during infancy and childhood. The frequency of household cleaning determines the indoor microbial agent concentrations, and the use of cleaning products may involve inhalation exposure to airway irritants. This thesis aims to explore the relationship between early life exposure to indoor biocontaminants and household use of cleaning products, and respiratory, allergic and neuropsychological and behavioural development in children participating in several European population-based birth cohorts.

This thesis has been written between 2008 and 2013 and supervised by Prof. Jordi Sunyer and Dr Jan-Paul Zock. It consists of a compilation of scientific publications co-authored by the PhD candidate and in agreement with the regulation of the Doctoral Programme in Biomedicine of the Department of Experimental and Health Sciences at the Pompeu Fabra University. Most of the work was performed at the Centre for Research in Environmental Epidemiology (Barcelona, Spain) and part of it at the Institute of Epidemiology I of the Helmholtz Zentrum München (Munich, Germany). This thesis includes an abstract, a general introduction, a rationale, the objectives, the methods, the results (a compilation of six scientific publications), an overall discussion section and final conclusions.

Four publications are based on data collected in the framework of the European HITEA project (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches), funded by the 7th Framework Program. This project includes populations from four European birth cohorts: LISA (influence of life-style factors on the development of the immune system and allergies in East and West Germany) in Germany, PIAMA (Prevention and Incidence of Asthma and Mite Allergy) in the Netherlands, INMA (INfancia y Medio Ambiente [Environment and Childhood]) in Spain, and LUKAS2 (Lapsuuden kasvuympäristö ja allergiat 2 [Childhood environment and allergies]) in Finland.

The remaining two publications are based on data from four birth cohorts of the INMA project and from two German birth cohorts: LISAplus (The influence of life-style factors on the development of the immune system and allergies in East and West Germany PLUS the influence of traffic emissions and genetics study) and GINIplus (the German

infant study on the influence of nutrition intervention PLUS environmental and genetic influences on allergy development study).

ABSTRACT

Indoor levels of microbial agents may play a role in the development of the immune system during early life. An impaired development of the immune system may lead to an increase of respiratory and allergic disorders. In addition, the use of domestic cleaning products is associated with respiratory disorders in adults and children. These chemicals reduce the levels of indoor microbial agents and contribute to increase the levels of indoor air chemicals. Furthermore, the central nervous system starts developing during pregnancy and the neurodevelopment process is not yet completed after puberty. Studies in animal models demonstrated that microbial agents derived from mould may have neurotoxic effects. Therefore, the exposure to these indoor factors during infancy or childhood may have important implications in the immunological, respiratory and neuropsychological development process. This thesis aims to assess the long term effects of indoor exposure to microbial agents and chemical based cleaning products on respiratory and mental health among children from birth to the age of 13 years old.

We used data from nine birth cohorts from four European countries (Finland, the Netherlands, Germany and Spain) that are part of three projects: the European HITEA project, the Spanish INMA project; and the German GINIplus and LISAplus birth cohort studies. Information on the exposure to indoor dampness and mould, pet ownership, the use of cleaning products and allergy and respiratory health was periodically collected through questionnaires in all the projects. In the HITEA project, living room dust samples were collected when the children were 2-3 months and analysed for bacterial endotoxin, fungal extracellular polysaccharides (EPS) from *Aspergillus* spp. and *Penicillium* spp, and mould β(1,3)-glucans. At the age of 8-13 years, FeNO measurements and forced spirometric testing were performed. The neuropsychological development was assessed through the administration of the McCarthy Scales of Children's Abilities (MCSA) and the California Preschool Social Competence Scale (CPSCS) at 4 years of age in one INMA cohort. In the German studies it was assessed at 10 years of age through the administration of the Strengths and Difficulties Questionnaire (SDQ). Multivariable regression models were used to assess the associations between the exposures and the health outcomes.

Our results showed that: 1) Concentrations of measured endotoxin, EPS and β(1,3)-D-glucan varied differently across the cohorts. 2) Season of dust sampling, dog

ownership, indoor report of dampness, and number of people living in the home is associated with the concentrations of microbial agents. 3) Early life exposure to endotoxin and dogs in the home is associated with lower FeNO at school age. 4) Domestic use of cleaning sprays, air fresheners and solvents during pregnancy is associated with a higher prevalence of wheezing and LRTI during the first year of life. 5) At school age, bystander exposure to domestic cleaning sprays increases FeNO, and exposure to air freshening sprays and solvents decreases the lung function. 6) Persistent exposure to indoor dampness during early life has negative effects on the cognitive function and social competences at 4 years old. 7) Exposure to visible mould, dampness and pet ownership during the first 10 years of life increases the risk of borderline or abnormal scores in the SDQ at the age of 10 years.

Overall, the results presented in this thesis suggest that indoor exposure to microbial agents during early life and exposure to chemical based cleaning products during pregnancy, infancy and childhood may play a role in the development of the respiratory, immune and central nervous systems.

RESUM

El desenvolupament dels sistemes immunitari, respiratori i nerviós central comença durant l'embaràs i continua al llarg la infància. Així, els primers anys de vida són moments crucials en que qualsevol insult ambiental pot resultar en un desenvolupament inadequat d'aquests sistemes i, a la llarga, contribuir al desenvolupament de malalties respiratòries, al·lèrgies o trastorns neuropsicològics. Respecte als sistemes immunitari i respiratori, la concentració d'agents microbiològics als espais interiors durant les primeres etapes de la vida podria jugar un paper important en el desenvolupament de patologies respiratòries i al·lèrgies. Els productes de neteja que utilitzem per mantenir la higiene de la nostra llar redueixen la concentració d'agents microbiològics, a més de contribuir a incrementar els nivells de contaminants químics a l'aire que respirem. A més, l'ús de productes de neteja, per se, està associat amb el desenvolupament trastorns respiratoris tant a la època adulta com en la infància. Respecte al desenvolupament neuropsicològic, estudis basats en models animals suggereixen que l'exposició a agents microbiològics derivats de la humitat podria tenir efectes neurotòxics. En resum, l'exposició durant la infància a aquests factors d'espais interiors pot tenir implicacions importants en els processos de maduració immunològic, respiratori i neuropsicològic. Aquesta tesi té com a objectiu avaluar els efectes a llarg termini de la exposició a agents microbiològics i a productes de neteja de base química a la llar, sobre la salut respiratòria i mental des del naixement fins a l'edat de 13 anys.

En aquesta tesi hem fet servir dades de nou cohorts de naixement de quatre països europeus (Finlàndia, Holanda, Alemanya i Espanya) que formen part de tres projectes: el projecte europeu HITEA, el projecte espanyol INMA, i els estudis alemanys GINIplus i LISAplus. La informació sobre la exposició a humitat i a fongs producte de la humitat, a animals de companyia, la utilització de productes de neteja, al·lèrgies i salut respiratòria es va recollir en tots els projectes mitjançant qüestionaris administrats als pares/mares. A més, al projecte HITEA es van recollir mostres de pols dels menjadors de les cases quan els nens/es tenien 2-3 mesos d'edat. Aquestes mostres es van analitzar per determinar les concentracions d'endotoxines bacterianes, polisacàrids extracel·lulars (EPS) d'*Aspergillus* spp. i *Penicillium* spp i β(1,3)-glucans fúngics. A l'edat de 8-13 anys, es van practicar proves respiratòries (òxid nitric exhalat (FeNO) i espirometries). El desenvolupament neuropsicològic es va avaluar amb l'administració

de diversos tests. A una de les cohorts INMA es van administrar els tests McCarthy Scales of Children's Abilities (MCSA) i California Preschool Social Competence Scale (CPSCS) a l'edat de 4 anys. A les cohorts alemanyes, es va administrar el qüestionari Strengths and Difficulties Questionnaire (SDQ) a l'edat de 10 anys. Per l'avaluació de les associacions entre exposicions i resultats de salut s'han desenvolupat models de regressió múltiple.

Els resultats mostren que: 1) Les concentracions d'endotoxines, EPS i $\beta(1,3)$-D-glucans mesurats en pols de les llars varien per cohort. 2) L'estació de l'any en que es recull la mostra de pols, la presència de gossos a la casa, reportar humitat a la casa i el número de persones que hi viuen estan associats amb la concentració d'agents microbiològics a la pols. 3) L'exposició durant els primers mesos de vida a endotoxines i gossos a la llar s'associa amb nivells baixos de FeNO a edat escolar. 4) L'ús d'esprais i dissolvents per la neteja de la casa, i ambientadors durant l'embaràs està relacionat amb un increment en la prevalença de sibilants i infeccions respiratòries de vies baixes al llarg del primer any de vida. 5) En edat escolar, la exposició passiva a productes de neteja domèstics utilitzats en forma d'esprais incrementa els nivells de FeNO, i l'exposició a esprais ambientadors i dissolvents per la neteja de la llar disminueix la funció pulmonar. 6) L'exposició persistent a humitat a la llar durant els 2 primers anys de vida té un efecte negatiu sobre la funció cognitiva i les competències socials mesurades als 4 anys d'edat. 7) L'exposició a fongs procedents de la humitat, a humitat i a animals de companyia a casa al llarg dels primers 10 anys de vida incrementa el risc de puntuacions "borderline" o anormals al questionari SDQ administrat als 10 anys.

En resum, els resultats presentats en aquesta tesi suggereixen que l'exposició a agents microbiològics durant els primers mesos de vida i l'exposició a productes de neteja durant l'embaràs i la infància juguen un paper important en el desenvolupament dels sistemes respiratori, immunològic i nerviós central.

CONTENTS

1 INTRODUCTION

The urban atmosphere contains multiple pollutants produced by combustion or industrial discharges that can have adverse effects on human health. Indoor air is not only affected by outdoor air pollutants, but also by other contaminants released from human activities. Gas cooking and heating, smoking inside our homes, as well as the use of certain cleaning products, are some of the sources of indoor air pollution. In addition, indoor sources of biocontaminants such as pet ownership or home dampness and mould may have an impact on human health. In particular, high levels of indoor microbial agents in the home may play a role in the development of certain disorders during infancy and childhood.

In the last decades, the prevalence of respiratory, allergic and neuropsychological disorders has increased. The respiratory, immune and central nervous systems start developing during pregnancy and reach maturity in adulthood. These systems are thus very vulnerable to any environmental inputs occurring during pregnancy, infancy and childhood. Because we spend most of our time indoors, evaluating the effects of early life exposure to indoor contaminants on the respiratory, health and neuropsychological development is a major issue of public health.

1.1 Microbial agents and determinants

Bacterial endotoxin, fungal extracellular polysaccharides (EPS) from *Aspergillus* spp. and *Penicillium* spp, and fungal β(1,3)-glucans are components or excretion products of microbial agents commonly present in house dust. Endotoxins (lipopolysacharides) are strong pro-inflammatory and immune-stimulatory components of the outer membrane of Gram-negative bacteria. The fungal EPS are carbohydrate polymers excreted by practically all mycelia of moulds. Finally, β(1,3)-glucans are glucose polymers present in the cell wall of a large variety of organisms including most fungi and yeasts, some bacteria, algae, most higher plants and many lower plants. They have pro-inflammatory properties and are associated with non-allergic respiratory health effects. These three microbial agents have been measured in dust from homes mainly to disentangle their potential role in the pathogenesis of respiratory disorders.

1.1.1 Geographical variation

Regional and country differences in concentrations of microbial agents have been reported. Thorne et al[1] found differences in endotoxin concentrations across the USA and according to the sampling location in the home. In Europe, three projects evaluated regional differences in indoor endotoxin concentrations, the ECRHS (European Community Respiratory Health Survey),[2] the AIRALLERG project,[3–6] that includes 3 European birth cohorts, and the ISAAC (International Study of Asthma and Allergies in Childhood) study.[7] They reported lower endotoxin concentrations in the Scandinavian regions and higher in the Alpine regions compared to the middle European regions. In addition, the ISAAC-study observed highest concentrations in Mediterranean regions such as Rome in Italy, and Tirana in Albania. Furthermore, the AIRALLERG project measured fungal EPS and β(1,3)-glucans. Interestingly, they reported higher concentrations of both fungal agents in the Scandinavian birth cohort, compared to the Dutch and the German cohorts. The differences in indoor microbial agents levels may be related to differences in climate and cultural behaviours across countries. For example, relative humidity and temperature vary across regions and may determine bacteria and mould proliferation in the environment. In addition, these climate differences influence housing conditions (e.g ventilation, central air conditioning, humidifiers) and cultural behaviour. Differences across regions in these factors may lead to differences in important determinants of indoor microbial agent concentrations.

1.1.2 Potential determinants

Multiple home and demographic characteristics are associated with the amount of microbial agents in house dust. To date, most studies have focused on the determinants of indoor endotoxin levels.[1,2,4,8–12] They found consistent associations between indoor endotoxin concentrations or loads and cat or dog ownership, indoor smoking and the number of people living in the home. However, while the percentage of variation explained by the region is large,[10,12] the variation explained by potential determinants in a single region is small.[4,11]

To date, a few studies have assessed the effects of potential determinants on the levels of EPS[5,10] or glucans[5,10,11,13] in indoor dust. In the case of EPS, frequency of cleaning, room ventilation, age of the house and gas cooking were found to be

significantly associated with indoor concentrations of this fungal agent. For glucans, a study performed in the US reported higher concentrations in summer than in winter.[11] The AIRALLERG concluded that the living room floor concentrations of glucan were largely determined by the type of floor.[5]

1.2 The use of household cleaning products

Cleaning products are used worldwide to maintain the functionality, appearance, and appropriate hygienic conditions of our homes. They prevent from surface degradation, and control the potential risk of infection by microorganisms and exposure to dust in general. A broad spectrum of cleaning agents has been developed to facilitate dust and dirt removal, disinfection and surface maintenance. For example, cleaning products containing disinfectants can be applied to reduce exposure to indoor microbial agents. Besides their hygienic effects, cleaning products contribute to the total burden of exposure to chemicals.

1.2.1 Geographical variation

To date, the country or regional variations in the use of the most common household cleaning products have not been specifically studied. Nevertheless, two studies based on data from the ECRHS showed that the prevalence of use of bleach and certain cleaning sprays (air fresheners and furniture and glass cleaners) at home was different across the involved European countries.[14,15] Interestingly, the frequency of use of these products was highest in Spain.

1.2.2 Persistence of cleaning products in indoor air

Household use of cleaning products and air fresheners is associated with higher levels of volatile organic compounds (VOC) in indoor air. Experimental studies performed in the US found that the use of cleaning products and air fresheners was associated with increased levels of glycol ethers and terpenes. Glycol ethers are toxic air contaminants and terpenes react with ozone to form secondary pollutants including formaldehyde and ultrafine particles.[16,17]

The relationship of home levels of VOC and the use of common domestic cleaning products was assessed in the British ALSPAC study. They measured the VOC levels in

the bedrooms and living rooms of the participants homes and asked for the use of 9 different cleaning products.[18] In particular, they assessed frequent (that is, once a week or more) use of glass cleaners, carpet cleaners, dry-cleaning fluids, white spirit, paint stripper, house paints or varnishes, pesticides, other aerosols or sprays and air fresheners. Out of these products, the use of domestic aerosol or sprays, air fresheners and carpet cleaners was associated with the higher levels of VOC in the home.

1.3 Child's respiratory health and allergies

1.3.1 Prevalence and time trends

Asthma and allergies in children are widespread in industrialized countries and their prevalence has notably increased in the last decades all over the world. Also, early life respiratory and allergic symptoms importantly contribute to the total burden of chronic pulmonary disease during adulthood.[19–21] The development of respiratory and allergic disorders during infancy and childhood is therefore a major public health issue.

The large International Study of Asthma and Allergies in Childhood (ISAAC)[22] was established during the 1990s to investigate worldwide trends in prevalence and severity of asthma and allergies. This project included 6-7 and 13-14 year old children from 155 centres in 56 industrialized and developing countries. In the first phase, the results showed variations across regions in the prevalence of respiratory and allergic disorders and symptoms. In particular, they observed differences in the prevalence of asthma, asthma symptoms, rhinitis, and eczema. They suggested that the differences were persistent among genetically similar populations.[23,24]

In a later phase, the study showed age- and population density-dependent time trends. The prevalence of asthma tended to increase after 5 years in the 6-7 years group. This trend was not clear for the 13-14 years group. The time change was small in most high prevalence countries, but high population density regions such as Africa, Latin America and parts of Asia observed significant increases. This indicates that the global burden of respiratory disorders is continuing to rise and that the global differences in prevalence tend to become smaller.[25,26]

1.3.2 Asthma, wheezing phenotypes, atopy and respiratory infections.

Asthma is a heterogeneous condition with different clinical expressions that occur at different ages. The majority of the cases of chronic asthma start with wheezing during pre-school ages. The association between wheezing and atopy, and the wheezing phenotypes identified in the first years of life differ in the risk and severity of asthma later in life.

In this regard, the first hypothesis-based study was conducted in the Tucson Children's Respiratory Study (USA) by Martinez et al.[27] They proposed the differentiation of three groups of children with wheezing according to the remission or persistence of wheezing episodes when reaching school age. Martinez et al. proposed a division between those whose episodes began early in life and remitted before school age (transient early wheezers), those whose episodes began very early in life and persisted at school age (persistent wheezers), and those whose episodes initiated after school age and persisted (late-onset wheezers). This classification has been further confirmed and extended in other birth cohort-based studies.

The Avon Longitudinial Study of Parents and Children (ALSPAC) in the UK and the Prevention and Incidence of Asthma and Mite Allery (PIAMA) study in the Netherlands used longitudinal latent class analyses to identify wheezing phenotypes.[28,29] The ALSPAC study subdivided the transient early wheezers and the late-onset wheezers into two groups. The PIAMA study only subdivided the late-onset wheezers into two groups. For transient early wheezers subdivision, the first group included those children whose symptoms remit at 18 months old (transient early) and the second group included those whose symptoms remit at 3 years old (prolonged early). For the late-onset wheezers subdivision, the first group included those starting at 18 months and rising from 18 months to 3 years (intermediate onset), and those who started at 3 years (late-onset).

To date, several studies have evaluated the associations between the wheezing phenotypes and the report of doctor diagnosed asthma, lung function, atopy and fractional exhaled nitric oxide (FeNO). Transient early wheezing is associated with a decline in the expiratory volumes,[29,30] however their risk of subsequent asthma is low[29] and the association with atopy almost inexistent.[29,30] On the contrary, the late onset

and persistent wheezing groups are positively associated with atopy,[29,30] asthma risk[29] and eosinophilic airway immlamation.[31] Lung function is not associated with the late onset of wheezing but a decline in the expiratory volumes is observed in most studies for the persistent wheezers group.[29,30,32,33]

Finally, wheezing during pre-school ages is associated with lower and upper respiratory tract infections (LRTI and URTI). In particular, LRTI caused by respiratory syncytial virus (RSV) and URTI caused by rhinovirus increase the risk of subsequent wheezing during school ages.[34–37] This suggests that the interaction between viral infections and T helper 2 (TH2) immunity predisposes to more severe acute responses to the virus and development of asthma.

1.3.3 Immunological mechanisms

Asthma is characterized by a chronic inflammation of the respiratory tract, which is mediated by increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. In addition, when the intensity of the chronic inflammation increases, acute episodes or asthma exacerbations occur.

The immunological mechanisms underlying this disease are shown in Figure 1. Inhaled allergens activate sensitized mast cells by cross-linking surface-bound IgE molecules, and the mast cells release several bronchoconstrictor mediators. Epithelial cells release stem-cell factor to maintain mucosal mast cells at the airway surface. Allergens are processed by myeloid dendritic cells, which attract TH2 cells. These cells have a central role in orchestrating the inflammatory response in allergy through the release of interleukin (IL)-4 and IL-13 (IgE sintetization by B cells), IL-5 (eosinophilic inflammation) and IL-9 (mast-cell proliferation).
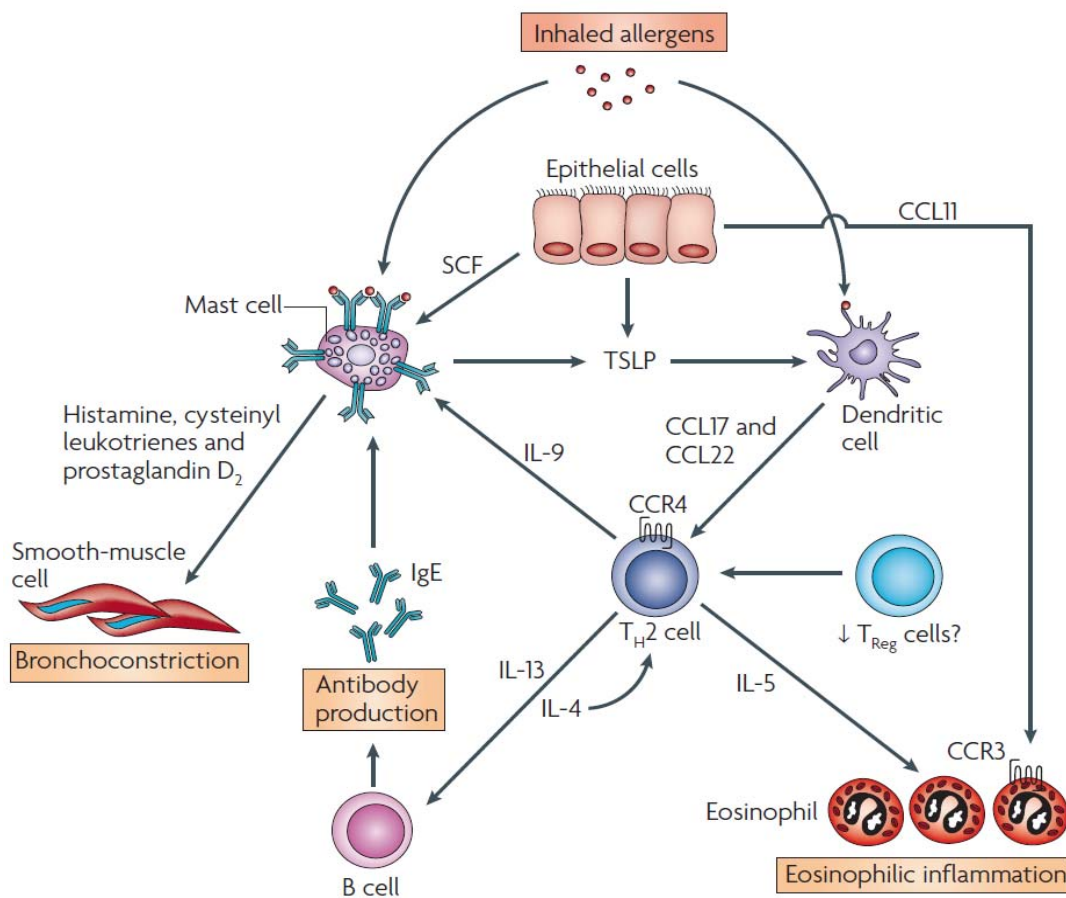
Figure 1. Inflammatory and immune cells involved in asthma. (adapted from Barnes et al[38])

1.3.4 Respiratory and immunological health effects of early life exposure to indoor biocontaminants.

Farming environment

In the late 1990s, Swiss researchers hypothesized that the farming environment in rural areas rather than the higher levels of air pollution in urban areas may be the cause of the disparities between rural and urban environments in asthma and allergy prevalence. In their study, they observed lower prevalence of atopic sensitization and allergy symptoms in children of farm workers.[39] The hypothesis of this study was further confirmed by other studies including rural areas from central European countries,[10,40–43] Scandinavian countries,[44–47] the USA[48] and Canada.[49,50] Furthermore, retrospective studies demonstrated that the farm protective effect persisted to adulthood.[51–53]

Moreover, some of these studies identified several factors that may contribute to the farm protective effect. Farm animal contact from early childhood, drinking farm milk during childhood and high microbial levels (endotoxin and EPS) in indoor dust were significantly associated with lower prevalence of asthma, asthma symptoms, allergy symptoms and atopic sensitization.[10,40–43,46,52]

The mechanisms potentially underlying the effects of farm exposure on the human immune symptoms are illustrated in Figure 2. In this model, the farm environment results in intense microbial pressure on the innate immune system. Microbial exposure facilitates the activation of the regulatory T (TReg) cell through the release of tumour necrosis factor (TNF) and IL-10 by the dendritic cell (DC). The TReg cell activation balances the adaptive immune responses and suppresses the allergic inflammation (allergen-induced TH2 cell-associated cytokine production and TH2 cell-dependent IgE synthesis).
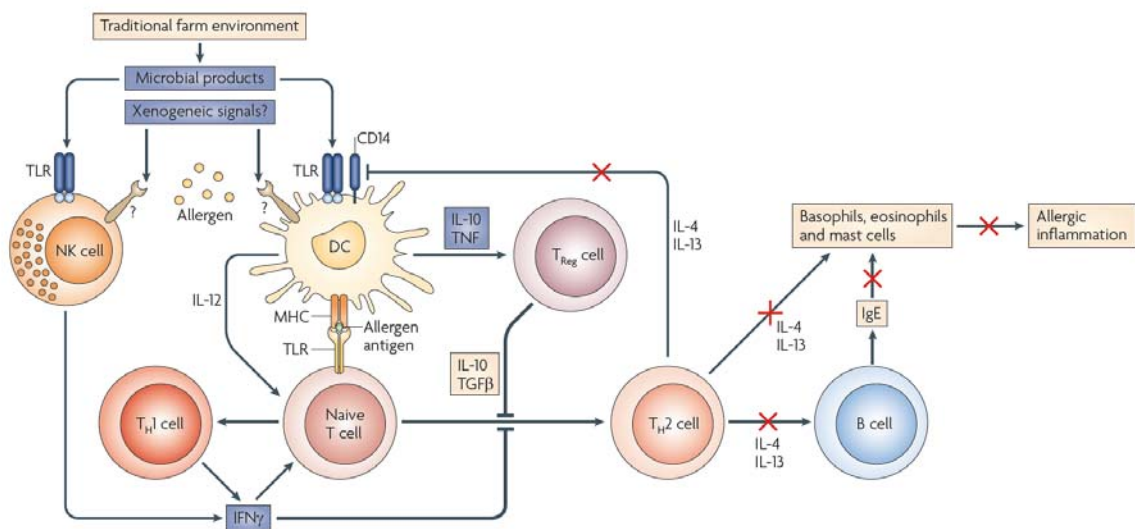
Figure 2. Mechanisms potentially underlying the impact of farm exposure on the immune system (from von Mutius et al[54]).

Indoor microbial agents in non-farm environments

After the publication of the first farm studies, a large number of studies tried to elucidate the relationship between exposure to high microbial agent concentrations during the first stages of life and the development of respiratory and allergic disorders.

A work performed in US children aged 9 to 24 months suggested that endotoxin exposure in the first two years of life protects against sensitization by enhancing T_H1 immunity.[55] Analyses from the German LISA birth cohort observed a U-shaped association between cord blood IgE and endotoxin concentration in maternal mattress dust, suggesting that prenatal exposure may affect the foetal development of the immune system.[56] In addition, they found that high endotoxin levels in child mattress dust during the first year of life protected against early life eczema.[57] A birth cohort in the USA of high risk children (that is, children with parental report of atopy) observed a protective effect of high β(1,3)-glucan concentrations on wheezing and on the asthma predictive index in pre-school age children.[58,59]

The results of longitudinal studies focusing on the early life (pre-school age) exposure to indoor microbial agents and school age health effects are not conclusive. They were performed in two birth cohorts including high risk children (PIAMA[60] and the Epidemiology of Home Allergens and Asthma Study[61]) and in a Norwegian population-based birth cohort.[62] The PIAMA study reported protective effects of early life high endotoxin and EPS concentrations on asthma and persistent wheezing at 4 years of age.[60] The study by Celedón et al[61] observed protective effects of early life endotoxin concentrations on atopy, but increased risk of wheezing at 7 years of age. Finally, the Norwegian Environment and Childhood Asthma did not find any association between indoor endotoxin and glucan measured at 2 years of age and asthma, lung function and atopy at the age of 10.[62] The work by Sordillo et al[63] suggests that the observed variations in the associations may be mediated by genetic factors.

The cross-sectional studies evaluating the association between microbial agent concentrations and respiratory health at school age are more consistent. The European AIRALLERG project that included populations from 4 European birth cohorts (PIAMA in the Netherlands, GINI and LISA in Germany and BAMSE in Sweden)[3,6] found a protective effect of endotoxin and EPS concentrations on rhinitis and doctor diagnosed asthma in the German cohorts at the age of 6 years. Moreover, a study performed within the ISAAC project including 9 to 12 year old children from Europe, New Zeeland and the United Kingdom observed similar results regarding the cross-sectional associations between endotoxin concentrations in living room floor dust and asthma.[7] Studies performed in high-risk infants and children in the USA and Canada showed similar associations between indoor endotoxin concentrations and asthma.[64]

Cat and dog ownership

Studies on pet ownership exposure, mainly focusing on cat and dog ownership, have found inconsistent results on the association between respiratory health and pet ownership. A study performed in the Finnish LUKAS2 birth cohort suggested that dog ownership during pregnancy and early life reduced immune system responses during early childhood. In particular, they observed a decreased capacity to produce TNF-α at birth and at 1 year of age.[65] In line with these results, an Australian study performed in high risk children observed that early life exposure to cats or dogs decreased the risk of atopy at the age of 5 years.[66] Moreover, a Canadian cross-sectional study found an inverse association between pet ownership and FeNO at 9 to 12 years of age.[67]

On the contrary, and the Canadian Primary Prevention of Asthma in Childhood study showed that early life cat or dog ownership were risk factors for asthma diagnosis at 7 years of age.[68] Moreover, two retrospective studies performed in the ISAAC project found that early life cat rather than dog ownership was a risk factor for asthma, wheezing, rhinoconjunctivitis and eczema at the age of 6-7 years.[69,70] In addition, current cat and dog or only dog exposure was associated with these health outcomes in adolescents.[70] Finally, the Swedish BAMSE birth cohort did not find any statistically significant association between these exposures in early life and asthma at the age of 4 years.[71]

Indoor dampness and mould

Unlike the results obtained for pet ownership, the associations observed between indoor dampness and mould and child's respiratory health are consistent through the literature. A recent systematic review focusing on children's respiratory health effects of mould and dampness concluded that home environments with visible mould and mould spore exposure increase the risk of allergic respiratory health outcomes in children.[6] The Pollution and the Young (PATY) study included pooled data from 12 cross-sectional studies conducted in Russia, America and Europe. They assessed the associations between the household report of dampness and eight respiratory and allergic symptoms. The conclusion of this huge meta-analysis was that indoor dampness was homogeneously associated with respiratory and allergic symptoms across regions.[72] Moreover, several longitudinal studies have shown that early life exposure to indoor dampness has adverse effects on asthma onset and respiratory

and allergic symptoms.[59,73–76] The mechanisms underlying these associations are not yet clear. Indoor dampness and mould are associated with higher levels of toxins,[77] VOCs[78,79] and allergens[80] in the house. Thus, a combination of the exposure to these three factors may be the cause of the associations between indoor dampness and mould and the respiratory and allergic disorders.

1.3.5 Respiratory health effects of exposure to household cleaning products.

Studies on the adverse effects of cleaning products started one decade ago focusing on respiratory effects associated with occupational exposure to cleaning products.[81–84] These studies reported that professional use of cleaning products is associated with asthma and other respiratory disorders. In particular, employment in domestic cleaning increases the risk of asthma and respiratory symptoms, and aggravates asthma in adults.[85–87] Regarding the effects of non-occupational domestic use of cleaning products, using spray applied products was associated with higher risk of asthma,[14,88] and the use of bleach with lower atopy but higher respiratory symptoms.[15]

The potential effects of passive exposure to cleaning products on children's respiratory health have not been evaluated in many studies. Two longitudinal analyses of data from the ALSPAC study suggested that frequent use of consumer chemicals, predominated by cleaning agents, during pregnancy increased the risk of persistent wheezing in pre-school and school ages.[89,90] These studies assessed the effects of a total chemical products score based on the frequency of use of 9 different household chemicals. The French PARIS (Pollution and Asthma Risk: an Infant Study) birth cohort focused on spray use and suggested that cleaning products applied by spray increased the risk of wheezing in the first 18 months of life.[91] Finally, a cross-sectional Belgian study reported protective effects of the domestic use of bleach on asthma and allergic sensitization in school-age children.[92]

1.4 Child's neuropsychological development

1.4.1 The development of the central nervous system

The development of the central nervous system or neurodevelopment is a genetically driven process composing several phases: neurulation, proliferation, migration,

myelination, and synaptic pruning. It is a structurally and functionally non linear process that starts during the prenatal period and continues until post-adolescence.[93]

The first phase of the neurodevelopment process is the neurulation. This phase takes place between the second and the forth week of gestation. It is characterized by the creation of the neural tube, the formation of the neurons and glial cells at the outside wall of the neural tube, and the development of areas such as the cerebral hemispheres, the olfactory bulb, and the pituitary gland. The cell proliferation and migration processes start after the fourth week of gestation and most neurons have migrated to their appropriate locations at birth. The migration of the neural cells to their proper location is regulated by physical and chemical processes.[94,95] Finally, the myelination and synaptic pruning processes start during the last weeks of gestation and continue until post-adolescence (Figure 3).

The postnatal period is characterised by increased cortical complexity.[96] Some neurogenesis that started in the foetal stages continues during the first year of life in the hippocampus, the olfactory bulb, and the cerebellum.[94,97] The brain continues to grow and specialize according to a precise genetic program which is modified by environmental influences. The brain mass increase is developed in irregular periods (growth spurts) and it is mainly due to the myelination process and the dendritic growth.[96]

Developmental course of human brain development

Experience-dependent synapse formation and dendritic arborization

Prefrontal cortex

Parietal and temporal association cortex

Sensorimotor cortex

Neurulation

Cell proliferation and migration

Synaptogenesis and synaptic pruning

Myelination

Conception

−8   −6   −4   −2   0   2   4   6   8   10   12   2   4   6   8   10   12   14   16   18   20

Months   Birth   Months   Years

Figure 3. Course of the human brain development (from Casey et al[93]).

The time courses of such neuronal and synaptic formation and elimination are considerably different among the diverse cortical areas. In particular, the neuroanatomical structure of the prefrontal cortex in humans undergoes considerable maturation during early childhood. This brain region is implicated in planning complex cognitive behaviour, personality expression, decision making and moderating social behaviour. Thus, the period from early childhood to preschool age is important in the development of the behavioural and cognitive functions related to the prefrontal cortex.[98–100]

1.4.2 Indoor factors and neuropsychological development.

Throughout an individual's lifespan, the brain receives multiple inputs that include environmental and social exposures. Early exposures can lead to an increased susceptibility in adults. Multiple or persistent exposures, in most cases at low doses, may promote disturbances or aberrant structural and/or functional consequences that can lead to significant problems later in life.

In particular, poor cognitive development in children was associated with several aspects related to the home. Although most epidemiological studies focus on psychosocial factors related to family structure and functioning,[101] illnesses,[102] time spent in front of the television or computer,[103] parental unemployment, and socio-economic status,[104] recent studies investigate the associations between neuropsychological development and physical and chemical indoor factors. For example, environmental tobacco smoke was found to be associated with poor cognitive development at four years of age[105] and with higher risk of hyperactivity/inattention problems at school age.[105–107] Indoor levels of nitrogen dioxide (NO_2) and gas appliances are suggested to have an adverse relation with cognition at 4 years of age.[108,109] In addition to these indoor factors, exposure to microbial agents present indoors and its sources (e.g pet ownership and home dampness or mould) might be related to neuropsychological development.

Microbial agents

Experimental studies performed in murine models suggest that pre-natal exposure to high endotoxin concentrations induces an immune activation that can inhibit neurogenesis in the pre-natal or early life periods.[110] Additional experimental studies in

animal and human models showed that the exposure to high endotoxin concentrations during adulthood lead to immune-activation involving increased TNF-α, IL-1 and IL-6. This activation of the immune system is related with mood disorders and cognitive disturbances.[111–115]

Mycotoxins are toxic secondary metabolites produced by molds that are present in higher concentrations in water damaged buildings.[77] Experimental studies in animal and human models show that high mycotoxin concentrations had inflammatory and neurotoxic effects.[116–121] In addition, the neurotoxic effect of mycotoxins is potentiated by the co-exposure to endotoxins.[122]

Cat and dog ownership

Specific associations between pet ownership during infancy and childhood and the neuropsychological development have not been studied. The assessment and interpretation of the relationship between pet ownership and human mental health is complex.[123–125] On the one hand, factors such as personality traits, age and economic or health status can impact on the decision to own a pet and on the type of pet chosen. Additionally, pet ownership may enhance social interactions, and provide emotional support, that would contribute to the family wellbeing, and as a consequence to the child's proper neurospychological development.[126] On the other hand, pet ownership, particularly cat and dog ownership, is associated with increased indoor biocontaminants including allergens and bacterial endotoxin[1,4,8,9] that may have an opposite impact on the child's neuropsychological development.

Indoor dampness and mould

The first epidemiological studies assessing mental health effects of living in damp homes were conducted in the UK during the 1980s. They reported higher prevalence of depression in adults and children living in water-damaged buildings. Nevertheless, they awarded a social explanation to the findings observed.[127,128] In 2007, a work performed in the Large Analysis and Review of European housing and Health Status Study reported a positive association between home dampness and mould and depression in adults. This association persisted after adjustment for several social-factors and was not mediated by physical illnesses.[129]

To date, the neuropsychological effects of living in water damaged homes in children have only been assessed in one longitudinal study. This study focused on persistent exposure to visible mould in the home during the first two years of life and cognitive development at 6 years of age.[130] They found that persistent early life exposure to indoor mould was associated with poorer cognitive function.

2 RATIONALE

The prevalence of respiratory, allergic and neuropsychological disorders in children has increased in the last decades. The respiratory, immune and central nervous systems start developing during pregnancy and reach maturity in adulthood. Therefore, early life exposure to environmental factors may play an important role in the development of respiratory, allergic and neuropsychological disorders during childhood and adulthood.

Exposure to indoor microbial agents during the first stages of life may play a role on the development of the immune and neuropsychological systems during infancy and childhood. Indoor factors like pet ownership or dampness contribute to higher levels of microbial agents in the home. In addition, the frequency of household cleaning and the use of certain specific cleaning agents determine levels of indoor microbial agents. Beyond the effects of cleaning products on the levels of indoor microbials, these chemicals have irritant effects on the respiratory system and contribute to the total burden of exposure to indoor chemicals.

Because we spend most of our times indoors and the prevalence of both respiratory and neuropsychological disorders has increased, the study of the health effects of indoor microbial agents, its determinants and the domestic use of cleaning products in children is of great relevance for public health.

3 OBJECTIVES

General aim

To evaluate long-term effects of indoor exposure to microbial agents and chemical based cleaning products on respiratory and mental health among children from birth to the age of 13 years old.

Specific objectives

1. To describe the concentrations of the non-viable microbial agents bacterial endotoxin, fungal extracellular polysaccharides (EPS) and β(1,3)-D-glucan in living room dust in homes from children aged 2-3 months.

2. To evaluate potential determinants of microbial agents (endotoxin, EPS and β(1-3)-glucans) concentrations in living room dust in homes from children aged 2-3 months.

3. To assess the associations between microbial agents concentrations (endotoxin, EPS and β(1-3)-glucans) in living room dust in homes from children aged 2-3 months, reported dampness and pet ownership and airway inflammation (FeNO) at school age.

4. To evaluate the effects of household use of cleaning products during pregnancy on respiratory symptoms and airway infections during the first year of life.

5. To assess the effects of household cleaning product use on airway inflammation (FeNO) and lung function (FVC and FEV1) in school age children.

6. To assess the effects of living in damp homes, pet ownership, and farm animal contact during early life, on cognitive function and social competences at 4 years of age.

7. To assess the effects of exposure to indoor visible mould, dampness and pet ownership on behavioural problems in children aged 10 years.

4 HYPOTHESES

The exposure to high levels of indoor microbial agents during early life and the passive exposure to cleaning products during pregnancy, infancy and childhood play a role on the development of the respiratory and central nervous systems during the first years of life.

The specific hypotheses are the following (see also figure 4):

a) Indoor factors such as dampness or mould, cat and dog ownership are associated with higher levels of indoor microbial agents.

b) Early life exposure to high indoor levels of microbial agents and to indoor factors (dampness, mould, cat and dog) protects from the risk of eosinophilic airway inflammation at school age. In addition, the exposure to household cleaning products at any age increases the risk of respiratory disorders.

c) Exposure to indoor factors and high indoor levels of microbial agents during infancy and childhood has a negative impact on the neuropsychological development.
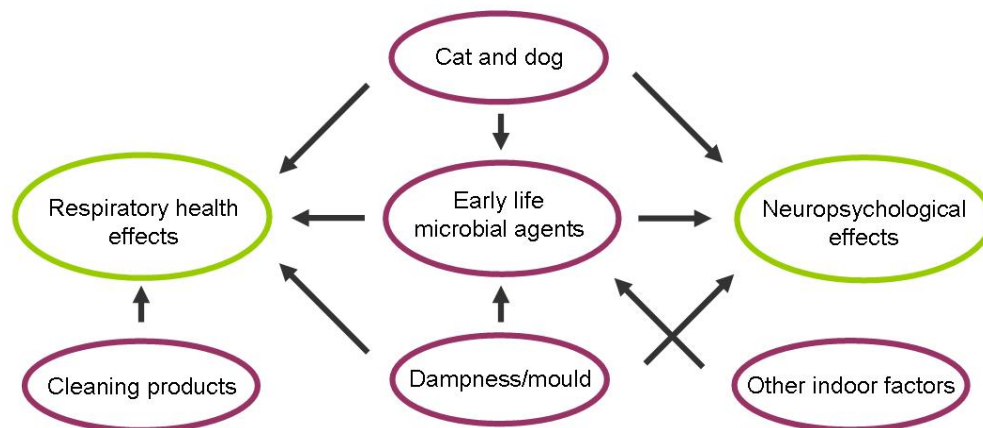
Figure 4. Associations to be considered in the interrelationship between early life microbial exposures, indoor dampness, pet ownership and cleaning products and respiratory health; and and neuropsychological development.

5 METHODS

This thesis is based on data from nine birth cohorts from four European countries (Finland, the Netherlands, Germany and Spain) that are part of three projects: the European HITEA project (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches), the Spanish INMA (INfancia y Medio Ambiente [Environment and Childhood]) project;[131] and the German GINIplus[132] (German infant study on the influence of nutrition intervention PLUS environmental and genetic influences on allergy development study) and LISAplus[56] (The influence of life-style factors on the development of the immune system and allergies in East and West Germany PLUS the influence of traffic emissions and genetics study) birth cohort studies. The HITEA project included data from the PIAMA cohort in The Netherlands, the LISA cohort in Germany, the INMA-Menorca cohort in Spain and the LUKAS2 cohort in Finland. The INMA project includes the birth cohorts from Asturias, Gipuzkoa, Sabadell, Valencia and Menorca. Finally, the GINIplus and LISAplus study include data from both German birth cohorts. All the studies included in this thesis were approved by the regional Research Ethical Committee, and informed consent was signed by all participants. The geographic and project location of the birth cohorts included in this thesis is shown in Figure 5. In addition, the methods used are summarised in table 1, at the end of this chapter.

a) Geographic location   b) Project location
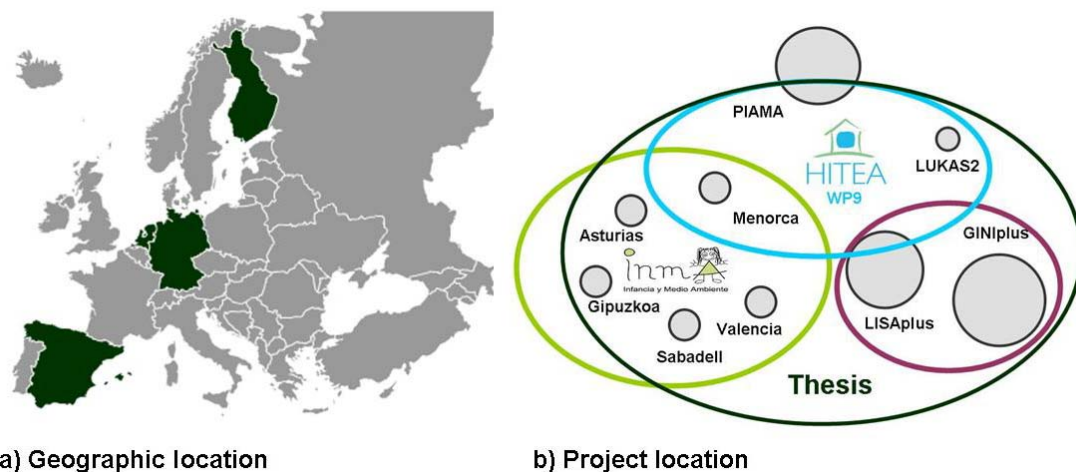
Figure 5. Geographic location (a) and project location (b) of the birth cohorts included in the thesis. Black circles represent the birth cohorts, the blue ellipse represents the birth cohort study in the HITEA project, the light green ellipse represents the INMA project, the purple ellipse is for the GINIplus and LISAplus study, and the dark green ellipse contains the data included in this thesis.

5.1 The HITEA project

The HITEA project (www.hitea.eu) is a European project funded by the European 7th Framework Program that started in 2008 and will finish in 2013. The overall aim of the HITEA project is to identify the role of indoor biological agents in the development of long term respiratory, inflammatory and allergic health impacts among children. The focus is on microbial exposures due to dampness problems of buildings. In addition, it studies the role of allergens, chemicals, cleaning agents, traffic exhaust and poor ventilation. The study consists of three main areas of research: indoor air in schools, indoor air in homes, and methods and mechanisms. The overall structure of the project is shown in Figure 6.
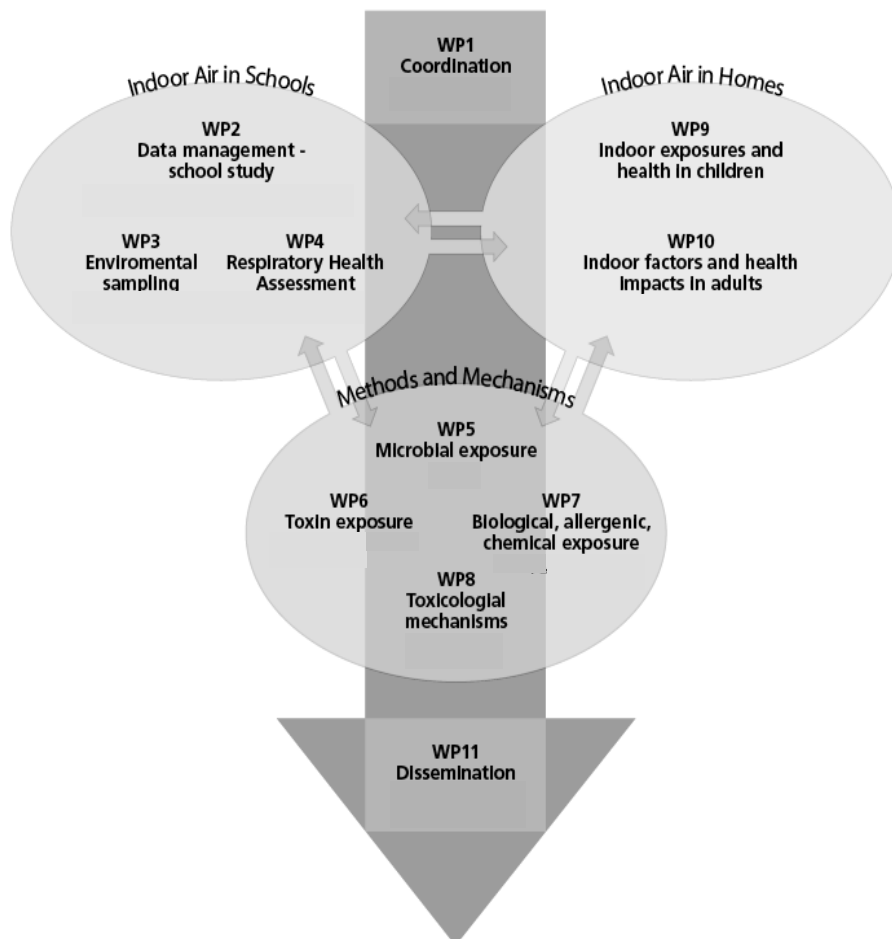
Figure 6. Overall structure and interdependencies of the components of the HITEA project.

This thesis includes the results from part of the indoor air in homes section: the indoor exposures and health in children work package (WP). The aim of this WP is to assess the long-term effects of perinatal exposures to indoor allergens and microbes in respiratory and immunological health in children. It includes populations from four ongoing European birth cohorts that started between 1996 and 2005: LISA[56] in Germany, PIAMA (Prevention and Incidence of Asthma and Mite Allergy) [60,133] in the Netherlands, INMA-Menorca[134] in Spain and LUKAS2 (Lapsuuden kasvuympäristö ja allergiat 2 [Childhood environment and allergies])[65] in Finland. Only individuals with house dust samples collected at 2-3 months of age were included in the HITEA study (LISA: n=395; PIAMA: n=696; INMA-Menorca: n=474; and LUKAS2: n=164).

Living room dust samples collected during early life (2-3 months old) were analyzed for non-viable microbial products (Endotoxin, EPS and β(1,3)-D-glucan) between 2009 and 2010 in the Institute for Risk Assessment Sciences (IRAS, Utrecht, NL) with the Limulus Amebocyte Lysate test (endotoxin) and a specific enzyme immunoassay () EPS and glucan) as in previous house dust analyses.[10] Between 2008 and 2011, when the children were 7 to 13 years old, new questionnaires on respiratory health and household use of cleaning products were administered and functional and biological respiratory health testing (FeNO and spirometry) was done in the LISA, PIAMA and INMA cohorts.

5.2 The INMA project

The INMA project (www.proyectoinma.org) is a network of birth cohorts in Spain that aims to study the role of environmental pollutants in air, water and diet during pregnancy and early childhood in relation to child growth and development. It includes pregnant women and their children from the general population in seven study areas (Figure 7). Three started on 1997-2000: Ribera d'Ebre (n=102), Menorca (n=482), Granada (n=668), and four started on 2004-2006 and followed the same protocol: Asturias (n=494), Gipuzkoa (n=638), Sabadell (n=657), and Valencia (n=855). This thesis includes data from the regions of Menorca (as part of the HITEA project), Asturias, Gipuzkoa, Sabadell and Valencia.

Extensive assessments were carried out in pregnant women and children. The information was gathered from a variety of sources: *ad hoc* administered health and environmental questionnaires in face-to-face interviews by trained INMA personnel,

clinical data, physical examinations, health tests, biological samples, diet determinants and environmental measurements (air pollution, water pollution and persistent and semi-persistent pollutants). Data collected at each wave varied slightly among cohorts according to local interests, but the main common variables were included in all cohorts.

Figure 7. Geographical location of the INMA cohorts in Spain.

In the INMA-Menorca cohort, the neuropsychological development was assessed when the child was 4 years old. A trained neuropsychologist administered the McCarthy Scales of Children's Abilities[135] (MCSA) to evaluate the cognitive development of the child. The social competences were measured by the teachers using the California Preschool Social Competence Scale[136] (CPSCS).

5.3 The GINIplus and LISAplus studies

The GINIplus study[132] was designed to investigate the influence of nutrition intervention in infancy, environmental exposures and genetic factors on the development of allergies. Between September 1995 and June 1998, a total of 5991 healthy full-term infants born in Munich and Wesel (Germany) were recruited. Children with a family history of allergy were invited to participate in a nutritional intervention trial. In total, 2252 children with a family history of allergy agreed to participate in the trial. In addition, 3739 children without a family history of allergy or whose parents declined to participate in the trial were recruited for the observational group. The nutrition intervention consisted of the administration of 3 hydrolyzed formulas, the standard formula, and dietary recommendations. The intervention study was performed only

during the first 12 months of life, after this period, no further intervention was applied to this group.

The LISAplus study[56] was created to evaluate the influence of life-style factors, traffic emissions and genetics on the development of the immune system and allergies. Newborns from obstetrical clinics in Munich, Leipzig, Wesel and Bad Honnef, in Germany, were invited to participate in the LISAplus birth cohort. A total of 3097 healthy full-term newborns were recruited in the study between December 1997 and January 1999.

Apart from the intervention study period in the GINIplus birth cohort, both cohorts followed similar protocols. The same health and environmental questionnaires and health tests were administered and the same biological samples were periodically taken. Moreover, environmental factors were measured equally for both studies.

For the assessment of behavioural problems, the Strengths and Difficulties Questionnaire (SDQ)[137–140] was administered in both birth cohorts when the children was 10 years old. This is a validated screening questionnaire to assess mental and behavioural strengths and difficulties in 3 to 16 year old children. The questionnaire was administered to the parents and identified the child's strengths and difficulties in 5 dimensions: "emotional symptoms", "conduct problems", "hyperactivity/inattention", "peer relationship problems" and "prosocial behaviour".

Table 1. Description of the methods used to achieve the specific objectives of the thesis.

Specific objectives	Project	Birth cohorts	Exposure (age)	Outcome (age)	Paper number
1. To describe the concentrations of microbial agents in home living room dust.	HITEA	LISA, PIAMA, INMA-Menorca and LUKAS2	Microbial agents[a] (2-3 months)	n/a	I
2. To evaluate potential determinants of microbial agents concentrations in home living room dust.	HITEA	LISA, PIAMA, INMA-Menorca and LUKAS2	Indoor factors[b] (2-3 months)	Microbial agents (2-3 months)	I
3. To assess the associations between microbial agents concentrations in early life home dust, reported dampness and pet ownership, and airway inflammation (FeNO) at school age.	HITEA	LISA, PIAMA and INMA-Menorca	Microbial agents[a] (2-3 months) and indoor factors[b] (birth to 10 years)	Airway inflammation (FeNO) (7-13 years)	II
4. To evaluate the effects of household use of cleaning products during pregnancy on respiratory symptoms and airway infections during the first year of life.	INMA	INMA-Asturias, INMA-Gipuzkoa, INMA-Sabadell and INMA-Valencia	Cleaning products (Pregnancy and 1 year)	Wheezing and LRTI (1 year)	III
5. To assess the effects of household cleaning products use on airway inflammation (FeNO) and lung function (FVC and FEV1) in school age children.	HITEA	INMA-Menorca	Cleaning products (10-13 years)	Airway inflammation (FeNO) and lung function (FVC and FEV1) (10-13 years)	IV
6. To assess the effects of living in damp homes, pet ownership, and farm animal contact during early life, on cognitive function and social competences at 4 years of age.	HITEA	INMA-Menorca	Indoor factors[b] (birth to 4 years)	Cognitive function (MSCA) and social competences (CPSCS) (4 years)	V
7. To assess the effects of the exposure to indoor visible mould, dampness and pet ownership on behavioural problems in children aged 10 years.	GINIplus and LISAplus	GINIplus and LISAplus	Indoor factors[b] (birth to 10 years)	Behavioural problems (SDQ) (10 years)	VI

[a]Endotoxin, Extracellular polisaccharides and β(1,3)-glucans; [b]Dampness and/or mould and pet ownership

6 RESULTS

Microbial agents and determinants

Paper I   Endotoxin, extracellular polysaccharides and β(1-3)-glucans concentrations in dust and their determinants in 4 European birth cohorts: results from the HITEA project

Respiratory health

Paper II   Early life microbial exposure and fractional exhaled nitric oxide in school-age children

Paper III   The use of household cleaning products during pregnancy and lower respiratory tract infections and wheezing during early life

Paper IV   Use of household cleaning products, exhaled nitric oxide and lung function in children

Neuropsychological development

Paper V   Early life exposures to home dampness, pet ownership and farm animal contact and neuropsychological development in 4 year old children: a prospective birth cohort study

Paper VI   Indoor factors and behavioural problems in children: the GINIplus and LISAplus birth cohort studies

6.1 PAPER I

Endotoxin, extracellular polysaccharides and β(1-3)-glucans concentrations in dust and their determinants in 4 European birth cohorts: results from the HITEA project

Casas L, Tischer C, Wouters IM, Valkonen M, Gehring U, Doekes G, Torrent M, Pekkanen J, Garcia-Esteban R, Hyvärinen A, Heinrich J and Sunyer J

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[*]This paper is reproduced according to the original print version. References of this paper are included in the references section of the thesis.

6.2 PAPER II

Early life microbial exposure and fractional exhaled nitric oxide in school-age children

Casas L, Tischer C, Wouters IM, Torrent M, Gehring U, Garcia-Esteban R, Thiering E, Postma D, de Jongste J, Smit AH, Borràs-Santos A, Zock JP, Hyvärinen A, Heinrich J, Sunyer J.

[Submitted][*]

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[*]This paper is reproduced according to the original submitted version. References of this paper are included in the references section of the thesis.

Early life microbial exposure and fractional exhaled nitric oxide in school-age children

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Abstract

Background: Early life exposure to microbial agents may have an effect on the development of the immune system and on respiratory health later in life. Inflammation is a key factor in the pathogenesis of respiratory diseases.

Objective: To evaluate the associations between early life microbial exposures, and the fractional exhaled nitric oxide (FeNO), a biomarker of eosinophilic airway inflammation, at school age.

Methods: In homes of participants of three prospective European birth cohorts (LISA, n=182; PIAMA, n=244; and INMA, n=355), endotoxin, EPS and β(1,3)-D-glucan were measured in living room dust collected at 2-3 months of age. Home dampness and pet ownership were periodically reported by the parents through questionnaires. FeNO was measured at age 8 for PIAMA and at age 10/11 for LISA and INMA. Cohort-specific associations between the indoor microbial exposures and FeNO were evaluated using multivariable regression analyses. Estimates were combined using random-effects meta-analyses.

Results: FeNO at school age was significantly lower in children exposed to endotoxin at age 2-3 months (β -0.054, 95% confidence interval (CI) -0.97;-0.01) and in children with reported dog ownership during the first two years of life (GM ratio 0.82, CI 0.70-0.96). FeNO was not significantly associated with early life exposure to EPS, β(1,3)-D-glucan, indoor dampness and cat ownership.

Conclusion: Early life exposure to bacterial endotoxin and early life dog ownership are associated with lower FeNO at school age. Further studies have to unravel the underlying effect mechanisms and possible clinical relevance of this finding.

Clinical implications/Key messages

Early life exposure to high concentrations of bacterial endotoxin and dog ownership decrease exhaled NO at school age, which may suggest a possible role of exposure in early life for immune development.

Capsule summary

Early life exposure to indoor bacterial endotoxin measured in living room dust and dog ownership during the first two years of life may reduce FeNO at school age.

Key words

Exhaled nitric oxide, eosinophilic inflammation, endotoxins, extracellular polysaccharide, beta-1,3-D-glucan, dampness, pet ownership, longitudinal, childhood.

Abbreviations

EPS: Extracellular polysaccharides; FeNO: Fractional exhaled nitric oxide; NO; inducible nitric oxide synthase; GM: Geometric mean; Gsd: Geometric standard deviation; sd: standard deviation; CI: 95% confidence interval; EU: Endotoxin units; EPSU: Extracellular polysaccharides units; LOD: Limit of detection; BBS: Borate buffered saline.

Introduction

The first years of life may be a crucial period for the development of the immune system and the onset of allergic and respiratory disorders.[149,150] In the last decade, numerous studies assessed associations between measured microbial agents and related indoor factors and reported respiratory and allergic outcomes in children.[3,6,7,41,43,57,58,60,61,63,64] Only few studies measured early-life microbial exposures and investigated health effects prospectively. They found that exposure to high levels of endotoxin in the first months of life may reduce the risk of eczema in infancy.[57] However, findings regarding wheezing and asthma are inconsistent.[60,61,151]. Regarding indoor factors associated with higher indoor microbial agents levels, previous studies observed that early life exposure to indoor mould and dampness increases the risk of asthma.[59,74,76] However, the direction of the associations between early life exposure to pet ownership and atopy, wheezing and asthma is ambiguous. Some studies found that the presence of a dog in the home during early life attenuated TNF-α production[65] and had a protective effect on atopy and wheezing.[66,152] Other studies observed positive or non-significant associations between home exposure to cats or dogs and atopy, respiratory symptoms and asthma. [62,68,69,153]

Inflammation is a key factor in the pathogenesis of respiratory diseases and FeNO is considered to be a non-invasive biomarker associated with eosinophilic airway inflammation.[154–156] High levels of NO in the airways are involved in the pathophysiology of asthma and other respiratory disorders[157] and are associated with respiratory symptoms and atopy in children.[31,158–160] In epidemiological studies, FeNO has the advantage of being continuous and easily measureable. FeNO is an objective variable, in contrast to self-reported categorical variables. Several epidemiologic studies showed associations between environmental exposures such as air pollution,[161,162] polycyclic aromatic hydrocarbons,[163] indoor allergens[164] and other indoor factors[67] and FeNO. However, the effects of early life exposure to microbial agents and indoor factors on eosinophilic airway inflammation have not been explored. In our study, we assessed the associations of microbial agents (endotoxin, EPS and β(1,3)-D-glucans) in house dust, reported dampness and pet ownership early in life with FeNO in school-aged children in three European birth cohorts participating in the European HITEA project (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches).

Methods

Study population and design: description of the three birth cohorts.

As part of the European HITEA project, the present study includes information from three ongoing European birth cohorts that started between 1996 and 1999: LISA (influence of life-style factors on the development of the immune system and allergies in East and West Germany) in Germany,[56] PIAMA (Prevention and Incidence of Asthma and Mite Allergy) in the Netherlands,[133] and INMA (INfancia y Medio Ambiente [Environment and Childhood]) in Spain.[134] Written informed consent was obtained from all parents and the studies were approved by the local ethics committees in each cohort region. A description of the participating birth cohorts is given in the supplementary material and in the paper describing the exposures under study.[165] The present study includes children with measured endotoxin, EPS and/or β(1,3)-D-glucans in living room dust collected during early life and FeNO measurements at school age (182 from LISA, 244 from PIAMA, and 355 from INMA).

Fractional exhaled Nitric Oxide (FeNO)

FeNO was measured in LISA at 10 years of age, in PIAMA at 8 years of age and in INMA at 10 to 13 years of age, according to the American Thoracic Society guidelines[166] using the NIOX MINO® (Aerocrine, Solna, Sweden; http://www.aerocrine.com).

Dust collection, extraction and analyses

Living room dust samples were collected at the child's age of 2-3 months in the homes of the participants, using vacuum cleaners equipped with ALK filterholders containing a paper filter and the date of sampling was recorded. Samples were collected on living room floors in LISA and PIAMA, and on living room sofa in INMA. Collected samples were stored at -20ºC, and analyzed for microbial agents at in the Institute for Risk Assessment Sciences (IRAS, Utrecht, NL). Endotoxin has been determined with the Limulus Amebocyte Lysate test and glucan and EPS with specific enzyme immunoassays as in previous house dust analyses.[10] Levels were expressed in Endotoxin Units (EU), EPS Units (EPSU), and μg of β(1,3)-D-glucan per mL, with as lower limit of detection (LOD) 10 EU/mL (2 EU/mL in INMA), 180 EPSU/mL, and 2 μg/mL, respectively, and converted into concentrations in the original dust samples (in EU/mg, EPSU/mg and μg/mg). Samples with non-detectable amounts of endotoxin,

glucans and EPS were assigned a value of 2/3 of the LOD. Detailed information on the dust collection, extraction and analyses and a description of the values below the LOD is shown elsewhere.[165]

Reported indoor factors: dampness at home and cat and dog ownership

Questions about housing characteristics and potential exposures in the home environment were taken from questionnaires administered to the parents from birth to the child's age of 8 (PIAMA) and 10 years (LISA and INMA). Dampness at home was reported by the parents at the child's ages of 3 months, 1 and 2 years in the three cohorts. We combined these data into a single binary exposure variable on ever reported dampness during the first 2 years of life. Cat and dog ownership was reported at birth and at the child's ages of 1, 2, 4, 6 and 10 (8 in PIAMA) years in the three cohorts. For the statistical analyses, we computed separate 3-category variables for cat and for dog, describing the timing of the first cat/dog ownership. These variables indicated "Never" (no report of ownership), "Ever during the first 2 years of life" (at least one report of ownership in the first 2 years of life), and "Ever after the first 2 years of life, but not during the first 2 years of life" (at least one report of ownership after the first 2 years of life, but no report during the first 2 years) exposure.

Potential confounders and effect modifiers

The questionnaires administered during pregnancy or at birth and at the child's ages of 1, 2, 4, 6 and 10 (8 in PIAMA) years included socio-demographic, health and environmental data such as: parental education (low, medium or high); medical data of the parents; parental smoking; housing characteristics including the location of the home (area population density) and moving to another home. Information about the child's allergic and respiratory health was obtained from parental reports of hay fever, rhinitis, eczema, wheezing, asthma medication and doctor diagnosed asthma. Two new variables, one for asthma and another including reported allergies, were computed and considered as potential effect modifiers. Regarding asthma, cases were children with report of two out of three of the following questions per survey: doctor diagnosed asthma or wheezing or asthma medication reports from 4 to 10 (8 in PIAMA) years of age. For the reported allergies variable, cases were children with at least one report per survey of the mentioned questions on hay fever, rhinitis and eczema from 4 to 10 (8 in PIAMA).

Statistical analyses

The distribution of the FeNO, endotoxin, EPS and glucan measurements was right-skewed and therefore their levels were natural log-transformed. Generalized additive models (GAM)[167] were used to assess the functional relationship between the concentrations of microbial agents and FeNO stratified per cohort and in cohort-adjusted pooled analyses. With descriptive propouses, in bivariate analyses, we computed quartiles of endotoxin concentrations and tertiles of EPS and glucan concentrations to show the distribution of FeNO according to the levels of microbial agents. A description of the quantiles is shown in Table 1 of the online supplement. Nevertheless, the main analyses are conducted using the microbial agents concentrations as continuous variables.

To assess the adjusted associations between FeNO and the exposure variables by cohort, we performed multivariable linear regression models. Potential confounders were a-priori identified from the literature and selected based on their relationship with FeNO and the exposure variables in the present study. The concentration of each microbial agent (endotoxin, EPS and glucans) and target indoor factor (dampness and cat and dog ownership) was included separately in the models. In order to facilitate interpretation, the resulting regression coefficients for categorical variables were back-transformed to their exponential (exp(β)), yielding the ratio of the GM of FeNO of each exposure category vs the reference category. For continuous natural log-transformed variables, we present the coefficients (β) in the tables. To facilitate the interpretation of these coefficients in the text, we transformed the coefficients to $(2^\beta-1)*100$. This transformation allows interpreting the coefficient as the percentage of increase in FeNO when doubling the dose of each microbial agent. In order to assess the possibility of effect modification of studied associations with the report of allergy, we included interaction terms between the report of allergy and the exposure variables and we performed stratified analyses.

Finally, we performed random-effects meta-analyses including the three cohort-specific estimates. We obtained combined GM ratios and their CI for each microbial agent and indoor factor. Potential heterogeneity between cohorts was examined using the Cochran Q statistics (p-value<0.05). Data analysis was conducted with STATA SE 10.0 statistical software (Stata Corporation, College Station, TX, USA).

Results

A description of the socio-demographic, respiratory health and indoor exposure characteristics of the HITEA participants included and not included in this study is shown in Table 1. Children included were not different from those not included regarding most characteristics. Across the participating cohorts, we observed statistically significant differences for all variables except sex and doctor diagnosed asthma. Higher parental education was observed in the LISA cohort, higher asthma prevalence in PIAMA and higher smoking report in INMA compared to the other cohorts. The percentage of children with allergy report was higher in the PIAMA cohort whereas the age at FeNO measurement and FeNO was lower. Regarding the exposure variables, in INMA, endotoxin concentrations were lower and the prevalence of dampness and dog ownership in the first two years of life was higher. Cat ownership in the first two years of life was higher in PIAMA.

Geometric means of FeNO were lower in individuals classified in the highest endotoxin quartile in INMA, FeNO was lower in the mid tertile of EPS in LISA and INMA and similar trends were observed for glucans in LISA. Children exposed to cats and to dogs in the first two years had lower FeNO, except for cats exposure in LISA. However the differences were not statistically significant (p-values≥0.05) (Table 2).

Sex, age at FeNO measurement, asthma, reported allergies, indoor smoking and parental education were associated with FeNO after mutual adjustment (p-values<0.05) and thus included in the multivariate models. Parental education was also associated with the levels of indoor microbial agents, asthma and indoor smoking with pet ownership, and indoor smoking was additionally associated with reported dampness.

Table 1. Description of the HITEA project participants from the LISA, PIAMA and INMA birth cohorts, not included and included in the study.

	LISA		PIAMA		INMA	
	Not included n=213	Included n=182	Not included n=309	Included n=244	Not included n=126	Included n=355
Population characteristics						
Sex (female): n(%)	104 (48.8)	76 (41.8)	139 (45)	127 (52.1)	57 (45.2)	177 (49.9)
Age at FeNO measurement (years): mean (sd)	-	10.2 (0.2)	-	8 (0.3)	-	11.5 (0.7)
FeNO (ppb): GM (Gsd)	-	14.7 (2)	-	10.2 (2)	-	12.4 (2.3)
Asthma (4 to 10 years old*): n (%)	9 (8.9)	10 (6.1)	20 (14.5)	19 (10.6)	33 (10.3)	41 (10.8)
Reported allergies (4 to 10 years old*): n (%)[ac]	50 (39.4)	46 (27.1)	64 (41.6)	86 (39.8)	41 (51.3)	117 (33.9)
Parental education: n (%)[c]						
Secondary school	38 (18.4)	20 (11.4)	98 (37.3)	80 (33.3)	38 (31.7)	130 (38.4)
Higher than secondary school	159 (76.8)	148 (84.6)	131 (49.8)	140 (58.3)	12 (10)	67 (19.8)
Parental asthma: n (%)	25 (12.3)	21 (11.9)	93 (30.8)	84 (35)	17 (14.1)	34 (9.6)
Parental smoking at the age of 10 years*: n (%)	9 (8.7)	11 (6.4)	9 (6.1)	10 (5)	28 (45.2)	115 (35.8)
Indoor exposures						
Endotoxin concentrations (EU/mg): GM (Gsd)	22.6 (2.5)	23.5 (2.4)	23.2 (3.7)	20.6 (3.2)	4 (6.3)	3.1 (6.4)
EPS concentrations (U/mg): GM (Gsd)	46.6 (2.5)	48.4 (2.1)	29.1 (2.8)	32.9 (3.5)	119.9 (2.7)	116.4 (2.5)
Glucan concentrations (ug/mg): GM (Gsd)[b]	1.9 (1.7)	2 (1.7)	1.6 (2.1)	1.8 (2.4)	-	-
Dampness at home (first 2 years of life)[b]	74 (35.1)	62 (34.3)	159 (64.9)	121 (54.8)	72 (57.6)	221 (63.3)
Cat ownership: n (%)[b]						
Ever during the first 2 years of life	25 (20.2)	23 (13.8)	79 (42)	59 (27.2)	15 (20.3)	68 (19.3)
Ever after the first 2 years of life	17 (13.7)	26 (15.6)	12 (6.4)	19 (8.8)	10 (13.5)	30 (8.5)
Dog ownership: n (%)[abc]						
Ever during the first 2 years of life	16 (13.5)	8 (5)	51 (28.5)	31 (14.5)	45 (47.9)	143 (40.9)
Ever after the first 2 years of life	16 (13.5)	16 (10.1)	32 (17.9)	16 (7.5)	19 (20.2)	45 (12.9)

sd: standard deviation; GM: geometric mean; Gsd: geometric standard deviation; FeNO: fractional exhaled nitric oxide; Reported allergies: eczema, rhinitis or hay fever. *8 years in PIAMA; [a] p-value<0.05 of the difference between included and not included in LISA; [b] p-value<0.05 of the difference between included and not included in PIAMA; [c] p-value<0.05 of the difference between included and not included in INMA

Table 2. Description of the the FeNO (ppb) according to the exposure per cohort (geometric mean (geometric standard deviation)).

	LISA	PIAMA	INMA
	n=182	n=244	n=355
	GM (Gsd)	GM (Gsd)	GM (Gsd)
Endotoxin concentrations (EU/mg)			
Q1 (<3.54 EU/mg)	12.6 (1.4)	7.8 (1.5)	12.3 (2.4)
Q2 (>3.54-12.4 EU/mg)	17.2 (1.9)	11.5 (2.2)	13 (2.2)
Q3 (>12.4-30.6 EU/mg)	14.3 (2.0)	10.1 (1.9)	11.8 (2.1)
Q4 (>30.6 EU/mg)	14.4 (2.0)	9.7 (1.8)	11.5 (2.2)
EPS concentrations (U/mg)			
<LOD	14.2 (2.3)	9.5 (2.0)	10.4 (1.8)
T1 (<42 U/mg)	15.2 (2.0)	10.6 (2)	11.8 (2.3)
T2 (>42-101 U/mg)	13.8 (1.9)	9.9 (1.9)	12.1 (2.1)
T3 (>101 U/mg)	16.2 (2.2)	12.5 (1.8)	13.2 (2.4)
Glucan concentrations (ug/mg)			
<LOD	-	9.4 (2.1)	-
T1 (<1.55 µg/mg)	15.8 (2.0)	10.9 (1.9)	-
T2 (>1.55-2.35 µg/mg)	13.6 (1.9)	10.3 (1.9)	-
T3 (>2.35 µg/mg)	15.4 (2.1)	10.7 (1.8)	-
Dampness at home			
Never	14.9 (2.0)	10.7 (1.9)	12.9 (2.3)
Ever during the first 2 years of life	14.3 (2.1)	10 (2.0)	12.1 (2.3)
Cat ownership			
Never	15.5 (2.0)	10.6 (1.9)	12.7 (2.3)
Ever during the first 2 years of life	16.1 (2.1)	9.7 (1.8)	10.8 (2.2)
Ever after the first 2 years of life	11.4 (1.7)	9.9 (2.4)	15.1 (2.5)
Dog ownership			
Never	15.1 (2.0)	10.5 (2.0)	13.3 (2.4)
Ever during the first 2 years of life	13.4 (1.6)	9 (1.8)	11.6 (2.3)
Ever after the first 2 years of life	15.5 (1.6)	10.9 (2.1)	12.3 (2.1)

Q: quartile; T: tertile; EPS: extracellular polysaccharides; <LOD: below the limit of detection

The smooth association between FeNO and the microbial agents concentrations, per cohort and combined, showed that the associations were linear (p-values>0.05 in all cases). The results from the GAM plots for microbial agents concentrations are shown in Figure 1.

Figure 1. Smoothed association between log transformed FeNO coefficients and log transformed microbial agents concentrations by cohort and pooled (Combined). Adjusted for sex, age of FeNO measurement, asthma, reported allergies (hay fever, rhinitis or eczema), parental smoking, parental education and season of dust sampling. The EPS and glucan graphs were additionally adjusted for the values below the limit of detection. The Combined graph was additionally adjusted for cohort.
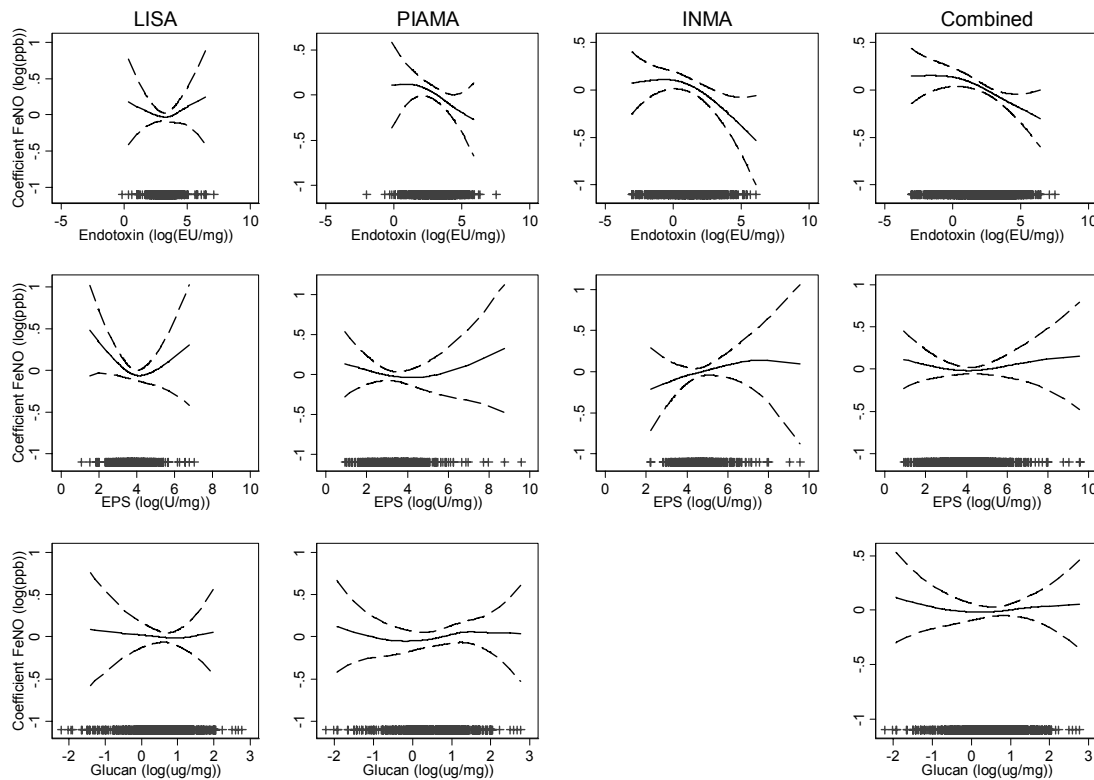
The combined random-effects adjusted coefficients for microbial agents concentrations were statistically significant for endotoxin, showing a 3.7% decrease in FeNO when doubling the dose of endotoxin (β=-0.054; 95% CI:-0.97; -0.01). In addition, combined random-effects adjusted GM ratios of FeNO were lower for individuals with reported dog ownership during the first 2 years of life. Association estimates for EPS, glucan, dampness and cat ownership were not statistically significant (p-values≥0.05). P-values for heterogeneity were above 0.1 in all cases (Table 3).

Table 3. Fractional exhaled nitric oxide (FeNO) adjusted associations per cohort and combined random-effects estimates resulting from meta-analyses for the total study population and stratified by reported allergies.

	LISA	PIAMA	INMA	Combined (random effects)		
				Total	Not reported allergies	Reported allergies
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Microbial agents concentrations						
Endotoxin (log(EU/mg))	0.01 (-0.11; 0.13)	-0.08 (-0.17; 0.01)	-0.06 (-0.11; 0)	-0.05 (-0.1; -0.01)	-0.06 (-0.11; -0.01)	-0.06 (-0.14; 0.03)
EPS (log(U/mg))	-0.03 (-0.18; 0.13)	0.01 (-0.1; 0.11)	0.06 (-0.05; 0.17)	0.02 (-0.05; 0.09)	0.08 (0; 0.17)	0.09 (-0.05; 0.22)
Glucan (log(μg/mg))	-0.07 (-0.28; 0.15)	0.02 (-0.1; 0.13)	-	0 (-0.1; 0.1)	0 (-0.21; 0.21)	0.04 (-0.15; 0.24)
	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)
Dampness at home						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	1.01 (0.8-1.26)	0.89 (0.73-1.1)	1.03 (0.85-1.24)	0.97 (0.87-1.1)	0.93 (0.81-1.06)	1.14 (0.8-1.63)
Cat ownership						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	0.97 (0.71-1.33)	0.89 (0.7-1.13)	0.83 (0.65-1.06)	0.89 (0.76-1.03)	0.98 (0.78-1.22)	0.72 (0.51-1.04)
Ever after the first 2 years of life	0.87 (0.64-1.19)	0.96 (0.65-1.43)	1.22 (0.88-1.69)	1.01 (0.82-1.24)	1.11 (0.9-1.37)	0.83 (0.52-1.32)
Dog ownership						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	1.04 (0.65-1.68)	0.89 (0.66-1.2)	0.75 (0.62-0.92)	0.82 (0.7-0.96)	0.88 (0.74-1.04)	0.69 (0.51-0.94)
Ever after the first 2 years of life	0.83 (0.57-1.21)	1.18 (0.8-1.76)	1 (0.75-1.33)	0.99 (0.81-1.21)	1.02 (0.82-1.27)	1.28 (0.5-3.29)

Adjusted for sex, age of FeNO measurement, asthma, reported allergies, parental smoking, and parental education. Models including endotoxin, EPS and glucan measurements were additionally adjusted for season of dust sampling. Models including EPS and glucan were additionally adjusted for values below the limit of detection. Models stratified by reported allergies were not adjusted for reported allergies. All the heterogeneity tests were not significant (p-values≥0.05)

The association estimates obtained for each exposure variable were homogeneous across cohorts (heterogeneity p-values≥0.05). Stratification by child reported allergy did not show significant differences (Table 2, online supplement). Moreover, adjusted GM ratios in non-asthmatic population (Table 3, online supplement) showed similar results as those presented in Table 3.

Discussion

Higher endotoxin concentrations and presence of dogs in the home during early life were associated with lower FeNO at school age. We investigated the association of FeNO as a biomarker of eosinophilic airway inflammation and microbial exposure variables, independently of any association with asthma or atopy. Our results do not suggest association with disease, but pertain to potential long-term effects of early life exposures on the immune system. Experimental studies in murines previously showed that endotoxin and allergen exposure during pregnancy may modulate airway inflammation in offspring later in life, resulting in reduced allergen-induced immune and airway responses.[168,169]

We observed that higher endotoxin exposure levels and dog ownership were associated with lower FeNO, but this was not the case for EPS and glucan exposure, cat ownership and reported home dampness. This is in line with previous epidemiological studies focusing on the health effects of residential exposure to microbial agents and pets in children. These studies mainly evaluated the associations with respiratory symptoms, asthma and atopy. Most previous studies reported that endotoxin may protect against allergen sensitization, eczema, wheezing and asthma later in life.[57,60,142,170] Similarly, no statistically significant associations have been found between exposure to fungal agents and respiratory symptoms in line with previous studies.[60,62] In addition, in line with our results, early life exposure to dogs was suggested to have protective effects against allergen sensitization and respiratory symptoms.[65,66,170]

Asthma and atopy are known to be associated with high FeNO in children.[158,171,172] Moreover, children with persistent and late onset wheezing phenotypes are more likely to show higher FeNO at school age[31] and have higher risk of atopy and asthma.[29,30] FeNO is one of the few tests that has diagnostic value in asthma, and its role in asthma monitoring remains to be defined.[173] In line with the previous studies, FeNO in our study was higher in asthmatic and allergic children (data not shown). However, we did

not find differences in the associations between endotoxin and dog exposures and FeNO according to the report of asthma or allergy.

Our longitudinal study is the first epidemiologic study assessing the long-term effects of early life exposure to indoor microbial agents and related indoor factors on FeNO measured at school age, as a biomarker of eosinophilic airway inflammation. Our study includes data from subpopulations of three European birth cohorts in three geographically spread locations and benefits from the availability of objective measurements of exposure during early life (2-3 months) and objective measurements of airway inflammation at school age, in addition to reported information periodically collected through questionnaires administered from pregnancy or birth to school age.

A few limitations must be considered when interpreting our results. The study populations involved in the HITEA project were selected differently depending on the cohort. Only subsamples of LISA and PIAMA cohorts were included in the project. Inclusion was based on the availability of dust samples. In LISA, children included in the present study never moved to another home since early life and lived only in high population density areas. In PIAMA, the HITEA project includes children from the intervention study (high risk children of allergic mothers). In addition, several differences existed between cohorts in subject characteristics and dust sampling and analyses. In descriptive analyses FeNO values and age were lowest in PIAMA, the cohort with the highest number of allergic children. Results from multivariable regression analyses showed that FeNO, after age- and reported allergy adjustment, were 40% lower in INMA compared to PIAMA (GM ratio=0.6; 95% CI: 0.4-0.9), and that FeNO in PIAMA children was not different from that in LISA children (GM ratio=0.9; 95% CI: 0.7-1.2). Regarding dust sampling and analyses, INMA dust samples were taken from sofa instead of floor because of the lack of carpets or rugs in the homes of the Menorca island. Furthermore, INMA samples were extracted in borate buffered saline (BBS) for earlier analysis of house dust mite allergens, which is not the standard extraction fluid used for endotoxin analyses, additionally the lack of heat-extraction impeded glucan quantification from these samples.

Overall, the association estimates differed somewhat by cohort. This might be explained by differences in inclusion criteria, in participants characteristics and in dust sampling and analyses. For these reasons, we explored to what extent our results are driven by only one or two cohorts by performing sensitivity analyses where we excluded one cohort at a time from the meta-analysis. The results from this sensitivity

analyses showed that the combined coefficients of endotoxin exposure and for early life dog ownership are statistically significant when excluding LISA from the meta-analyses but not significant when excluding PIAMA or INMA. Nevertheless, the magnitude and direction of the coefficients do not change. The number of individuals included in the LISA cohort is lower and population in this cohort than in the other cohorts. Therefore, the lack of statistical significance in the estimates when including LISA and only one of the other participating cohorts may be related to the statistical power and with the narrow range of exposures in LISA. In addition, all LISA homes were located in a high density area while in the PIAMA and INMA cohorts the density of the area where homes were located varied. This may also explain the differences observed in the coefficients for endotoxin exposure and dog ownership.

Apart from the differences across cohorts, other limitations related with the associations between the exposures, the outcome and the respiratory symptoms and allergy should be considered. FeNO is associated with atopy and asthma.[154,155,158,171,172] On the other hand, asthma and atopy may be associated with early life exposure to microbial agents and related indoor factors.[59–61] In order to assess the possibility of effect modification by reported allergy, we included interaction terms between reported allergy and the exposure variables in the models. Associations for the interaction terms between reported allergy and endotoxin and dog ownership were not statistically significant (p-values>0.1). Moreover, stratified analyses did not show differences in the effect estimates according to the disease group. However, because of the limited power after stratification, these results must be cautiously interpreted.

In conclusion, exposure to high concentrations of bacterial endotoxin in the first months of life and dog ownership during the first two years of life, but not exposure to fungal microbial agents or indoor dampness, was associated with FeNO at school age. Further studies are needed to assess how this association is biologically explained, and what the clinical relevance could be.

Acknowledgments

Supplementary material

Description of the study population

LISA. In the city of Munich (Germany), a total number of 1467 neonates were recruited between December 1997 and January 1999. Living room floor dust samples were collected at the child's age of 3 months and information regarding child's health was obtained through yearly questionnaires and health exams from birth to the age of 4 and at the child's ages of 6 and 10 years. The HITEA project included a randomly selected sample of those LISA participants with a high response rate from birth to the age of 10 years and with living room dust samples available (n=448). From the selected children, 182 had FeNO measurements at 10 years of age.

PIAMA. The PIAMA study is conducted in the Netherlands and consists of a natural history cohort and an intervention study. Early life microbial exposure was only assessed in the intervention study, as described previously.[60] The HITEA study thus included children from the intervention study only. In this part of the study, mothers with self-reported allergies, asthma or both were invited to participate. Of the 810 participants: one group (intervention group) was supplied with mite-impermeable mattress and pillow covers and the other (placebo group) was supplied with placebo cotton covers for use both on the infants' and their parents' beds. Samples for microbial analysis were obtained from 696 participants, nicely divided over the intervention (51%) and control (49%) group. Living room floor dust samples were taken at the age of 2-3 months. Information regarding child's health was obtained through yearly questionnaires and health exams from birth to the age of 4 and at the child's ages of 6, and 8 years. FeNO was measured to 244 children aged 8 years approximately and with early life dust samples.

INMA. In Menorca island (Spain), all pregnancies from the general population were selected at the third trimester between September 1997 and January1999 (n=486). Living room sofa dust samples were taken at the age of 2-3 months. Information regarding child's health was obtained through yearly questionnaires and health exams from birth to the age of 4 and at the child's ages of 6 and 10 years. All participants in the INMA-Menorca cohort with living room dust samples were included in the HITEA project (n=474). FeNO was measured in 355 participants aged 10 to 13 years.

Supplementary tables

Table 1. Description (number and percentage) of the quantiles of microbial agents concentrations in the study population.

	LISA n=182 n(%)	PIAMA n=244 n(%)	INMA n=355 n(%)	Total n=781 n(%)
Endotoxin concentrations (EU/mg)				
Q1 (<3.54 EU/mg)	4 (2.2)	12 (5.2)	174 (50.7)	190 (25.1)
Q2 (>3.54-12.4 EU/mg)	30 (16.5)	66 (28.3)	93 (27.1)	189 (24.9)
Q3 (>12.4-30.6 EU/mg)	82 (45.1)	67 (28.8)	41 (12.0)	190 (25.1)
Q4 (>30.6 EU/mg)	66 (36.3)	88 (37.8)	35 (10.2)	189 (24.9)
EPS concentrations (U/mg)				
<LOD	7 (3.9)	69 (30.1)	10 (3.0)	86 (11.5)
T1 (<42 U/mg)	70 (38.5)	69 (30.1)	31 (9.1)	170 (22.7)
T2 (>42-101 U/mg)	77 (42.3)	60 (26.2)	112 (33)	249 (33.2)
T3 (>101 U/mg)	28 (15.4)	31 (13.5)	186 (54.9)	245 (32.7)
Glucan concentrations (ug/mg)				
<LOD	0 (0)	92 (40.4)	-	92 (22.4)
T1 (<1.55 µg/mg)	47 (25.8)	59 (25.9)	-	106 (25.9)
T2 (>1.55-2.35 µg/mg)	75 (41.2)	31 (13.6)	-	106 (25.9)
T3 (>2.35 µg/mg)	60 (33)	46 (20.2)	-	106 (25.9)

Q: quartiles; T: tertiles, EPS: extracellular polysaccharides, <LOD: below the limit of detection

Table 2. Fractional exhaled nitric oxide adjusted associations per cohort stratified by reported allergy (ever rhinitis, hay fever, or eczema).

	LISA		PIAMA		INMA	
	Not reported allergies n=124	Reported allergies n=46	Not reported allergies n=130	Reported allergies n=86	Not reported allergies n=228	Reported allergies n=117
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Microbial agents concentrations[a]						
Endotoxin (log(EU/mg))	-0.06 (-0.2; 0.08)	0.23 (-0.06; 0.51)	-0.03 (-0.13; 0.07)	-0.16 (-0.32; 0)	-0.07 (-0.13; 0)	-0.03 (-0.13; 0.08)
EPS (log(U/mg))	0.02 (-0.16; 0.2)	-0.04 (-0.43; 0.36)	0.05 (-0.09; 0.19)	0.01 (-0.19; 0.21)	0.15 (0.02; 0.28)	-0.04 (-0.26; 0.18)
Glucan (log(μg/mg))	-0.13 (-0.39; 0.12)	-0.09 (-0.55; 0.36)	0.09 (-0.05; 0.22)	-0.08 (-0.3; 0.15)	-	-
	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)
Dampness at home						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	0.97 (0.75-1.25)	1.17 (0.71-1.93)	0.95 (0.76-1.19)	0.83 (0.55-1.26)	0.85 (0.67-1.09)	1.49 (1.04-2.13)
Cat ownership						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	1.03 (0.74-1.43)	0.93 (0.31-2.73)	1.14 (0.88-1.46)	0.54 (0.32-0.89)	0.78 (0.58-1.06)	0.88 (0.56-1.39)
Ever after the first 2 years of life	0.99 (0.72-1.37)	0.55 (0.18-1.7)	1.19 (0.77-1.84)	0.65 (0.31-1.35)	1.22 (0.83-1.79)	1.18 (0.63-2.24)
Dog ownership						
Never	1	1	1	1	1	1
Ever during the first 2 years of life	1.05 (0.67-1.65)	-	0.95 (0.69-1.31)	0.73 (0.42-1.28)	0.79 (0.63-1.01)	0.68 (0.47-0.98)
Ever after the first 2 years of life	1.03 (0.64-1.64)	0.67 (0.33-1.37)	0.99 (0.69-1.42)	6.69 (1.64-27.28)	1.05 (0.73-1.49)	0.9 (0.54-1.49)

Adjusted for sex, age of FeNO measurement, asthma, parental smoking, and parental education. Models including microbial agents measurements were additionally adjusted for season of dust sampling. Models including EPS and glucans were adjusted for values below the limit of detection.

Table 3. Fractional exhaled nitric oxide adjusted associations per cohort in children without reported asthma.

	LISA	PIAMA	INMA	Combined (random effects)
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Microbial agents concentrationsa				
Endotoxin (log(EU/mg))	-0.02 (-0.14; 0.1)	-0.06 (-0.15; 0.03)	-0.05 (-0.1; 0.01)	-0.05 (-0.09; 0)
EPS (log(U/mg))	-0.05 (-0.2; 0.1)	-0.03 (-0.15; 0.09)	0.08 (-0.03; 0.19)	0.01 (-0.07; 0.09)
Glucan (log(µg/mg))	-0.09 (-0.3; 0.13)	0.01 (-0.11; 0.13)	-0.21 (-0.51; 0.09)	-0.02 (-0.12; 0.09)
	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)	GM ratio (95%CI)
Dampness at home				
Never	1	1	1	1
Ever during the first 2 years of life	1.06 (0.85-1.33)	0.89 (0.72-1.1)	0.97 (0.8-1.18)	0.97 (0.86-1.09)
Cat ownership				
Never	1	1	1	1
Ever during the first 2 years of life	0.99 (0.73-1.34)	0.92 (0.71-1.19)	0.9 (0.7-1.17)	1 (0.82-1.21)
Ever after the first 2 years of life	0.92 (0.68-1.26)	0.97 (0.65-1.44)	1.12 (0.8-1.56)	0.84 (0.72-0.98)
Dog ownership				
Never	1	1	1	1
Ever during the first 2 years of life	1.07 (0.68-1.69)	0.88 (0.65-1.21)	0.78 (0.64-0.95)	0.84 (0.72-0.98)
Ever after the first 2 years of life	1 (0.68-1.48)	1.17 (0.79-1.74)	1.08 (0.81-1.44)	1.08 (0.89-1.32)

Adjusted for sex, age of FeNO measurement, reported allergies, parental smoking, and parental education. Models including microbial agents measurements were additionally adjusted for season of dust sampling. Models including EPS and glucans were adjusted for values below the limit of detection..

6.3 PAPER III

The use of household cleaning products during pregnancy and lower respiratory tract infections and wheezing during early life.

Casas L, Zock JP, Carsin AE, Fernandez-Somoano A, Esplugues A, Santa-Marina L, Tardón A, Ballester F, Basterrechea M, Sunyer J.

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6.4 PAPER IV

Use of household cleaning products, exhaled nitric oxide and lung function in children

Casas L, Zock JP, Torrent M, García-Esteban R, Gracia-Lavedan E, Hyvärinen A, Sunyer J.

[Submitted][*]

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Use of household cleaning products, exhaled nitric oxide and lung function in children

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Abstract

We aimed to study the effects of the use of 10 cleaning products on fractional exhaled nitric oxide (FeNO) and spirometric lung function during childhood.

In a Spanish birth cohort, parents reported the frequency of use of household cleaning products. FeNO was measured and Forced Vital Capacity (FVC) and Forced Expiratory Volume in the first second (FEV1) were determined by spirometry when the child was 10-13 years old. Associations between the use of specific products and respiratory outcomes were evaluated using multivariable regression analyses.

FeNO was higher when cleaning and/or air refreshing sprays were used at least once a week (Geometric Mean (GM) ratio 1.5; 95% confidence interval (CI) 1.2-1.9). FVC and FEV1 tended to be lower when different types of cleaning products were used. FVC was 135 mL (CI 17-252) lower when solvents were frequently used and FEV1 was 98 mL (CI 9-187) lower when air refreshing sprays were used. Findings were similar in children without allergies or asthma.

In conclusion, the use of household cleaning agents including different sprays and solvents may have an adverse effect on airways inflammation and lung function in school-age children. Further studies are required to identify the underlying pathophysiological effect mechanisms.

Keywords: Airway inflammation; childhood; cohort studies; household chemicals; indoor air; spirometry.

Introduction

Household cleaning products are used worldwide to maintain appropriate hygienic conditions of our homes, including the reduction of indoor exposure to microbial agents.[11] However, the use of cleaning products contributes to the exposure to chemicals.[18] There is vast evidence that occupational exposure to cleaning chemicals has adverse effects on respiratory health [82]. In addition, domestic cleaning agents use increases the risk of asthma and respiratory symptoms,[87] and aggravates asthma in adults,[86] in particular when products in spray-form are applied.[14,88]

Despite the findings from studies in adults and the wide-spread use of these products, the potential effects of passive exposure to cleaning chemicals on children's respiratory health have not been extensively explored. Two longitudinal analyses of data from the Avon Longitudinal Study of Parents and Children (ALSPAC) suggested that frequent use of chemical-based products, predominated by cleaning agents, during pregnancy increased the risk of persistent wheezing at school age.[89,90] However, they did not evaluate the effects of specific cleaning products but rather a composite score.

The PARIS (Pollution and Asthma Risk: an Infant Study) birth cohort suggested that cleaning products applied as spray increased the risk of wheezing in the first 18 months of life.[91] In addition, the four younger cohorts of the INMA – *INfancia y Medio Ambiente* [Environment and Childhood] project evaluated pre- and post-natal exposure to 12 commonly used cleaning products. This study showed that the use of sprays, solvents and air fresheners increased the risk of lower respiratory tract infections and wheezing during the first year of life.[181] Finally, a Belgian study reported protective effects of bleach use at home on the prevalence of asthma and allergic sensitization at school age [92].

Thus, the independent effects of several cleaning products have only been evaluated in one study.[181] However, in this study respiratory health outcomes were restricted to the first year of life and not confirmed by objectively measured respiratory health indicators. In our study, we aimed to assess the effects of the use of 10 common cleaning products on the fractional exhaled nitric oxide (FeNO) indicative of eosinophilic airway inflammation and on spirometric lung function during childhood in a Spanish birth cohort.

Methods

Study design and population

Our study is based on a population-based birth cohort in Menorca Island. This cohort was established within the Asthma Multicenter Infant Cohort Study,[134] is part of the INMA project[131] and is involved in the European HITEA project.[165] Pregnant women were enrolled during pregnancy at public primary health care centres or public hospitals in the island over a 12-month period starting in mid-1997. A total of 482 children (94% of those eligible) were enrolled at birth. Written informed consent was obtained from all participants and the study was approved by the Ethics Committees of the Institut Municipal d'Investigació Mèdica, Barcelona.

Interviewer-led questionnaires on the use of household cleaning products, and FeNO and lung function tests were administered once when the child was 10-13 years old. Health questionnaires were administered repeatedly from birth to 10 years of age. A total of 295 individuals (61%) completed the 10 year follow up visit and had information on the use of household cleaning products, FeNO and/or lung function. Children included were not different from those not included regarding most characteristics (sex, atopy, asthma, parental asthma and parental smoking at home). However, mothers of participants were more likely to have had a higher education and higher concentrations of endotoxin in house dust in early life (data not shown).

The use of household cleaning products

The questionnaire administered at 10-13 years of age included questions about the use of domestic cleaning products that are common in Spain: bleach, ammonia, polishes or waxes (for floor or furniture), acids (including decalcifiers and liquid scale removers), solvents (including stain removers), furniture sprays, glass cleaning sprays, degreasing sprays (including oven cleaning sprays), air refreshing sprays, and air refreshing plug-in devices (or other electric air fresheners). Information on the frequency of use of each cleaning product was obtained (never, <1day/week, 1-3days/ week and 4-7days/week).

We computed dichotomous variables for each cleaning product (<1day/week and ≥1day/week). We used a combined spray variable comprising furniture sprays, glass cleaning sprays, degreasing sprays, and air refreshing sprays. This approach is further justified by the fact that the application of cleaning products through spraying is

associated with higher indoor total volatile organic compounds concentrations [18] and is likely to facilitate respiratory exposure [14]. Finally, we computed a frequency score for each product being the mean of the reported days per week (never=0, <1day/week=0.5, 1- 3days/week=2 and 4-7days/week=5.5). The frequency scores for all products were summed providing a semi-quantitative total score for cleaning products use ranging from 0 (no exposure) to 55 (expected to all 10 products 4-7days/week).

Exhaled nitric oxide and lung function

FeNO was measured and forced spirometry testing was performed once at the child's age of 10-13 years. FeNO Measurements were performed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines[166] using the NIOX MINO®, a handheld device to measure FeNO at a constant expiratory flow of 50 mL/s. Lung function was assessed according to the ATS/ERS guidelines[182] using an NDD EasyOne® spirometer. Forced Vital Capacity (FVC) and Forced Expiratory Volume in the first second (FEV1) were obtained and expressed in L at body temperature and ambient pressure saturated with water vapour. Differences between exposed and non-exposed groups were expressed in mL. Standing height and weight were measured during the visit.

Potential confounders and effect modifiers

The questionnaires administered during pregnancy or at birth and at the child's ages of 1, 2, 4, 6 and 10 years included socio-demographic, health and environmental data such as: maternal education (primary school or less, secondary school or university), parental asthma and parental smoking at home. Data on child's allergic and respiratory health were obtained from parental report of hay fever, rhinitis, eczema, wheezing, asthma medication and doctor diagnosed asthma.

We created new variables for lifetime wheezing, lifetime asthma and lifetime reported allergies. For reported wheezing, children were classified as never wheeze, ever wheeze in the first four years of life but not later (transient early wheezing), and ever wheeze from 5 to 10 years of age (persistent and late onset of wheezing). This variable was based on that described by Martinez et al in the Tucson Children's Respiratory Study.[27] Cases of reported asthma from 4 to 10 years of age were considered when at

least two out of three of the following variables were reported from 4 to 10 years of age: wheezing, doctor diagnosed asthma and asthma medication. We defined allergy as at least one report of hay fever, rhinitis and eczema from the age of 4 years. These three variables were considered potential effect modifiers in the associations between exposure to cleaning products, FeNO and lung function.

Dust samples form the living room sofa had been taken at the child's age of 2-3 months and analysed for endotoxin with the Limulus Amebocyte Lysate test and a specific enzyme immunoassay.[10,165] Levels were expressed in Endotoxin Units (EU) per mL and converted into concentrations in the original dust samples (in EU/mg).

Statistical analyses

Multivariable linear regression models were developed to predict FeNO, FVC and FEV1. The distribution of FVC and FEV1 was normal, however the distribution of the FeNO measurements was right-skewed and therefore FeNO was natural log-transformed. The coefficients obtained from the log-FeNO linear models were exponentiated to obtain geometric mean (GM) ratios for FeNO values.

Potential confounders were *a priori* selected based on their known relationship with the outcomes and the exposure variables described in the literature. We used generalized additive models to assess the functional relationship between the outcomes and the potential confounders. No indication of non-linear relationships was observed. In order to assess the possibility of effect modification by children's lifetime wheezing, asthma or reported allergies, we performed stratified analyses and evaluated the p-value for interaction. All statistical analyses were conducted with STATA SE 10.0 statistical software (Stata Corporation, College Station, TX, USA).

Results

The main characteristics of the study population are shown in Table 1. The GM of FeNO was 13.7 ppb and the average lung function was 2.9L and 2.4L for FVC and FEV1, respectively. The prevalence of reported persistent or late onset wheezing was 14%, more than 1/3 of the participants reported ever hay fever, eczema or rhinitis from 4 years of age, and asthma from 4 to 10 years of age was reported for 9% of the children.

Table 1. Description of the study population (n=295)

	n (%)
Sex (female)	144 (49)
Age (years): mean (sd)	11.9 (0.4)
Maternal education	
Primary school or less	151 (52)
Secondary school	94 (32)
University	48 (16)
Parental asthma	30 (10)
Parental smoking indoors (10 years old)	96 (36)
Wheezing	
Early transient (birth to 4 years old)	55 (19)
Persistent or late onset (birth to 10 years old)	40 (14)
Reported allergies (4 to 10 years old)[a]	103 (36)
Reported asthma (4 to 10 years old)	27 (9)
FeNO (ppb): GM (Gsd)[a]	13.7 (2.2)
FEV1 (L): mean (sd)[b]	2.4 (0.4)
FVC (L): mean (sd)[b]	2.9 (0.5)

[a]Reported allergies: eczema, hay fever or rhinitis

The most commonly used product was bleach (weekly use in 2/3 of the homes). Glass cleaning sprays, furniture sprays and degreasing sprays were used ≥1day/week in more than 1/4 of the homes. Spray use ≥1day/week was reported in almost 3/4 of the homes. On the contrary, acids and ammonia were the least used cleaning agents (15% and 16%, respectively). FeNO was higher among children of homes, where acids, spray, and furniture sprays were used (p-values<0.05). FVC and FEV1 volumes were significantly lower (p-values<0.05) in children of homes of air refreshing spray users (Table 2).

FeNO GM ratios after adjustment were significantly higher for individuals exposed to sprays (GM ratio=1.5 CI:1.2; 1.9) and furniture sprays (GM ratio=1.3 CI:1.1; 1.6). Although the GM ratios were not statistically significant, the FeNO values after adjustment were 18 to 27% higher for children exposed to acids, solvents, glass cleaning and air refreshing sprays. There was an 8% increase in FeNO associated with a change of the score of days-product use over interquartile range of 6.5 days-product/week. FVC and FEV1 were lower when cleaning products were used at home. This was more apparent for the FEV1 than for the FVC. A lower FEV1 was associated with the use of all products except bleach. Nevertheless, although the associations were not statistically significant, both outcomes decreased when increasing from the 25th to the 75th percentile of the score of days-product use (39mL for FVC and 26mL for FEV1). The use of solvents was significantly associated with a 135 ml lower FVC

(95% CI -252 to -17) and the use of air refreshing sprays with a 98 ml lower FEV1 (95% CI -187 to -9) (Table 3).

Table 2. Distribution of fractional exhaled nitric oxide (FeNO) and lung function (FEV1 and FVC) according to the weekly use of cleaning products (n=295).

	n (%)	FeNO GM (Gsd)	FVC Mean (sd)	FEV1 Mean (sd)
Bleach				
< 1/week	98 (33)	13.1 (2.2)	2.87 (0.52)	2.42 (0.42)
≥ 1/week	197 (67)	14.0 (2.2)	2.88 (0.50)	2.46 (0.43)
Ammonia				
< 1/week	246 (84)	13.8 (2.2)	2.85 (0.51)	2.43 (0.44)
≥ 1/week	46 (16)	13.0 (2.4)	3.01 (0.50)	2.54 (0.37)
Polishes or waxes (for floor or furniture)				
< 1/week	218 (76)	14.0 (2.2)	2.90 (0.52)	2.45 (0.43)
≥ 1/week	69 (24)	12.4 (2.2)	2.82 (0.49)	2.44 (0.43)
Acids, including decalcifiers and liquid scale removers				
< 1/week	248 (85)	13.2 (2.1)	2.89 (0.50)	2.45 (0.42)
≥ 1/week	44 (15)	17.3 (2.4)*	2.86 (0.52)	2.42 (0.46)
Solvents, including stain removers				
< 1/week	242 (82)	13.4 (2.1)	2.90 (0.52)	2.46 (0.44)
≥ 1/week	52 (18)	15.3 (2.4)	2.75 (0.46)	2.39 (0.39)
Spray[a]				
< 1/week	79 (27)	10.5 (2.0)	2.87 (0.51)	2.46 (0.44)
≥ 1/week	209 (73)	14.8 (2.2)*	2.88 (0.51)	2.44 (0.42)
Furniture sprays				
< 1/week	183 (64)	12.5 (2.2)	2.88 (0.52)	2.47 (0.45)
≥ 1/week	104 (36)	15.6 (2.2)*	2.87 (0.50)	2.40 (0.39)
Glass cleaning sprays (for windows or mirrors)				
< 1/week	147 (52)	12.6 (2.1)	2.86 (0.49)	2.44 (0.43)
≥ 1/week	138 (48)	14.8 (2.3)	2.90 (0.53)	2.45 (0.43)
Degreasing sprays, including oven cleaning sprays				
< 1/week	205 (72)	13.2 (2.2)	2.89 (0.50)	2.46 (0.43)
≥ 1/week	81 (28)	14.5 (2.1)	2.87 (0.54)	2.44 (0.43)
Air refreshing sprays				
< 1/week	234 (80)	13.5 (2.2)	2.91 (0.51)	2.49 (0.43)
≥ 1/week	57 (20)	14.2 (2.0)	2.75 (0.50)*	2.29 (0.41)*
Plug-in or other electric air fresheners				
< 1/week	220 (75)	13.8 (2.3)	2.88 (0.50)	2.45 (0.43)
≥ 1/week	75 (25)	13.3 (1.9)	2.87 (0.53)	2.42 (0.43)
Score of days-product use				
0.5 to 5.5 days-product/week	71 (27)	12.0 (2.3)	2.97 (0.51)	2.52 (0.43)
6 to 8 days-product/week	63 (24)	15.4 (2.3)	2.81 (0.48)	2.40 (0.41)
8.5 to 12 days-product/week	62 (24)	13.0 (2.3)	2.83 (0.52)	2.45 (0.45)
12.5 to 32.5 days-product/week	64 (25)	13.5 (1.9)	2.92 (0.56)	2.45 (0.46)

FeNO: Fractional exhaled Nitric Oxide; FVC: Forced Vital Capacity; FEV1: Forced Expiratory Volume in the first second. [a]Spray: furniture, glass cleaning, degreasing or air refreshing sprays
*p-value<0.05

Table 3. Adjusted[a] associations between FeNO (geometric mean (GM) ratios), FVC and FEV1 (β coefficients) and the use of cleaning products (reference: less than once per week).

	FeNO (ppb) GM ratio (95% CI)	FVC (ml) β (95% CI)	FEV1 (ml) β (95% CI)
Bleach	1.08 (0.87; 1.34)	5 (-90; 100)	47 (-29; 123)
Ammonia	0.94 (0.70; 1.24)	6 (-122; 133)	-35 (-136; 67)
Polishes or waxes (for floor or furniture)	0.84 (0.66; 1.07)	-94 (-202; 15)	-22 (-109; 65)
Acids, including decalcifiers and liquid scale removers	1.27 (0.95; 1.69)	-72 (-199; 55)	-72 (-173; 29)
Solvents, including stain removers	1.18 (0.91; 1.54)	-135 (-252; -17)	-52 (-146; 42)
Spray[b]	1.52 (1.20; 1.91)	12 (-92; 117)	-35 (-119; 48)
Furniture sprays	1.31 (1.06; 1.62)	21 (-74; 117)	-46 (-122; 31)
Glass cleaning sprays (for windows or mirrors)	1.19 (0.97; 1.46)	-6 (-98; 86)	-27 (-100; 46)
Degreasing sprays, including oven cleaning sprays	1.08 (0.85; 1.36)	-35 (-139; 69)	-26 (-109; 57)
Air refreshing sprays	1.20 (0.94; 1.54)	-50 (-163; 62)	-98 (-187; -9)
Plug-in or other electric air fresheners	1.02 (0.82; 1.28)	2 (-100; 103)	-38 (-119; 43)
Score of days-product use per week[c]	1.08 (0.94; 1.23)	-39 (-101; 22)	-26 (-75; 23)

[a] Adjusted for sex, age at the moment of the respiratory test, parental smoking, parental asthma and parental education. The FVC and FEV1 models also

include height. The FeNO models also include the early life endotoxin concentrations.

[b] Spray: furniture, glass cleaning, degreasing or air refreshing sprays

[c] Change in FeNO, FVC and FEV1 per IQR increase of the score (IQR=6.5 days-product/week)

127

In stratified analyses, higher GM ratios for FeNO were observed in the group of reported allergies. Bleach use increased 47% FeNO in allergic children. The use of polishes reduced FeNO in allergic children. Acid, spray and furniture and glass cleaning sprays use GM ratios were higher among allergic children. Regarding FVC and FEV1, ammonia use decreased the volumes in non-allergic children while volumes were higher in allergics. Similar differences in the direction of the adjusted estimates between the allergic and the non-allergic groups were observed for the use of sprays (Table 4). However, none of the estimates were statistically significant. P-values for the interaction terms between the use of each specific cleaning product and reported allergies for FeNO, FVC and FEV1 were below 0.1 for the use of bleach and polishes for FeNO, and the use of ammonia for FEV1.

Regarding asthma and wheezing, p-values for the interaction terms were below 0.1 for polishes, acids, sprays, furniture sprays, air refreshing sprays and the score of cleaning products use (data not shown). The use of air refreshing sprays in both groups (non-wheezing and non-asthmatic) significantly increased FeNO (38% and 34%, respectively). An increase of the interquartile range in the cleaning products score significantly increased FeNO (26% in non-wheezing children and 18 non-asthmatic children). Regarding FVC and FEV1, all the effect estimates remained similar in both groups compared to the results in all the study population. Only the FEV1 volumes significantly decreased (141mL) in children exposed to acids of the never wheezing group (Table 1, online supplement).

Table 4. Adjusted[a] associations between FeNO (geometric mean (GM) ratios), FVC and FEV1 (β coefficients) and the use of cleaning products (reference: less than once per week) according to the report of allergy (hay fever, eczema and/or rhinitis) from 4 to 10 years old.

	FeNO (ppb) GM ratio (95% CI)		FVC (ml) β (95% CI)		FEV1 (ml) β (95% CI)	
	Reported allergies	No reported allergies	Reported allergies	No reported allergies	Reported allergies	No reported allergies
Bleach	1.47 (0.98; 2.22)	0.95 (0.73; 1.23)	145 (-7; 297)	-18 (-142; 106)	116 (-14; 245)	49 (-49; 147)
Ammonia	1.21 (0.69; 2.11)	0.78 (0.57; 1.08)	65 (-151; 280)	-90 (-247; 66)	107 (-75; 289)	-109 (-231; 14)
Polishes or waxes (for floor or furniture)	0.55 (0.34; 0.90)	1.01 (0.76; 1.33)	-65 (-252; 123)	-92 (-227; 42)	35 (-124; 193)	-51 (-157; 56)
Acids, including decalcifiers and liquid scale removers	1.67 (1.02; 2.73)	1.02 (0.7; 1.49)	-167 (-352; 18)	-2 (-177; 174)	-172 (-328; -16)	-5 (-144; 134)
Solvents, including stain removers	1.10 (0.67; 1.82)	1.24 (0.91; 1.69)	-203 (-387; -19)	-85 (-234; 64)	-75 (-234; 85)	-28 (-146; 90)
Spray[b]	1.73 (1.09; 2.73)	1.39 (1.06; 1.83)	98 (-80; 276)	-21 (-153; 110)	15 (-137; 167)	-43 (-147; 61)
Furniture sprays	1.41 (0.93; 2.14)	1.27 (0.99; 1.63)	12 (-150; 173)	9 (-112; 130)	-56 (-192; 81)	-31 (-127; 65)
Glass cleaning sprays (for windows or mirrors)	1.48 (1.00; 2.17)	1.06 (0.83; 1.36)	21 (-137; 179)	-35 (-148; 78)	-5 (-137; 126)	-43 (-134; 47)
Degreasing sprays, including oven cleaning sprays	0.88 (0.57; 1.36)	1.16 (0.88; 1.54)	-79 (-254; 96)	-40 (-170; 91)	-15 (-164; 133)	-30 (-133; 74)
Air refreshing sprays	1.24 (0.76; 2.02)	1.28 (0.96; 1.73)	23 (-175; 221)	-65 (-204; 74)	-157 (-322; 8)	-75 (-185; 34)
Plug-in or other electric	1.14 (0.73; 1.77)	1.03 (0.79; 1.34)	-31 (-207; 144)	3 (-124; 129)	-30 (-179; 118)	-28 (-128; 71)
Score of days-product use per week[c]	1.08 (0.87; 1.35)	1.10 (0.93; 1.31)	-33 (-127; 62)	-45 (-126; 37)	-22 (-100; 56)	-14 (-79; 52)

[a]Adjusted for sex, age at the moment of the respiratory test, parental smoking, parental asthma and parental education. The FVC and FEV1 models also include height. The FeNO models also include the early life endotoxin.

[b]Spray: furniture, glass cleaning, degreasing or air refreshing sprays

[c]Change in FeNO, FVC and FEV1 per IQR increase of the score (IQR=6.5 days-product/week)

Discussion

Our study suggests that bystander exposure to domestic cleaning sprays, in particular furniture sprays, may have adverse effects on school-age children's airway inflammation by increasing FeNO. In addition, exposure to air freshening sprays and solvents at home may decrease lung function apparent at school age.

Domestic cleaning involves exposure to a large variety of cleaning products containing both irritants and sensitizers that can have an effect on both airway inflammation and lung function.[183] Cleaning products contain volatile organic compounds (VOCs) that can evaporate into the air as gas or vapour. The levels of these compounds are increased after cleaning and can persist over a period of a couple of days.[184] Aerosols and air fresheners have been identified as determinants of VOCs in homes.[18] As suggested in adult studies, the use of sprays for domestic cleaning may lead to a higher degree of inhalatory exposure compared with liquid products in active users.[14,88,185] Children are not active users of cleaning products. Thus, in this specific population, the application form may become crucial in the exposure assessment. Therefore, the use of household sprays may lead to a higher passive exposure compared with the use of similar products in other application forms. In addition, the periodical exposure to irritants from cleaning products that persist in indoor air may affect the development of the respiratory system.

Our results are consistent with those reported in infants of the younger INMA cohorts and the PARIS and ALSPAC cohorts.[89,91,181] Although respiratory symptoms during infancy do not necessarily lead to an onset of asthma, most children with asthma and reductions in lung function at school age begin with wheezing and frequent LRTI during the first years of life.[27] In addition, persistent wheezing starting during early life is associated with higher FeNO during school age.[31] Like in the young INMA cohorts and PARIS studies, we observed positive associations between the use of sprays and reported wheezing and asthma (data not shown) which are supported by the higher FeNO and the decline in FEV1 in children of spray users. However, the associations for lung function were not statistically significant. In addition, in the four younger INMA cohorts[181] we showed associations between reported wheezing and LRTI in infants and the use of solvents and air fresheners at home. In the present study, we observed a decline in lung function that was statistically significant for FVC and solvents exposure and for FEV1 and air refreshing sprays.

The ALSPAC studies[89,90] showed positive associations between pre-natal exposure to a composite score of cleaning products and persistent wheezing at 3 and 7 years of age, and lower spirometric volumes at 8.5 years of age. In our study, although not statistically significant, an increase in our score was associated with increased FeNO and decreased lung function. This points towards a potential dose-response effect on airway inflammation and lung function, although we may have limited power due to the sample size to test this.

Finally, a cross-sectional study based in school-age children observed a protective effect of bleach use for home cleaning on asthma report and allergic sensitization.[92] The association was not confirmed by the results on FeNO or lung function. In our study, we did not find significant associations between the bleach and FeNO in all study participants. However, the exposure to bleach increased 47% the FeNO in children with reported allergies. The adjusted estimates for bleach and lung function suggested a protective effect (increase of 47mL in FEV1) but the associations did not reach statistical significance. The increase in the FVC and FEV1 was higher in children with reported allergies (145mL and 116mL, respectively).

Our cross-sectional study is nested in a longitudinal birth cohort with a long follow-up that includes data collected prospectively, starting during the third trimester of pregnancy and ongoing for more than 10 years. This prospective design provides our cross-sectional analysis of more detailed information on environmental exposures and health outcomes. Moreover, it gives our study objective measurements of early life exposure to microbial agents that may be associated with respiratory health,[56,57,186] and with the hygiene of the home environment in the first stages of life.[11]

In addition, our study benefits from the objective measurement of the outcomes. FeNO as a biomarker of eosinophilic airway inflammation[154,155] and FVC and FEV1 as a indicators of general respiratory health[182] were measured at the moment of the exposure assessment and the results obtained were consistent with those for the reported respiratory outcomes. This consistency between reported and objective outcomes strengthened our findings.

Nevertheless, we must consider a few limitations when interpreting our results. Because of the cross-sectional design of our study, some parents who reported child's wheezing or asthma may have over-reported the use of some cleaning products. The

use of cleaning products may vary according to the asthmatic or atopic status of the parents or the child,[88] and child asthma and wheezing are associated with airway inflammation[31,187] and lung function.[188] For these reasons, we performed sensitivity analyses stratifying by wheezing, asthma, parental asthma and child reported allergies.

The number of individuals with report of wheezing, asthma and parental asthma was low. Therefore we were not able to assess the adjusted associations in these groups. Nevertheless, we could study the associations in the groups of never wheeze, never asthma and never parental asthma (data not shown). In all cases, the direction of the effect estimates did not change. In addition, stratification by reported allergy did not show significant differences in the associations obtained between both groups. These results suggest that these variables do not modify the relationship between the report of cleaning products and the outcomes.

In conclusion, domestic use of cleaning sprays, in particular furniture sprays, may increase child's airway inflammation, and the use of solvents and air freshening sprays may have an adverse effect on children's lung function. The use of cleaning products in private homes is common and many of these cleaning products are applied in spray formulation. Therefore, our findings may have significant implications for public health. Further investigation is required to obtain a better exposure assessment and validation of the reported exposure, as well as to identify the underlying pathophysiological mechanisms.

Acknowledgements

Table 1. Adjusted[a] associations between FeNO and the use of cleaning products (reference: less than once per week) among never wheezing group and non asthmatic children (4 to 10 years old).

	FeNO (ppb) GM ratio (95% CI)		FVC (ml) β (95% CI)		FEV1 (ml) β (95% CI)	
	Never wheezing	Non asthmatics	Never wheezing	Non asthmatics	Never wheezing	Non asthmatics
Bleach	1.11 (0.89; 1.39)	1.13 (0.91; 1.39)	-3 (-120; 115)	5 (-100; 111)	27 (-68; 121)	42 (-43; 127)
Ammonia	0.99 (0.75; 1.31)	0.96 (0.72; 1.27)	-65 (-213; 83)	-30 (-170; 110)	-90 (-208; 29)	-62 (-175; 50)
Polishes or waxes (for floor or furniture)	0.96 (0.74; 1.25)	0.9 (0.7; 1.15)	-60 (-198; 78)	-93 (-211; 26)	-22 (-133; 89)	-57 (-153; 39)
Acids, including decalcifiers and liquid scale removers	1.43 (1.06; 1.94)	1.23 (0.92; 1.64)	-110 (-267; 46)	-86 (-230; 57)	-141 (-266; -16)	-95 (-210; 21)
Solvents, including stain removers	1.17 (0.88; 1.55)	1.2 (0.93; 1.55)	-129 (-274; 17)	-142 (-268; -16)	-61 (-179; 57)	-58 (-161; 44)
Spray[b]	1.53 (1.21; 1.93)	1.42 (1.13; 1.78)	9 (-115; 133)	-11 (-124; 102)	-30 (-130; 70)	-47 (-139; 44)
Furniture sprays	1.50 (1.21; 1.86)	1.49 (1.21; 1.83)	45 (-74; 163)	14 (-93; 120)	-43 (-138; 53)	-58 (-144; 28)
Glass cleaning sprays (for windows or mirrors)	1.09 (0.88; 1.34)	1.07 (0.87; 1.31)	9 (-105; 123)	-40 (-142; 63)	3 (-89; 95)	-38 (-121; 44)
Degreasing sprays, including oven cleaning sprays	1.10 (0.86; 1.40)	1.15 (0.91; 1.45)	-76 (-202; 51)	-60 (-176; 55)	-69 (-171; 32)	-40 (-133; 54)
Air refreshing sprays	1.38 (1.07; 1.78)	1.34 (1.05; 1.71)	-39 (-179; 101)	-42 (-167; 82)	-114 (-225; -2)	-87 (-187; 13)
Plug-in or other electric	1.13 (0.90; 1.42)	1.13 (0.91; 1.41)	-49 (-171; 72)	-32 (-142; 78)	-76 (-173; 22)	-43 (-132; 46)
Score of days-product use per week[c]	1.26 (1.08; 1.47)	1.18 (1.03; 1.34)	-45 (-125; 35)	-39 (-108; 30)	-43 (-108; 21)	-30 (-86; 26)

[a] Adjusted for sex, age at the moment of the respiratory test, parental asthma and parental education. The FVC and FEV1 models also include height. The FeNO models also include the early life endotoxin concentrations.

[b] Spray: furniture, glass cleaning, degreasing or air refreshing sprays

[c] Change in FeNO concentration, FVC and FEV1 per IQR increase of the score (IQR=6.5 days-product/week)

6.5 PAPER V

Early life exposures to home dampness, pet ownership and farm animal contact and neuropsychological development in 4 year old children: a prospective birth cohort study

Casas L, Torrent M, Zock JP, Doekes G, Forns J, Guxens M, Täubel M, Heinrich J, Sunyer J.

6.6 PAPER VI

Indoor factors and behavioural problems in children: The GINIplus and LISAplus birth cohort studies.

Casas L, Tiesler C, Thiering E, Brüske I, Koletzko S, Bauer CP, Wichmann HE, von Berg A, Berdel D, Krämer U, Schaaf B, Lehmann I, Herbarth O, Sunyer J, Heinrich J; for the GINIplus and LISAplus Study Group.

[*] This paper is reproduced according to the original print version. References of this paper are included in the references section of the thesis.

7 DISCUSSION

This section is complementary to the discussion sections included in the papers with the aim to provide a broader and more integrated interpretation of the entire study project.

7.1 What does this thesis add to the current knowledge?

7.1.1 Indoor microbial agents

In the HITEA project, we measured the concentrations of three different microbial agents: bacterial endotoxin and fungal EPS and β(1,3)-D-glucan in living room dust samples of 1572 homes of 4 geographically spread European regions. Specifically, we measured microbial agents concentrations in 164 dust samples from Finish homes, 553 from Dutch homes, 395 from German homes, and 460 from Spanish homes. We found that the concentrations of measured microbial agents varied differently across the cohorts. The concentrations of microbial agents were similar in the Dutch and German cohorts. Endotoxin concentrations were lower in the Spanish and Finish cohorts, EPS concentrations were higher in the Spanish cohort and glucan concentrations were higher in the Finish cohort. However, we need to be cautious when comparing the results across cohorts. The INMA dust samples were collected from sofa and previously analysed for allergens. In the other cohorts, dust samples were collected from living room floor and first analysed for microbial agents. In line with our results, several studies have reported geographical variation of indoor levels of microbial agents. Nevertheless, most studies focused on endotoxin levels,[1–4,6–8,11] and few described the levels of fungal agents.[3,5,6,11]

Moreover, the number of samples per region included in the HITEA project was higher than the number of samples per region included in previous studies. Thus, we had a greater statistical power to identify potential determinants of the levels of indoor microbial agents per region and to assess the heterogeneity of the associations. In our study, season of dust sampling, dog ownership, indoor report of dampness, and number of people living in the home were significantly associated with the concentrations of at least one of the microbial agents. Our findings were in line with other studies reporting indoor determinants of endotoxin,[1,2,4,8–12,55] EPS[5,10] and

glucan.[5,10,11,13] In addition, our study is the first evaluating heterogeneity across cohorts in the effect of the assessed exposure determinants. We observed that the effect of indoor factors on the indoor concentrations of microbial agents varied across the cohorts.

7.1.2 Household cleaning products

This thesis describes the use of several domestic cleaning products in the homes of 5 geographically spread regions in Spain. These regions correspond to 5 birth cohorts participating in the INMA project. Overall, reported use of all different cleaning products was higher in the Valencia cohort. However, the prevalence of specific use of spray applied products was higher in the Menorca cohort. Studies within the ECRHS also showed differences between European countries in the report of cleaning products. Zock et al[14,15] reported country differences in the prevalence of use of bleach and sprays. In their studies, weekly use of household bleach and of sprays was more prevalent in Spain as compared to other European countries. The prevalence of use of bleach and sprays in Spain was similar to that we observed in Valencia. Nevertheless, caution needs to be exercised when comparing these results. The questionnaire administered in Asturias, Gipuzkoa, Sabadell and Valencia did not include frequency of use. Thus, the prevalence of use is based on any use ever of each specific cleaning agent at home. On the contrary, the questionnaire used in Menorca was similar to that used in the ECRHS. Here, the report of each specific cleaning product refers to a frequency of cleaning product use of least once a week.

Furthermore, we observed differences in the pre-natal and post-natal report of cleaning products use in Asturias and Valencia birth cohorts. For example, approximately 75% and 50% of the parents reported changes in the use of sprays and air fresheners, respectively, when comparing the pre-natal with the post-natal periods. The ALSPAC study[89] also had pre-natal and post-natal information on the use of household cleaning products. Unlike in the INMA project, the use of cleaning products at both time periods in ALSPAC was highly correlated. Considering the lack of information on the frequency of use of each cleaning product, we can not discard that the variations observed in our study may be due to the report of cleaning products that are very infrequently used at home.

7.1.3 Respiratory health

Microbial agents, indoor factors and respiratory health

Based on data from the HITEA project, this thesis presents the first longitudinal study assessing the long-term effects of early life exposure to measured indoor microbial agents and related indoor factors on FeNO levels measured at school age. We observed that early life exposure to high endotoxin concentrations and dog ownership may decrease FeNO at school age. These associations were not modified by the allergic status of the child. In addition, the same effects were observed in non-asthmatic children

To date, studies performed in animal models have shown that endotoxin and allergen exposure during pregnancy may modulate airway inflammation later in life.[168,169,215] But these effects in humans were never assessed before. Epidemiological studies mainly focused on reported respiratory and allergic diseases. Cross-sectional studies in rural areas and in general population suggested that the exposure to high indoor endotoxin levels may protect against asthma, atopy and respiratory and allergic symptoms.[6,7,41,43,64] In addition, studies including early life exposure to endotoxin concluded that this exposure may be protective against allergen sensitization, atopic eczema, wheezing and asthma.[57,60,142,170] Regarding dog ownership, previous birth cohort studies suggested that early life dog ownership had a protective effect against allergen sensitization.[65,66,170] In addition, a cross-sectional study evaluating the effects of multiple indoor factors on FeNO in school age children found that dog ownership was associated with lower FeNO, regardless the atopic or asthmatic status of the child.

High FeNO in school-age children is associated with asthma,[158,171] atopy,[172] and persistent and late onset wheezing phenotypes.[31] Asthma is a disease based on clinical diagnosis and its background is often, but not always, due to eosionphilic inflammation. FeNO is thus not diagnostic of asthma, but may indicate asthma due to airway eosinophilia. In this thesis, we investigated the role of FeNO as a biomarker of epithelial inflammation, independently of its association with asthma or atopy. Our results are thus not indicative of the risk of disease but of the maturation of the immune system in the child's respiratory system. Further investigation is needed to evaluate the clinical relevance of these findings.

Domestic use of cleaning products and respiratory health

The results reported in this thesis suggest that exposure during pregnancy, infancy and childhood to certain commonly used cleaning products, such as furniture cleaners, solvents or air fresheners and the use of cleaning products in spray form may be harmful for child's respiratory health. In particular, exposure during pregnancy was associated with a higher prevalence of wheezing and LRTI during the first year of life. This association remained apparent when sprays or air fresheners were used during pregnancy but not during the first year of life, suggesting a potential effect on foetal development. In addition, exposure to solvents, air fresheners and sprays during pregnancy and the first year of life increased the odds of LRTI and wheezing. Finally, exposure to cleaning products during school age resulted in harmful effects on airway inflammation and lung function. Children exposed to domestic cleaning sprays, in particular to furniture sprays, during school age had higher FeNO, and those exposed to air freshener sprays and solvents had a lower lung function.

Our results are in line with those reported in infants and children of the ALSPAC and PARIS birth cohorts.[89–91] The ALSPAC cohort assessed the effects of using a composite score of household cleaning products during pregnancy on wheezing from birth to 7 years old and lung function at 8.5 years of age.[89,90] However, they did not assess the effects of each cleaning product separately and they could not determine whether the association was due to pre- or a post-natal exposure.[18,89] Furthermore, the PARIS study only focused on the use of cleaning sprays and the respiratory health effects in the first year of life, focusing on wheezing. Thus, the work presented in this thesis adds to the current knowledge a comprehensive assessment of the respiratory health effects of the use of specific cleaning products and application forms at three time points: pregnancy, infancy and childhood. In addition, the respiratory health assessment presented in this work does not only include reported symptoms or disorders from birth to school age, but also objective measurements of airway inflammation and lung function at school age.

7.1.4 Neuropsychological development

Our findings suggest that exposure to indoor dampness, visible mould and pet ownership may have adverse effects on the neuropsychological development of the child. In particular, persistent exposure to indoor dampness during the first two years of

life decreases the cognitive function and social competences scores at 4 years of age. In addition, exposure to visible mould, dampness and pet ownership during the first 10 years of life increases the risk of borderline or abnormal scores in the SDQ at the age of 10 years; in particular they increased the risk of borderline or abnormal scores in the "emotional symptoms" dimension of the questionnaire. The results in both studies persisted even after considering several social factors as potential confounders. We hypothesise that the associations between exposure to indoor dampness, mould and pet ownership during infancy and childhood and neuropsychological development could be mediated by the neurotoxic and pro-inflammatory effects of the microbial agents.

Experimental studies suggest that pre-natal and early life immune activation through exposure to endotoxins can inhibit neurogenesis.[110–115] In addition, exposure to mycotoxins from mould show inflammatory and neurotoxic effects.[116–121] However, we need to be cautious when comparing results from experimental studies with those from observational studies, since the exposures assessed in experimental studies are much higher than the actual exposures that can be expected in indoor environments. To date, only one epidemiological study assessed the specific association between the early life exposure to indoor dampness and cognitive function during childhood.[130] Their results were in line with one of the results presented in this thesis: persistent exposure to indoor dampness during the first two years of life has a negative effect on cognitive function. Nevertheless, they did not assess the effects on social competences and behavioural problems of longer exposure.

The long-term effects of pet ownership associated with high indoor microbial agent levels on neuropsychological development at 4 and 10 years of age are assessed for the first time in the studies presented in this thesis. The assessment and interpretation of the relationship between pet ownership and human health is complex.[123–125] Several co-factors associated with pet ownership may also be positively associated with the neuropsychological development. For example, pet ownership may enhance social interactions, and provide emotional support, that would contribute to the family wellbeing. Although some of these factors have been considered in the studies presented in this thesis, these positive co-factors of pet ownership could compensate potentially negative effects on the neuropsychological development. Consequently, the results obtained regarding pet ownership and neuropsychological development must be interpreted with caution.

7.2 Strengths

The work presented in this thesis is based on combinations of data from nine European birth cohort studies that started with the same aim: to investigate the impact of pre- and post-natal exposures to environmental contaminants on child's growth, health and development, from pregnancy or birth to adolescence. Data in these birth cohorts were collected prospectively and they followed very similar protocols. The questions included in the questionnaires were in most cases comparable across cohorts and the periodical surveys were performed at similar ages of their participants. In addition, similar sampling procedures were performed in order to obtain exposure and health measurements.

Another important issue is that the nine birth cohorts participate in three collaborative projects: the European HITEA project, the Spanish INMA project and the German GINIplus and LISAplus studies. The collaborative participation of single cohorts in projects allows for standardizing methods for new data collection across cohorts, increases the power of the statistical analyses, and permits the analyses of potential geographical differences in the results.[216]

Regarding the new data collection, the early life dust samples collected in the birth cohorts participating in the HITEA project were analyzed in the same laboratory using the same techniques. In the INMA project and in the LISAplus and GINIplus study, their respective participant birth cohorts administered the same questionnaires and health assessment instruments. For example, the same cleaning products and respiratory health questionnaires were administered in the Asturias, Guipuzkoa, Sabadell and Valencia birth cohorts; and the same behavioural problems assessment tool (SDQ) was administered in both German birth cohorts.

Geographical differences across regions in the prevalence of the studied exposures and the respiratory outcomes exist. The collaborative projects that include data from different regions and countries allow studying the differences, not only in the studied exposure or outcomes, but also in the effect estimates. Within the HITEA project, we could evaluate the regional differences in Europe in levels of indoor microbial agents, in the effects of their potential determinants, and in the effect estimates on respiratory health outcomes. In addition, the INMA project provided information on the regional differences in Spain in the frequency of use of cleaning products, in the prevalence of

reported respiratory health outcomes and in the effect estimates of cleaning products use and respiratory health.

Last but not least, this thesis benefits from the availability of objective measurements of both exposures and health outcomes, from a validated and reliable instrument for the assessment of the neuropsychological development and from the longitudinal design of the projects and birth cohorts that provides of repeated information on exposure, health outcomes and co-variables along the study periods.

Three microbial agents (Endotoxin, EPS and β(1,3)-D-glucan) were measured in living room dust form HITEA participant's homes. The dust samples were collected during early life. During this period, parents reported information on potential determinants of high microbial agent levels in the home. Thus, we could evaluate the association between reported and measured exposures in the HITEA studies included in this thesis that assess early life exposures. In addition, we used two objectively measured respiratory health outcomes to assess the associations between respiratory health and early life microbial exposure and cleaning products at school age: spirometric lung function variables and FeNO as a marker of eosinophilic airway inflammation.

Finally, the repeated information obtained from the longitudinal design permitted to evaluate the direction of causality. For example, exposure to pets, visible mould and dampness, and the use of cleaning products were reported periodically in most birth cohorts. Thus, we had the possibility to assess respiratory health and neuropsychological effects of these exposures only during pregnancy, only during early life or only during childhood.

7.3 Limitations

A few general limitations of the work presented in this thesis must be considered. As explained in the first paper of this thesis, the populations participating in the HITEA project were selected differently depending on the cohort. In addition, because the birth cohorts participating in the HITEA project started years before the beginning of the project, there are minor differences in some questions regarding confounding variables and in the dust sampling and analyses. For these reasons, we performed meta-analyses and showed the random effects estimates in all HITEA studies. Meta-analyses techniques help avoid potential biases due to differences in the study

populations. Nevertheless, compared to pooled analyses, it limits the statistical power of the study. In particular, we had limited power to perform stratified analyses for health outcomes such as asthma because of the low number of asthmatic children in each cohort. Therefore, we can not determine if FeNO is an intermediate factor between exposure to indoor microbial agents or whether the finding is independent of the clinical disorder.

Furthermore, although we had several objective measurements of exposure and health outcomes, these measurements could not be used to achieve all objectives of the thesis. Therefore, some of the work presented is based only on reported or other subjective variables. We did not have any objective measurement of the use of household cleaning products that could validate the parental report. Also, respiratory health assessment in the first year of life was exclusively based on reported questionnaires. Consequently, the evaluation of the respiratory health effects of cleaning products during early life was based only in parental reports and we need to assume the potential for diagnostic or recall bias in this study. These biases may have resulted in an overestimation of our associations. Nevertheless, the early life findings were in line with those we obtained at school age, when objective respiratory health outcomes (lung function and FeNO) were measured.

Regarding our studies on neuropsychological development, the exposure to reported indoor dampness could not be confirmed by any objective measurement. Previous studies comparing dampness report with observations found evidence of systematic reporting bias.[217] In addition, the assessment of behavioural problems with the SDQ and of the social competences with the CPSCS may be subjective. We may think that parents who tend to overeport water damage problems in their buildings may tend to give worse scores in the behvarioural and social compentences tests of their children. This may have resulted in a bias away from the null. However, the assessment of the cognitive function in the INMA-Menorca birth cohort was based on a validated and reliable test (the MSCA). Although the SDQ used in the German cohorts and the CPSCS used in INMA do not measure the same as the MSCA test used in INMA and the ages at testing were different across regions, the findings in both cohorts point towards a negative effect of living in water damaged buildings on the neuropsychological development. Thus, we may assume that the effects observed are beyond the potential subjective responses given when using the SDQ or the CPSCS.

7.4 Evidence of causation: the Bradford Hill criteria

The Bradford Hill criteria of causality provide a reference frame to make judgements on the strengths of the empirical evidence of the associations obtained in epidemiological studies. In order to evaluate the causality of the associations reported in chapter 6 and their relationship with the hypotheses formulated in chapter 4 (figure 8), we will apply the following criteria:

(1) Strength: Is the association estimate strong?

(2) Consistency: Has the association been repeatedly observed by different studies, in different circumstances, places and times?

(3) Specificity: Is a single exposure producing the outcome?

(4) Temporality: Does the exposure precede the outcome?

(5) Biological gradient: Is there a dose-response effect?

(6) Plausibility: Does the association agree with theoretical biological mechanisms?

(7) Coherence: Is the association compatible with the existing theoretical mechanisms?

(8) Experiment: Do experimental studies agree with the observed associations?

(9) Analogy: Is an alternative explanation of the association possible?
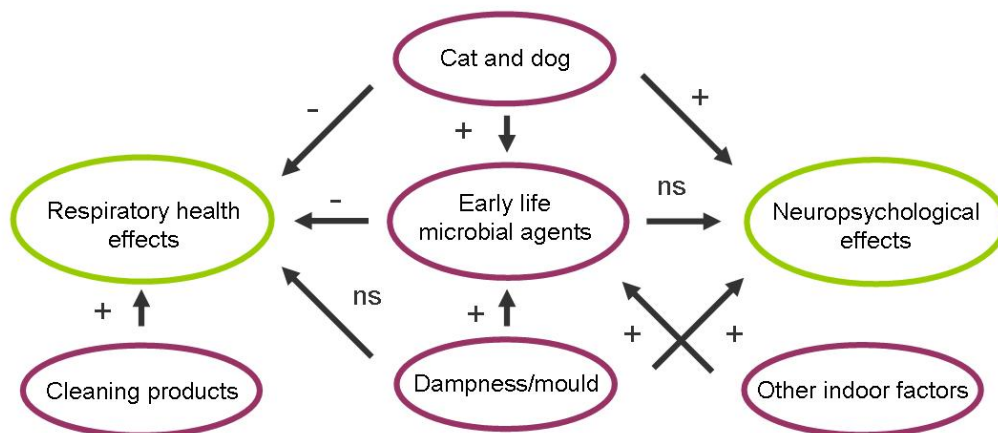

Figure 8. Associations considered in the interrelationship between early life microbial

exposures, indoor dampness, pet ownership and cleaning products and respiratory health; and and neuropsychological development. The + and − signs indicate positive and negative association, respectively; ns indicates non significant association..

The following sections evaluate the first 8 criteria of causation according to the three main hypotheses described in the hypotheses section: 1) Indoor microbial agents and potential determinants, 2) respiratory health and microbial agents, indoor factors and cleaning products, and 3) neuropsychological development and indoor factors. The last section discusses the analogy of the three main association groups.

7.4.1 Indoor microbial agents

The associations between the levels of microbial agents and the study region were significant and **strong (1),** however, the strength of the associations between potential determinants and the levels of microbial agents varied across cohort and determinant. Our results were **consistent (2)** with those reported in previous studies regarding geographical differences in the levels of indoor microbial agents[1–8,11] and their associations with indoor factors.[1,2,4,5,8–13,55] Nevertheless, multiple indoor and outdoor factors may influence the indoor levels of microbial agents. In our study, we considered indoor factors that had previously been described in the literature. Therefore, it is possible that other indoor and outdoor factors contribute to increase or decrease the levels of microbial agents (**specificity (3)**).

Information on the assessed indoor factors was collected at one year of age and referred to the first year of the child's life. The levels of indoor microbial agents were measured in living room dust collected at 2-3 months of age. Therefore, we could not guarantee **temporality (4)** in the associations between indoor factors and microbial agents. In addition, the **dose-response effect (5)** could only be tested for one indoor factor: the number of people living in the home. In all cases, the higher the number of people, the stronger the effect was.

Endotoxin is a component of the wall of Gram-negative bacteria, EPS and glucans come from fungi. These microorganisms may be transferred from outdoors to indoors through persons and animals or house ventilation, or may grow in water-damaged buildings[77] (**plausibility (6)**). Our findings are **coherent (7)** with this theory. Unfortunately, there are no **experimental (8)** studies that assess the association between indoor factors and the levels of microbial agents.

7.4.2 Respiratory health

Microbial agents, indoor factors and respiratory health

We observed a 3.7% decrease in FeNO when doubling the endotoxin dose and a 18% decrease in children exposed to dogs during early life (**strength (1)**). Our results for endotoxin and dog ownership were **consistent (2)** in two of the three participating birth cohorts (INMA and PIAMA). The differences observed in LISA compared to PIAMA and INMA may be due to low statistical power. Although the associations between the assessed indoor exposures and the respiratory health outcomes were strong, other indoor factors (e.g. cleaning products use) and outdoor factors (e.g. air pollution) may be influencing these associations (**specificity (3)**).

The assessed exposures **temporally (4)** preceded the respiratory outcome. Dust samples were collected at 2-3 months of age, dog ownership was reported periodically from birth to school age, and FeNO was measured at school age. In addition, we observed a **dose-response (5)** relationship between the endotoxin measurements and FeNO. However, we could not evaluate this effect for dog ownership.

The exposure to environments with high concentrations of microbial agents contributes to the proper maturation of the immune system and decreases the risk of asthma and allergies later in life[54,141] (**plausibility (6)**). Our findings are **coherent (7)** with this theory. In addition, **experimental (8)** studies in murine models showed that the exposure to endotoxins during pregnancy was associated with lower airway inflammation.[168,215]

Domestic use of cleaning products and respiratory health

The use of certain household cleaning products and application forms during pregnancy and infancy was significantly and **strongly (1)** associated with wheezing and LRTI during infancy (exposure to sprays, air fresheners and solvents increased 30 to 40% the odds of wheezing or LRTI). During childhood, the use of household cleaning products was strongly associated lung function (solvents and air refreshing sprays exposure decreased approximately 100mL the FVC and the FEV1, respectively) and FeNO (50% increase in children exposed to sprays). The associations between the use of cleaning products and respiratory health outcomes were **consistent (2)**

throughout our studies and with previous epidemiological studies.[89–91] Associations were consistent across cohorts and ages of exposure and outcome assessment. Nevertheless, despite the strength and consistency of the associations, other risk factors may be influencing these associations (**specificity (3)**).

The use of household cleaning products is common and constant. Therefore, it is difficult to assess **temporal (4)** relationships between this exposure and the health outcomes. Nevertheless, early life exposure to household cleaning products was assessed during pregnancy and at 1 year of age. This allowed investigating the effects of the exposure according to a time window. In most cases, the associations remained strong when the exposure happened only during pregnancy. At school age, exposure was assessed at the same moment as the respiratory outcomes; in this case, the exposure did not precede the outcome. Nevertheless, we observed a consistent **dose-response (5)** relationship between the use of cleaning products and the respiratory health effects. During early life, the effects were stronger when children were exposed to sprays, solvents and air fresheners at both time windows (during pregnancy and at one year of age) compared to the exposure only during pregnancy or only at one year of age. At school age, we evaluated the association between the respiratory health outcomes and a composite score that included the number of cleaning products used and the frequency of use. Although this association was not statistically significant., the estimate was in line with the results observed for the exposure to specific cleaning products.

Cleaning products are chemical based products containing both irritants and sensitizers that can have an effect on respiratory health[183] (**Plausibility (6)**). Our results are **coherent (7)** with this hypothesis. In addition, e**xperimental (8)** studies performed in murine models support this hypothesis.[218,219]

7.4.3 Neuropsychological development

The associations between indoor factors (indoor dampness, mould and pet ownership) and the neuropsychological development were **strong (1)**. The strength of the associations was independent of the age at neuropsychological testing, the instrument used to measure the outcome and the study region: persistent exposure to home dampness during early life decreased 5 points the general cognitive index of the MSCA.; in addition, ever living in a water-damaged home and pet ownership from birth

to 10 years increased a 50% the odds of abnormal scores in the SDQ. The results presented in both studies were **consistent (2)** with those reported in previous studies[129,130] regardless the differences in the study region, age, neuropsychological outcome and instrument. Nevertheless, despite the strength and consistency of the associations, other risk factors such as the socio economic status of the family and the family structure may be influencing these associations. Therefore, the **specificity (3)** of our associations may be weak.

In our studies, parents reported indoor dampness and pet ownership years before the measurement of the neuropsychological development (**temporality (4)**). Moreover, **dose-response (5)** relationships were observed for the association between early life dampness and cognitive function and social competences: statistically significant associations were only observed when the report of dampness was persistent during the first two years of life.

Brain development starts during pregnancy and is still developing after puberty; therefore, the brain is particularly susceptible to any environmental insult during the first two decades of life, especially during early life.[189] Exposure to high levels of microbial agents during early life may have a negative impact on the neurpsychological development.[116,122] (**plausibility (6)**). Our results are **coherent (7)** with this hypothesis and consistent with the **experimental (8)** studies performed in murine models. These studies suggest that pre-natal and early life immune activation through the exposure to endotoxins can inhibit neurogenesis, and that mycotoxins may be neurotoxic.[110,116–120]

7.4.4 Analogy

The indoor and outdoor environments consist of a complex mixture of factors that are constantly interacting and that significantly vary across regions. Therefore, it is very difficult to assign specific determinants to a particular exposure and a specific exposure to a particular health effect. In this thesis, we considered a large variety of potential confounders. However, several indoor and outdoor contaminants and regional and personal characteristics could not be taken into account.

For example, the regional climate may influence the house structure, the potential for water damage, and the social behaviour of the house inhabitants. Therefore, we would expect analogous associations for regional temperature and humidity, for frequency of

home ventilation or for the ratios of outdoor/indoor activities, to the observed for indoor factors and microbial agents. Moreover, indoor endotoxin concentrations, dog ownership and the use of household cleaning products may be only partly responsible for the observed respiratory health effects. The exposure to other indoor factors such as indoor aeroallergens or the exposure to outdoor air pollution may result in analogous associations. Finally, several psychosocial factors may influence the persistent exposure to dampness, pet ownership and the neuropsychological development. Our findings regarding the neuropsychological development may be partly explained by factors such as family structure and functioning, parental unemployment, household income, and socio-economical status.

7.5 Implications

7.5.1 Public health relevance

Since the foetal stages of life we are exposed to microbial agents in indoor environments. The levels of microbial agents vary across regions and according to the presence of common indoor factors such as pet ownership or water damage. In addition, we use a broad range of domestic cleaning agents, weekly or even daily, to maintain the appearance and hygiene of our homes. The diversity of cleaning products and the application forms in the developed countries is high. In particular, the use of cleaning products in spray form is highly prevalent in our homes. This common practice may contribute to reduce the levels of microbial agents at home and to increase the total amount of toxic chemicals in indoor air. Exposure to microbial agents and cleaning products may be involved in the development of respiratory, allergic and neuropsychological disorders. In the last decades, the prevalence of these disorders has increased. The environment to which we are exposed during infancy importantly contributes to the development of our immune and central nervous system. Thus, it plays an important role in the onset of respiratory, allergic and neuropsychological disorders later in life.

The results presented in this thesis have important implications for public health. First, our study provides highly relevant information on indoor factors associated with the indoor levels of microbial exposure, their distribution and differential effects across countries, and their impact on child's health. This information is essential to distribute to various stakeholders, such as professionals within city planning, building design,

construction, material manufacturing and building maintenance and, on the other hand, to patient and consumer organizations.

Second, this thesis adds evidence to that observed in adult studies that the use at home of certain cleaning products contributes to the total burden of respiratory disorders by increasing the risk of respiratory symptoms and infections during early life and by increasing the airway inflammation and decreasing the lung function at school age. The use of cleaning products in private homes is common and many of these cleaning products are applied in spray formulation. Therefore, general population should be educated regarding the potential harmful effects of cleaning products use and measures to avoid the harmful exposure should be implemented.

7.5.2 Open research questions for the scientific community

Several gaps in the current knowledge of the respiratory and neuropsychological health effects of exposure to indoor microbial agents and cleaning products warrant further research. Exposure assessment of indoor microbial agents and cleaning products should be further developed. In addition, further research on the long-term effects of indoor microbial exposure on child's respiratory health and neuropsychological development is needed. Also, the mechanisms involved in the associations presented in this thesis still need to be elucidated. Thus, future research in the field of environmental research with a focus in child health and indoor exposures should further investigate the following issues:

Microbial agents, indoor factors and cleaning products

- The evaluation of the potential temporal variation of the indoor levels of microbial agents across regions and the assessment of the potential indoor factors that may determine the temporal variation are needed. This work will be developed in the future with data from the four cohorts involved in the HITEA project.

- The home levels of measured neurotoxins and their association with indoor factors remains unexplored.

- It is important to obtain an objective measure of the domestic use of cleaning products. Therefore, studies on the association between the report of specific cleaning products and measurement of indoor VOCs are needed.

- The regional differences in the use of domestic cleaning products and the potential determinants and patterns of cleaning products use need to be further investigated.

- The association between the levels of indoor microbial agents and the use of specific domestic cleaning products, not only the frequency of cleaning, has not yet been investigated. This work will be developed in the future with data from the four cohorts involved in the HITEA project.

Respiratory health

- The assessment of the respiratory health effects of early life exposure to indoor microbial agents needs further investigation. The association between early life microbial agent concentrations and wheezing phenotypes, allergic sensitization, asthma and respiratory infections at school age will be assessed in three of the four cohorts involved in the HITEA project.

- The evaluation of the potential respiratory health effects at school age of temporal differences in the measurements of indoor microbial agents during early life and at school age is needed.

- Further research is needed to elucidate the mechanisms involved in the respiratory health effects of the exposure to early life indoor microbial agents, as well as of the exposure to cleaning products during pregnancy and childhood.

Neuropsychological development

- Further epidemiological studies on the effects of indoor environmental factors on neuropsychological development are needed.

- Epidemiological studies that evaluate the effects of early life exposure to measured neurotoxins in the homes on neuropsychological development have not yet been performed.

- Further research is needed to elucidate the mechanisms involved in the effects on neuropsychological development of exposure to early life indoor microbial agents and other indoor factors.

8 CONCLUSIONS

Overall, the results presented in this thesis suggest that indoor exposure to microbial agents during early life and exposure to chemical based cleaning products during pregnancy, infancy and childhood may play a role in the development of the respiratory, immune and central nervous systems.

More specifically, the following conclusions result from this thesis:

Microbial agents and potential determinants

- The levels of endotoxin, EPS and β(1,3)-D-glucan in home living room dust vary across European geographical regions.

- Endotoxin and glucan levels are associated with season, presence of dogs and the number of people living in the home. Endotoxin levels are in additional associated with home dampness. The relevance of these indoor factors is highly heterogeneous across regions.

Domestic cleaning products

- The use of cleaning products in private homes is common and many of these cleaning products are applied in spray formulation.

Exposure to microbial agents and respiratory health

- Exposure to high concentrations of bacterial endotoxin in the first months of life and dog ownership during the first two years of life is associated with airway inflammation, as assessed with FeNO.

Exposure to domestic cleaning products and respiratory health

- The use of household cleaning products such as solvents, cleaning sprays, air fresheners or degreasing products during pregnancy may increase the risk of respiratory symptoms and/or infections during the first year of life.

- Exposure to domestic use of cleaning sprays, in particular furniture sprays, at school age may increase child's airway inflammation. In addition the use of solvents

and air freshener sprays at school age may have an adverse effect on children's lung function.

Exposure to indoor factors and neuropsychological development

- Persistent exposure to indoor dampness during early life can have an adverse effect on cognitive function and social competences at the age of 4 years. Effects of comparable sources of microbial exposure such as pet ownership or farm animal contact are not apparent.

- Exposure to visible mould, dampness, and pet ownership during infancy and childhood increases the risk of emotional problems at the age of 10.

9 FUTURE RESEARCH PLANS

As previously mentioned, there are several gaps in the current knowledge of the characterization of the exposure to microbial agents and cleaning products, their interrelation and their effects on child respiratory health. In this regard, the HITEA project provides the necessary data to unravel some of the research questions proposed at the end of this thesis. In the coming months, I will be involved in the following HITEA studies:

- Evaluation of the potential temporal variation of the indoor levels of microbial agents in four European birth cohorts: LISA, PIAMA, INMA and LUKAS2.

- Assessment of the potential indoor factors that may determine the temporal variations in the indoor levels of microbial agents in four European birth cohorts: LISA, PIAMA, INMA and LUKAS2

- Evaluation of the interrelation between the levels of indoor microbial agents and the use of specific domestic cleaning products.

- Assessment of the association between early life microbial agent concentrations and wheezing phenotypes, allergic sensitization, asthma and respiratory infections at school age.

- Evaluation of the potential respiratory health effects at school age of temporal differences in the measurements of indoor microbial agents.

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