A three-generation study on the association of tobacco smoking with asthma

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Simone Accordini,[1,*] Lucia Calciano,[1] Ane Johannessen,[2] Laura Portas,[1] Bryndis Benediktsdóttir,[3] Randi Jacobsen Bertelsen,[4,5] Lennart Bråbäck,[6] Anne-Elie Carsin,[7,8,9] Shyamali C Dharmage,[10] Julia Dratva,[11,12] Bertil Forsberg,[6] Francisco Gomez Real,[4] Joachim Heinrich,[13] John W Holloway,[14] Mathias Holm,[15] Christer Janson,[16] Rain Jögi,[17] Bénédicte Leynaert,[18] Andrei Malinovschi,[19] Alessandro Marcon,[1] Jesús Martínez-Moratalla Rovira,[20,21] Chantal Raherison,[22] José Luis Sánchez-Ramos,[23] Vivi Schlünssen,[24,25] Roberto Bono,[26] Angelo G Corsico,[27] Pascal Demoly,[28,29] Sandra Dorado Arenas,[30] Dennis Nowak,[13,31] Isabelle Pin,[32,33,34] Joost Weyler,[35] Deborah Jarvis,[36,37,†] Cecilie Svanes,[2,5,†]; on behalf of the Ageing Lungs in European Cohorts (ALEC) Study.

*Corresponding author. †Equally contributed.

[1]Unit of Epidemiology and Medical Statistics, Department of Diagnostics and Public Health, University of Verona, Verona, Italy; [2]Centre for International Health, University of Bergen, Bergen, Norway; [3]Faculty of Medicine, University of Iceland, Reykjavik, Iceland; [4]Department of Clinical Science, University of Bergen, Bergen, Norway; [5]Department of Occupational Medicine, Haukeland University Hospital, Bergen, Norway; [6]Division of Occupational and Environmental Medicine, Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [7]ISGlobal, Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain; [8]Universitat Pompeu Fabra (UPF), Barcelona, Spain; [9]CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain; [10]School of Population and Global Health,

University of Melbourne, Melbourne, Australia; [11]ZHAW School of Health Professions, Winterthur, Switzerland; [12]Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel University, Basel, Switzerland; [13]Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, Inner City Clinic, University Hospital Munich, Ludwig-Maximilians-Universität Munich, Germany; [14]Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK; [15]Department of Occupational and Environmental Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [16]Department of Medical Sciences: Respiratory, Allergy and Sleep Research, Uppsala University, Uppsala, Sweden; [17]Lung Clinic, Tartu University Hospital, Tartu, Estonia; [18]INSERM UMR 1152, Pathophysiology and Epidemiology of Respiratory Diseases, Paris, France; [19]Department of Medical Sciences: Clinical Physiology, Uppsala University, Uppsala, Sweden; [20]Servicio de Neumología del Complejo Hospitalario Universitario de Albacete (CHUA), Servicio de Salud de Castilla-La Mancha (SESCAM), Albacete, Spain; [21]Facultad de Medicina de Albacete, Universidad de Castilla-La Mancha, Albacete, Spain; [22]INSERM U1219, Bordeaux University, Bordeaux, France; [23]Department of Nursing, University of Huelva, Huelva, Spain; [24]Department of Public Health, Aarhus University, Aarhus, Denmark; [25]National Research Center for the Working Environment, Copenhagen, Denmark; [26]Department of Public Health and Pediatrics, University of Turin, Turin, Italy; [27]Division of Respiratory Diseases, IRCCS 'San Matteo' Hospital Foundation-University of Pavia, Pavia, Italy; [28]Département de Pneumologie et Addictologie, Hôpital Arnaud de Villeneuve, University Hospital of Montpellier, Montpellier, France; [29]UMR-S 1136 INSERM, IPLESP, UPMC, Sorbonne Universités, Paris, France; [30]Unit of Pneumology, Galdakao-Usansolo Hospital, Galdakao, Spain;

[31]Comprehensive Pneumology Center Munich, Member of the German Center for Lung Research, Munich, Germany; [32]Pediatrie, CHU Grenoble Alpes, Grenoble, France; [33]INSERM 1209, Institute for Advanced Biosciences, Grenoble, France; [34]Université Grenoble Alpes, Grenoble, France; [35]Epidemiology and Social Medicine and the StatUA Statistics Centre, University of Antwerp, Antwerp, Belgium; [36]Faculty of Medicine, National Heart & Lung Institute, Imperial College, London, UK; [37]MRC-PHE Centre for Environment and Health, Imperial College, London, UK.

CORRESPONDING AUTHOR: Professor Simone Accordini, Sezione di Epidemiologia & Statistica Medica, Dipartimento di Diagnostica e Sanità Pubblica, Università degli Studi di Verona c/o Istituti Biologici II, Strada Le Grazie 8, 37134 Verona, Italy; phone: +39 045 8027657; e-mail: simone.accordini@univr.it

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ABSTRACT

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Background: Mothers' smoking during pregnancy increases asthma risk in their offspring. There is some evidence that grandmothers' smoking may have a similar effect, and biological plausibility that fathers' smoking during adolescence may influence offspring's health through transmittable epigenetic changes in sperm precursor cells. We evaluated the three-generation associations of tobacco smoking with asthma.

Methods: Between 2010-2013, at the European Community Respiratory Health Survey III clinical interview, 2233 mothers and 1964 fathers from 26 centres reported whether their offspring (aged ≤51 years) had ever had asthma and whether it had coexisted with nasal allergies or not. Mothers and fathers also provided information on their parents' (grandparents) and their own asthma, education, and smoking history. Multilevel mediation models within a multicentre three-generation framework were fitted separately within the maternal (4666 offspring) and paternal (4192 offspring) lines.

Results: Fathers' smoking before they were 15 [relative risk ratio (RRR) = 1.43, 95% confidence interval (CI): 1.01-2.01] and mothers' smoking during pregnancy (RRR = 1.27, 95% CI: 1.01-1.59) were associated with asthma without nasal allergies in their offspring. Grandmothers' smoking during pregnancy was associated with asthma in their daughters [odds ratio (OR) = 1.55, 95% CI: 1.17-2.06] and with asthma with nasal allergies in their grandchildren within the maternal line (RRR = 1.25, 95% CI: 1.02-1.55).

Conclusions: Fathers' smoking during early adolescence, grandmothers' and mothers' smoking during pregnancy may independently increase asthma risk in offspring. Thus, risk factors for asthma should be sought in both parents and before conception.
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Key words: asthma, mothers' smoking during pregnancy, grandmothers' smoking during pregnancy, fathers' smoking during puberty, multilevel mediation model, Ageing Lungs in European Cohorts (ALEC) Study.

KEY MESSAGES

- Fathers' smoking before they were 15 was associated with an increased risk of asthma without nasal allergies in their offspring, suggesting an effect of paternal pre-adolescent environment on the next generation.
- Grandmothers' smoking during pregnancy was associated with an increased risk of asthma with nasal allergies in their grandchildren within the maternal line, suggesting a multi-generation effect of tobacco smoking.
- A multi-generation perspective is needed to better understand major public health challenges, such as smoking and asthma, and to assess the value and feasibility of preventive interventions.

INTRODUCTION

Considerable resources are invested in smoking prevention, with substantial health benefits. Pregnant women are a target of such interventions, as consistent evidence has demonstrated the negative impact of prenatal exposures on offspring's health. In particular, it is widely accepted that mothers' smoking during pregnancy increases the risk of asthma and asthma-like symptoms in their offspring.[1–4] Indeed, nicotine exposure during the pre- and perinatal periods appears to permanently affect the development of the lungs, with adverse effects on their final structure and function.[5] These changes may increase the risk of asthma later in life and accelerate lung function decline with aging.[5–8]

The enhanced understanding of the heritable effects of tobacco smoking through transmissible epigenetic phenomena opens a new paradigm,[9,10] providing a biological basis for preventive interventions during pregnancy and even in young males. Animal studies support multi-generation effects of nicotine exposure during gestation and lactation on the lungs,[11] but evidence in humans is scarce and controversial. There are reports that the risk of asthma increases for a child if the maternal grandmother had smoked when pregnant with the child's mother, even if the child was not exposed to the mother's smoking *in utero*.[2,12,13] However, grandmothers' smoking was not associated with their grandchildren's respiratory outcomes through the maternal line in another population survey.[14] Tobacco smoking may have heritable effects also within the paternal line, as fathers' smoking during adolescence may cause epigenetic changes in sperm precursor cells that can be transmitted to later generations.[15] Supporting evidence to the effect of fathers'

smoking during puberty on offspring's health has been provided by the Respiratory Health in Northern Europe (RHINE) III study.[16]

The present study aims at investigating the pattern of associations between tobacco smoking and asthma across three generations [grandparents (F0), parents (F1), offspring (F2)], during different developmental stages within those generations (grandmothers/mothers' pregnancies, fathers' puberty). To fulfil this objective, we used data from the European Community Respiratory Health Survey (ECRHS).[17–19]

METHODS

Study population

The ECRHS is an international, population-based, cohort study on respiratory health in subjects aged 20-44 at the time of recruitment (ECRHS I; 1991-1993).[17] At baseline, each participant was sent a brief screening questionnaire (stage 1) and, from those who responded, a 20% random sample was invited to undergo a more detailed clinical examination (stage 2). Follow-up of the participants in stage 2 took place in 1998-2002 (ECRHS II)[18] and 2010-2013 (ECRHS III).[19] The participants underwent a standardized clinical interview, lung function tests, and laboratory exams on all occasions. An additional sample of adults with asthma-like symptoms recruited at baseline was not included in the present analyses. Ethical approval was obtained for each centre from the appropriate Ethics Committee and written consent was obtained from each participant.

The 4449 subjects (from 26 centres in Europe and Australia; Table 1S, available as supplementary data) who had participated in both the ECRHS I and III, and who had reported at least one offspring at the ECRHS III clinical interview, were eligible for the present analyses (Figure 1). Among these individuals, 2233 mothers and 1964 fathers provided complete information on gender, birth year, asthma, and nasal allergies (including hay fever) of their 4666 and 4192 offspring, respectively, as well as information on their parents' (grandparents) and their own asthma and smoking history.

Definitions

Offspring's asthma was classified as: "*ever asthma with nasal allergies*"; "*ever asthma without nasal allergies*"; "*never asthma*". Grandparental and parental ever asthma ("*present*" *vs* "*absent*") was reported by parents at baseline or at the ECRHS II and III (5.6% of grandparents and 8.7% of parents).

The parents provided detailed information on their own smoking history (including when they had started and quitted smoking) at each clinical interview. Mothers' smoking was classified according to the birth year of each offspring: "*smoking when the offspring was in utero*" (mothers smoked during their child's birth year and/or during the previous year; these mothers also smoked during other periods); "*smoking during other periods*" (mothers stopped smoking at least two years prior to their child's birth year -at least three months before conception- and/or started or restarted smoking after their child's birth year); "*never smoking*". Fathers' smoking was classified as: "*smoking initiation before 15 years of age*" (before the mean age of completed puberty in boys);[20] "*smoking initiation at 15 years of age or older*"; "*never smoking*". At ECRHS I, the parents provided information on their mother's smoking during the period around their birth. Consequently, grandmothers' smoking was categorised as: *"smoking when the parent was in utero*"; "*smoking during other periods (or unknown smoking period)*"; "*never smoking*".

Grandparents' education level was parent-reported and considered low if both grandparents had only studied up to the minimum school leaving age. Mothers' and fathers' education level was self-reported and considered low if less than or equal to the minimum school leaving age in their country before the start of the ECRHS.[21] An "*unknown*" category was used when no information on education was available.

Statistical analyses

Mediation models[22] within a hierarchical framework were used to investigate the multi-generation pattern of associations between tobacco smoking and asthma within the maternal and paternal lines. Our data have a hierarchical structure (see the online supplementary appendix) because we evaluated multiple offspring (level 1 units) from the same parent (i.e. the participants in the ECRHS III; level 2 units), and because many parents had been sampled from each of the different centres (level 3 units).

The following variables were included in the mediation models (the paths investigated in the analyses are represented in Figure 2 and Figure 3):

- offspring's ever asthma with or without nasal allergies as the multinomial-distributed outcome;
- maternal/paternal ever asthma as the Bernoulli-distributed mediator;
- grandmother's and grandfather's ever asthma, grandmother's smoking, grandparents' education level, and maternal/paternal age as the potential predictors of the mediator;
- grandmother's smoking, maternal/paternal smoking and education level, and offspring's gender and age as the potential predictors of the outcome.

Both mediation models had a complex two-level structure, in which the predictors of the mediator and the mediator were measured at level 2 (parent), whereas the outcome was measured at level 1 (offspring). This type of mediation models has been labelled "*2→2→1*" in the literature.[23] Random intercept terms at level 2 were included in the models. Cluster-robust standard errors were computed in order to take the correlation among parents within each of the different centres (cluster variable) into account.

Due to the complex mediation pattern (see the online supplementary appendix), only controlled direct effects[24] (i.e. the effects of exposures on the outcome that would be observed if the mediator were controlled uniformly at a fixed value) were calculated. In particular, the direct effects on the Bernoulli-distributed mediators and the direct effects on the multinomial-distributed outcome were summarised as odds ratios (ORs) and relative risk ratios (RRRs), respectively. The interactions of the offspring's gender with maternal/paternal smoking and asthma were evaluated by testing the significance of the extra parameters in the models.

The statistical analyses were carried out using STATA 14.2 (StataCorp, College Station, TX) and Mplus 8 (Muthén & Muthén, Los Angeles, CA).

Sensitivity analyses

Sensitivity analyses (see the online supplementary appendix) were performed in order to check if:

- the covariates included in the models represent the "*minimal sufficient adjustment set*" (i.e. the group of measured covariates that needs to be included in order to eliminate confounding) through a directed acyclic graph (DAG; Figure 1S, available as supplementary data),[25] using DAGitty (*http://dagitty.net*);
- the inclusion of one unmeasured confounder in the models[26] changes the estimate of the direct effects of grandmothers' smoking on offspring's asthma, using the Umediation package (*https://github.com/SharonLutz/Umediation*) in R3.4.1.

RESULTS

Main characteristics of the subjects

The 2233 mothers and 1964 fathers included in the present analyses were of similar age, and their parents had similar education levels (Table 1). Mothers, compared to fathers, were more likely to have ever had asthma (18.3 *vs* 12.7%), to report that their mothers (11.0 *vs* 7.6%) and fathers (9.2 *vs* 7.4%) had ever had asthma, and to report that their mothers had smoked during their pregnancy (10.5 *vs* 6.7%).
Half of the parents had two offspring, and 24.0% of the mothers and 22.5% of the fathers had only one child (Table 2S, available as supplementary data). The 4666 offspring in the maternal line (females: 50.3%; age range: 1-51 years) were more likely to have ever had asthma with or without nasal allergies (6.8 *vs* 6.0% and 8.2 *vs* 4.8%, respectively) than the 4192 offspring in the paternal line (females: 49.1%; age range: 0-48 years; Table 2). Of all the offspring, 12.5% were born to the 239 fathers (12.2%; Table 1) who had started smoking before they were 15, and 29.2% had been exposed to their mother's smoking during pregnancy (Table 2).

Recurrence of asthma across three generations

The risk of mothers' asthma (generation F1) was higher if their parents (generation F0) had ever had asthma (grandmothers' asthma: OR = 2.24; grandfathers' asthma: OR = 2.60; Table 3). The risk of asthma with or without nasal allergies in offspring (generation F2) was higher if the offspring's mother had ever had asthma (RRR = 2.50 and 1.69, respectively). Similar results were found within the paternal line (Table 4). Whether the offspring was a boy or a girl did not modify the association of parents' asthma with the offspring's asthma (tests for interaction: p-value >0.9).

These estimates did not change when grandparental/parental smoking and education level were excluded from the models (Table 3S and Table 4S, available as supplementary data).

Associations of tobacco smoking with asthma across three generations

Grandmothers' smoking when mothers were *in utero* (generation F0) was significantly associated with maternal asthma (generation F1; OR = 1.55; Table 3). In turn, mothers' smoking when the offspring was *in utero* (generation F1) was significantly associated with asthma without nasal allergies in their offspring (generation F2; RRR = 1.27). Within the paternal line, we did not find any association between grandmothers' smoking during pregnancy and fathers' asthma (Table 4). However, if fathers had started smoking before they were 15, the risk of asthma without nasal allergies in their offspring was higher (RRR = 1.43). The associations of parental smoking with asthma without nasal allergies in their offspring were not significantly different whether the offspring was a boy or a girl (tests for interaction: p-value >0.2).

Grandmothers' smoking when mothers were *in utero* (generation F0) was positively associated with asthma with nasal allergies in their grandchildren (generation F2; RRR = 1.25; Table 3). This association did not reach statistical significance when fathers were *in utero*.

Sensitivity analyses

The DAG analysis supported the assumption that the measured covariates included in the models represent the "*minimal sufficient adjustment set*" (see the online supplementary appendix). In addition, the simulation analyses showed that the

inclusion of one unmeasured confounder in the models had a limited impact on the estimate of the direct effects of grandmothers' smoking on offspring's asthma (Figure 2S, available as supplementary data).

DISCUSSION

We have shown that fathers' smoking during early puberty is associated with a higher risk of asthma without nasal allergies in their offspring, suggesting an effect of paternal pre-adolescent environment on the next generation. We have also shown that grandmothers' smoking when mothers were *in utero* is a possible risk factor for asthma with nasal allergies in their grandchildren, suggesting a multi-generation effect of tobacco smoking. Finally, we have confirmed the higher risk of asthma in the offspring of mothers who smoked during their pregnancy and the recurrence of asthma across generations. Our findings have considerable public health implications with regard to the environment of male adolescents and to forecast the health of future generations.

Recurrence of asthma across three generations

We have found that asthma susceptibility recurred from grandparents to grandchildren, irrespective of the parent/offspring's gender. These results support the well-established evidence that the offspring of asthmatic parents are at a higher risk of asthma.[28] Although some case-control and cross-sectional surveys on asthma recurrence have shown that this was more marked for mothers,[29] a longitudinal study has found a comparable risk in the parental lines,[30] in agreement with our findings.

The association of mothers' asthma with their offspring's asthma can be explained through a combination of genetic and non-genetic factors *in utero* (e.g. genetic imprinting, the trans-placental passage of Th2 cytokines and immunologic cells[31]), maternally dependent post-natal exposures, such as breastfeeding,[32] and

hormonal factors.[33] Asthma phenotypes, which mainly depend on the effect of paternal asthma, are likely mediated either by hormonal mechanisms or through imprinting.[33]

Associations of tobacco smoking with asthma across three-generations

Tobacco smoking has adverse effects on human fertility, reproduction, and early development.[34,35] The most consistent association with offspring's asthma has been found for maternal smoking during pregnancy,[1–4] which may permanently affect the lungs.[5] Animal studies have shown that nicotine can penetrate the placental barriers and disturb alveolar development,[36] expression of nicotinic receptors,[37] and lung function.[38] In agreement with this knowledge, we have found that grandmothers' smoking during pregnancy was associated with asthma in their sons and daughters and, in turn, maternal smoking during pregnancy was associated with asthma in their offspring, irrespective of the offspring's gender.

A key-finding is the association of grandmothers' smoking when the mother was *in utero* with asthma with nasal allergies in their grandchildren, irrespective of maternal asthma and smoking status during pregnancy. This is consistent with previous studies on humans.[2,12,13] Epigenetic changes may be a potential explanation for this association (see the online supplementary appendix).[39,40] In fact, tobacco smoking may cause heritable modifications of the epigenome, particularly in the prenatal period and shortly after birth.[41] Animal data have shown that these epigenetic changes may be inherited by second-generation offspring, and affect lung function.[42] One study on humans has highlighted a link between prenatal smoke exposure, DNA methylation changes, and asthma-related lung function.[43] An alternative explanation is that the association between grandmothers' smoking and

grandchildren's asthma might be due to confounding effects of other lifestyle and environmental factors. However, we controlled for education level, which may act as a proxy for some of these factors. The results pertaining to the education level of parents/grandparents are discussed in the online supplementary appendix.

A ground-breaking finding of our study is that paternal smoking before 15 years of age was associated with asthma without nasal allergies in their offspring, irrespective of gender. This is of particular concern as smoking in 11- to 15-year-old boys has increased in Europe over recent decades (Alessandro Marcon. Data presented at the European Respiratory Society International Congress 2016). At present, public health strategies do not focus on the environment of male adolescents with regard to the health of their future offspring and to do so would represent a paradigm-shift in preventive policies. Our results are consistent with findings from the RHINE study,[16] a questionnaire-based postal follow-up of the ECRHS subjects from the 7 Nordic centres listed in Table 1S (available as supplementary data). A minority of the parents evaluated in RHINE (11.5%) also underwent clinical examinations as part of the ECRHS and are included in this report. The present work is based on clinical interview data from these Nordic centres and 19 additional centres (located in other parts of Europe and Australia). One report from the Avon Longitudinal Study of Parents and Children (ALSPAC), showing that body fat increases in the sons of fathers who had started smoking in early puberty, also supports the hypothesis that paternal lifestyle and exposures well before conception may influence the health of their offspring.[44] The heritable effect of smoking in young males seems biologically plausible. Male adolescence represents a critical period for the germ line development[15] and for the susceptibility to tobacco-related DNA damage. Reproductive cells in male adolescents are characterised by an increased number of

cell divisions, and they have a six-fold higher risk of DNA mutations than female oocytes.[45] Smokers have altered spermatozoal mRNA profiles compared to non-smokers.[46] Tobacco smoking could also induce changes in the miRNA profiles of spermatozoa, leading to harmful phenotypes that are hypothesised to be transmitted to future generations through the male germ line.[47] Altered miRNA is involved in perturbation of cell death and apoptosis pathways.[47] Spermatozoal miRNA could be transferred to oocyte at fertilization[48] and to target epigenetic compounds, which are important in DNA methylation and histone modification, and it could mediate gene expression during embryogenesis and alter phenotypes in future progeny. Curiously, in our study, grandmothers' smoking during pregnancy was associated with asthma with nasal allergies in their grandchildren, whereas maternal smoking during pregnancy and paternal smoking during puberty were associated with asthma without nasal allergies in their offspring. We speculate that parental smoking may have a detrimental effect on lung growth and function during foetal development, whereas grandmothers' smoking could give rise to epigenome changes that alter the expression of inflammatory genes or regulate immune development.

Strengths and weaknesses

The information in the present study was available from three generations of subjects. The parents were selected from the general population in different countries and they were interviewed in clinical settings following a highly standardised protocol. Moreover, the analyses were carried out using appropriate statistical methods for evaluating the complex pattern of associations among variables in different generations.

There are very few epidemiological studies with detailed information on respiratory health across generations and, in the ECRHS centres involved in the Ageing Lungs in European Cohorts (ALEC) study (see *www.alecstudy.org*), this work is being extended to include health assessment of children and registry-based collection of grandparents' health status. However, the ECRHS is not a family-based study. It recruited a representative sample of men (fathers) and women (mothers), but their partners (co-parent of the offspring) did not participate in the study. Moreover, the information regarding grandparents and offspring was parent-reported, rather than directly assessed. This could have generated an information bias across generations and between the parental lines (see the online supplementary appendix).

It is also possible that important confounders [e.g. parental socio-economic status (SES) and offspring's smoking history] were not included in the models. However, as we believe that an early start of smoking is one of the major SES-related exposures responsible for the influence of SES on health, we might expect that adjusting for this would attenuate our smoking-related associations. Moreover, in our simulations, unmeasured confounding had a limited impact on the estimated associations of grandmothers' smoking with offspring's asthma.

Finally, we investigated the associations of grand-maternal/parental smoking with offspring's atopic/non-atopic asthma, rather than conditioning on offspring's nasal allergies. Indeed, the inclusion of offspring's asthma as the outcome and offspring's nasal allergies as a mediator in the models would induce spurious exposure-outcome associations (collider bias), if we assume:[49] (i) an effect of grand-maternal/parental smoking on offspring's nasal allergies; (ii) the possibility of unmeasured confounders associated with offspring's asthma and nasal allergies (but not with grand-

maternal/parental smoking); (iii) a probable link between nasal allergies and asthma in offspring.

Conclusions

The present analyses suggest that smoking during pregnancy and male puberty may increase the risk of asthma in the next generation, and that the effect of smoking during pregnancy may continue into a further generation within the maternal line. Our results provide further evidence on asthma recurrence across multiple generations. Therefore, risk factors for asthma should be sought before conception in men and women to improve the health of future generations.

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REFERENCES

1. Jaakkola JJ, Gissler M. Maternal smoking in pregnancy, fetal development, and childhood asthma. Am J Public Health 2004; 94:136-140.
2. Li YF, Langholz B, Salam MT, Gilliland FD. Maternal and grandmaternal smoking patterns are associated with early childhood asthma. Chest 2005; 127:1232-1241.
3. Skorge TD, Eagan TM, Eide GE, Gulsvik A, Bakke PS. The adult incidence of asthma and respiratory symptoms by passive smoking in utero or in childhood. Am J Respir Crit Care Med 2005; 172:61-66.
4. Fuentes-Leonarte V, Estarlich M, Ballester F, et al. Pre- and postnatal exposure to tobacco smoke and respiratory outcomes during the first year. Indoor Air 2015; 25:4-12.
5. Maritz GS, Harding R. Life-long programming implications of exposure to tobacco smoking and nicotine before and soon after birth: evidence for altered lung development. Int J Environ Res Public Health 2011; 8:875-898.
6. Svanes C, Omenaas E, Jarvis D, Chinn S, Gulsvik A, Burney P. Parental smoking in childhood and adult obstructive lung disease: results from the European Community Respiratory Health Survey. Thorax 2004; 59:295-302.
7. Maritz GS. Perinatal exposure to nicotine and implications for subsequent obstructive lung disease. Paediatr Resp Rev 2013; 14:3-8.
8. Dratva J, Zemp E, Dharmage SC, et al. Early life origins of lung ageing: early life exposures and lung function decline in adulthood in two European cohorts aged 28-73 years. PLoS One 2016; 11:e0145127.

9. Pembrey M, Saffery R, Bygren LO, Network in Epigenetic Epidemiology. Human transgenerational responses to early-life experience: potential impact on development, health and biomedical research. J Med Genet 2014; 51:563-572.
10. DeVries A, Vercelli D. Epigenetic mechanisms in asthma. Ann Am Thorac Soc 2016; 13(Suppl 1):S48-50.
11. Maritz GS, Mutemwa M. The effect of grand maternal nicotine exposure during gestation and lactation on lung integrity of the F2 generation. Pediatr Pulmonol 2014; 49:67-75.
12. Magnus MC, Håberg SE, Karlstad Ø, Nafstad P, London SJ, Nystad W. Grandmother's smoking when pregnant with the mother and asthma in the grandchild: the Norwegian Mother and Child Cohort Study. Thorax 2015; 70:237-243.
13. Lodge CJ, Bråbäck L, Lowe AJ, Dharmage SC, Olsson D, Forsberg B. Grandmaternal smoking increases asthma risk in grandchildren: A nationwide Swedish cohort. Clin Exp Allergy. 2017 Sep 19. doi: 10.1111/cea.13031. [Epub ahead of print].
14. Miller LL, Henderson J, Northstone K, Pembrey M, Golding J. Do grandmaternal smoking patterns influence the etiology of childhood asthma? Chest 2014; 145:1213-1218.
15. Soubry A, Hoyo C, Jirtle RL, Murphy SK. A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. Bioassays 2014; 36:359-371.
16. Svanes C, Koplin J, Skulstad SM, et al. Father’s environment before conception and asthma risk in his children: a multi-generation analysis of the Respiratory Health In Northern Europe study. Int J Epidemiol 2017; 46:235-245.

17. Burney PG, Luczynska C, Chinn S, Jarvis D. The European Community Respiratory Health Survey. Eur Respir J 1994; 7:954-960.
18. European Community Respiratory Health Survey II Steering Committee. The European Community Respiratory Health Survey II. Eur Respir J 2002; 20:1071-1079.
19. Amaral AF, Newson RB, Abramson MJ, et al. Changes in IgE sensitization and total IgE levels over 20 years of follow-up. J Allergy Clin Immunol 2016; 137:1788-1795.
20. Håkonsen LB, Olsen J, Støvring H, et al. Maternal cigarette smoking during pregnancy and pubertal development in sons. A follow-up study of a birth cohort. Andrology 2013; 1:348-355.
21. Hill M. Social policy: a comparative analysis. London: Prentice-Hall/Harvester Wheatsheaf, 1996.
22. Muthén BO, Muthén LK, Asparouhov T. Regression and mediation analysis using Mplus. Los Angeles: Muthén & Muthén, 2016.
23. Krull JL, MacKinnon DP. Multilevel modeling of individual and group level mediated effects. Multivariate Behav Res 2001; 36:249-277.
24. Pearl J. Direct and indirect effects. In: Proceedings of the Seventeenth Conference on Uncertainty in Artificial Intelligence. San Francisco: Morgan Kaufmann, 2001; pp. 411-420.
25. Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. Epidemiology 1999; 10:37-48.
26. Lutz SM, Thwing A, Schmiege S, et al. Examining the role of unmeasured confounding in mediation analysis with genetic and genomic applications. BMC Bioinformatics 2017; 18:344.

27. Satorra A, Bentler PM. Ensuring positiveness of the scaled difference chi-square test statistic. Psychometrika 2010; 75:243-248.
28. Thomsen SF. Genetics of asthma: an introduction for the clinician. Eur Clin Respir J 2015; 2:24643.
29. Lim RH, Kobzik L, Dahl M. Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. PLoS One 2010; 5:e10134.
30. Paaso EM, Jaakkola MS, Lajunen TK, Hugg TT, Jaakkola JJ. The importance of family history in asthma during the first 27 years of life. Am J Respir Crit Care Med 2013; 188:624-626.
31. Lim RH, Kobzik L. Maternal transmission of asthma risk. Am J Reprod Immunol 2009; 61:1-10.
32. Guilbert TW, Stern DA, Morgan WJ, Martinez FD, Wright AL. Effect of breastfeeding on lung function in childhood and modulation by maternal asthma and atopy. Am J Respir Crit Care Med 2007; 176:843-848.
33. Raby BA, Van Steen K, Celedón JC, Litonjua AA, Lange C, Weiss ST. Paternal history of asthma and airway responsiveness in children with asthma. Am J Respir Crit Care Med 2005; 172:552-558.
34. Rogers JM. Tobacco and pregnancy. Reprod Toxicol 2009; 28:152-60.
35. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. Nat Rev Urol 2017; 14:470-485.
36. Maritz GS, Dennis H. Maternal nicotine exposure during gestation and lactation interferes with alveolar development in the neonatal lung. Reprod Fertil Dev 1998; 10:255-261.

37. Sekhon HS, Jia Y, Raab R, et al. Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. J Clin Invest 1999; 103:637-647.
38. Sekhon HS, Keller JA, Benowitz NL, Spindel ER. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. Am J Respir Crit Care Med 2001; 164:989-994.
39. Skinner MK. Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. Mol Cell Endocrinol 2014; 398:4-12.
40. Arshad SH, Karmaus W, Zhang H, Holloway JW. Multigenerational cohorts in patients with asthma and allergy. J Allergy Clin Immunol 2017; 139:415-421.
41. Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. Reprod Toxicol 2011; 31:363-373.
42. Rehan VK, Liu J, Naeem E, et al. Perinatal nicotine exposure induces asthma in second generation offspring. BMC Med 2012; 10:129.
43. Patil VK, Holloway JW, Zhang H, et al. Interaction of prenatal maternal smoking, interleukin 13 genetic variants and DNA methylation influencing airflow and airway reactivity. Clin Epigenetics 2013; 5:22.
44. Pembrey ME, Bygren LO, Kaati G, et al. Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet 2006; 14:159-166.
45. Forster P, Hohoff C, Dunkelmann B, et al. Elevated germline mutation rate in teenage fathers. Proc Biol Sci 2015; 282:20142898.
46. Linschooten JO, Van Schooten FJ, Baumgartner A, et al. Use of spermatozoal mRNA profiles to study gene-environment interactions in human germ cells. Mutat Res 2009; 667:70-76.

47. Marczylo EL, Amoako AA, Konje JC, Gant TW, Marczylo TH. Smoking induces differential miRNA expression in human spermatozoa: a potential transgenerational epigenetic concern? Epigenetics 2012; 7:432-439.
48. Amanai M, Brahmajosyula M, Perry AC. A restricted role for sperm-borne microRNAs in mammalian fertilization. Biol Reprod 2006; 75:877-884.
49. Greenland S. Quantifying biases in causal models: classical confounding vs collider-stratification bias. Epidemiology 2003; 14:300-306.

FIGURE LEGENDS

Figure 1. Study population of parents and offspring, according to the parental line.

Detailed legend:

* Six mothers were excluded because their age at their child's birth was <13 years. † Complete information on offspring's gender, birth year, asthma, and nasal allergies (including hay fever).

Figure 2. Two-level mediation model within the maternal line.

Detailed legend:

The two ellipses represent: (i) the level 2 unit (mother; the presence of arrows indicates the random intercept terms at level 2); (ii) the cluster variable (centre; the absence of arrows indicates that cluster-robust standard errors were computed in order to take the correlation among mothers within centres into account). * The "*unknown*" category is not shown. † Smoking during other periods (or unknown smoking period).

Figure 3. Two-level mediation model within the paternal line.

Detailed legend:

The two ellipses represent: (i) the level 2 unit (father; the presence of arrows indicates the random intercept terms at level 2); (ii) the cluster variable (centre; the absence of arrows indicates that cluster-robust standard errors were computed in order to take the correlation among fathers within centres into account). * The "*unknown*" category is not shown. † Smoking during other periods (or unknown smoking period).

Table 1. Main characteristics of the parents and grandparents, according to the parental line.

	Maternal line	Paternal line	
N° of parents	n = 2233	n = 1964	p-value*
Grandmother's ever asthma, %	11.0	7.6	<0.001
Grandfather's ever asthma, %	9.2	7.4	0.04
Grandparents' education level, %			0.56
low	45.7	47.0	
high	52.1	51.1	
unknown	2.2	1.9	
Grandmother's smoking, %			<0.001
when the parent was *in utero*	10.5	6.7	
during other periods (or unknown smoking period)	13.5	15.3	
not smoking	76.0	78.0	
Parent's age (years), median (range)	55 (40-67)	55 (40-67)	0.08
Parent's ever asthma, %	18.3	12.7	<0.001
Parent's education level, %			0.35
low	14.1	12.6	
high	82.2	83.4	
unknown	3.8	4.0	
Father's smoking initiation, %			-
<15 years of age	-	12.2	
≥15 years of age	-	51.3	
not smoking	-	36.6	

* Obtained by using Pearson chi-square and Wilcoxon-Mann-Whitney tests.

Table 2. Main characteristics of the offspring, according to the parental line.

	Maternal line	Paternal line	
N° of offspring	n = 4666	n = 4192	p-value
Offspring's gender (female), %	50.3	49.1	- *
Offspring's age (years), median (range)	26 (1-51)	24 (0-48)	- *
Offspring's ever asthma, %			<0.001†
with nasal allergies	6.8	6.0	
without nasal allergies	8.2	4.8	
never asthma	85.0	89.2	
Mother's smoking, %			-
when the offspring was *in utero*	29.2	-	
during other periods	26.2	-	
not smoking	44.6	-	

* Not computed because of the hierarchical data structure (offspring nested within parents). † Obtained by using the likelihood ratio test for the comparison of the goodness-of-fit of the following nested models: (i) two-level multinomial regression model (parent = level 2 unit) with the offspring's ever asthma as the outcome and the parental line as the covariate; (ii) the previous model with no covariates.

Table 3: Controlled direct effects[24] within the maternal line.

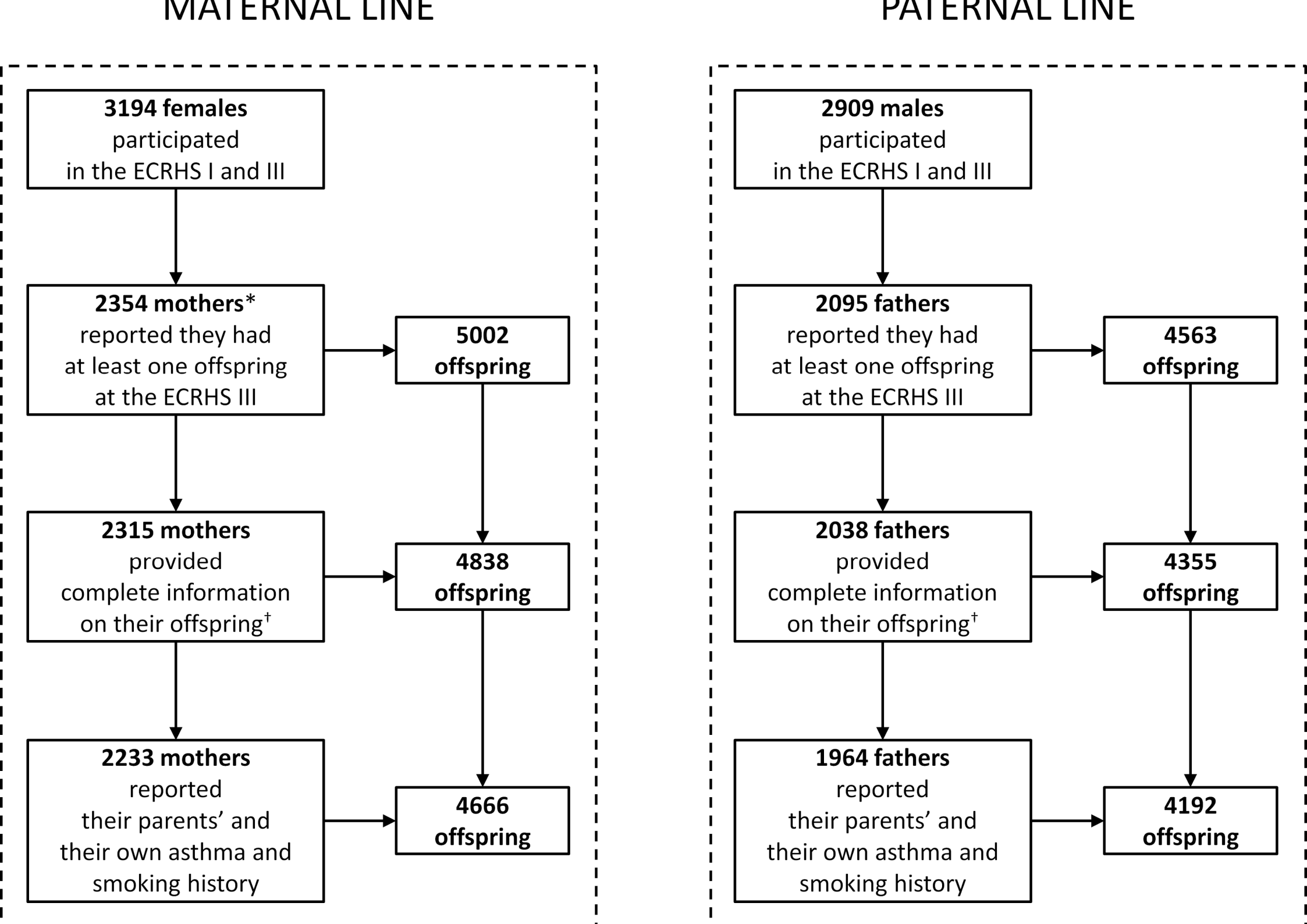
GENERATION		F1	F2	
		Mother's ever asthma	Offspring's ever asthma with nasal allergies	Offspring's ever asthma without nasal allergies
		OR (95% CI)	RRR (95% CI)	RRR (95% CI)
F0	Grandmother's ever asthma (present *vs* absent)	2.24 (1.58-3.17)	-	-
	Grandfather's ever asthma (present *vs* absent)	2.60 (1.98-3.42)	-	-
	Grandparents' education level* (low *vs* high)	0.71 (0.58-0.87)	-	-
	Grandmother's smoking (*vs* not smoking)			
	when the mother was *in utero*	1.55 (1.17-2.06)	1.25 (1.02-1.55)	1.31 (0.86-1.98)
	during other periods (or unknown smoking period)	1.12 (0.83-1.52)	1.20 (0.88-1.63)	1.12 (0.85-1.48)
F1	Mother's age (1-year increase)	1.00 (0.99-1.02)	-	-
	Mother's ever asthma (present *vs* absent)	-	2.50 (1.95-3.22)	1.69 (1.25-2.28)
	Mother's education level* (low *vs* high)	-	1.31 (0.93-1.83)	1.79 (1.26-2.55)
	Mother's smoking (*vs* not smoking)			
	when the offspring was *in utero*	-	1.06 (0.76-1.49)	1.27 (1.01-1.59)
	during other periods	-	0.87 (0.61-1.24)	0.96 (0.71-1.28)
F2	Offspring's gender (female *vs* male)	-	0.80 (0.66-0.97)	0.89 (0.71-1.12)
	Offspring's age (1-year increase)	-	0.98 (0.97-1.00)	0.96 (0.95-0.98)

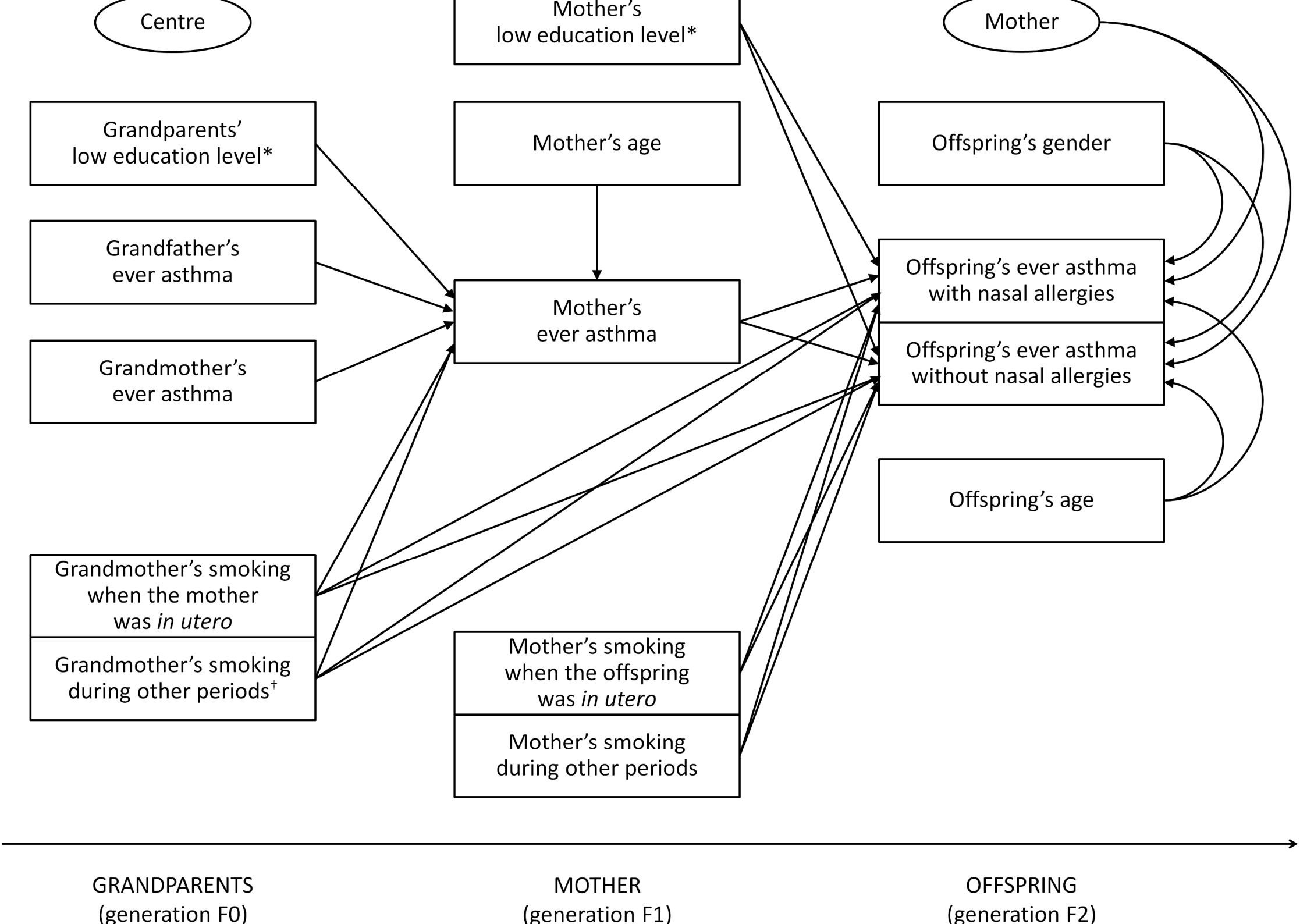
Comparison of the goodness-of-fit between the present mediation model and the cluster-robust (centre = cluster variable) two-level (mother = level 2 unit) multinomial regression model (outcome: offspring's ever asthma with or without nasal allergies; covariates: grandmother's smoking, mother's ever asthma, education level and smoking, offspring's gender and age): p-value (Satorra-Bentler scaled chi-squared test for nested models[27] with 8 degrees of freedom) <0.0001. OR: odds ratio; RRR: relative risk ratio; CI: confidence interval. * The estimates for the "*unknown*" category are not shown.

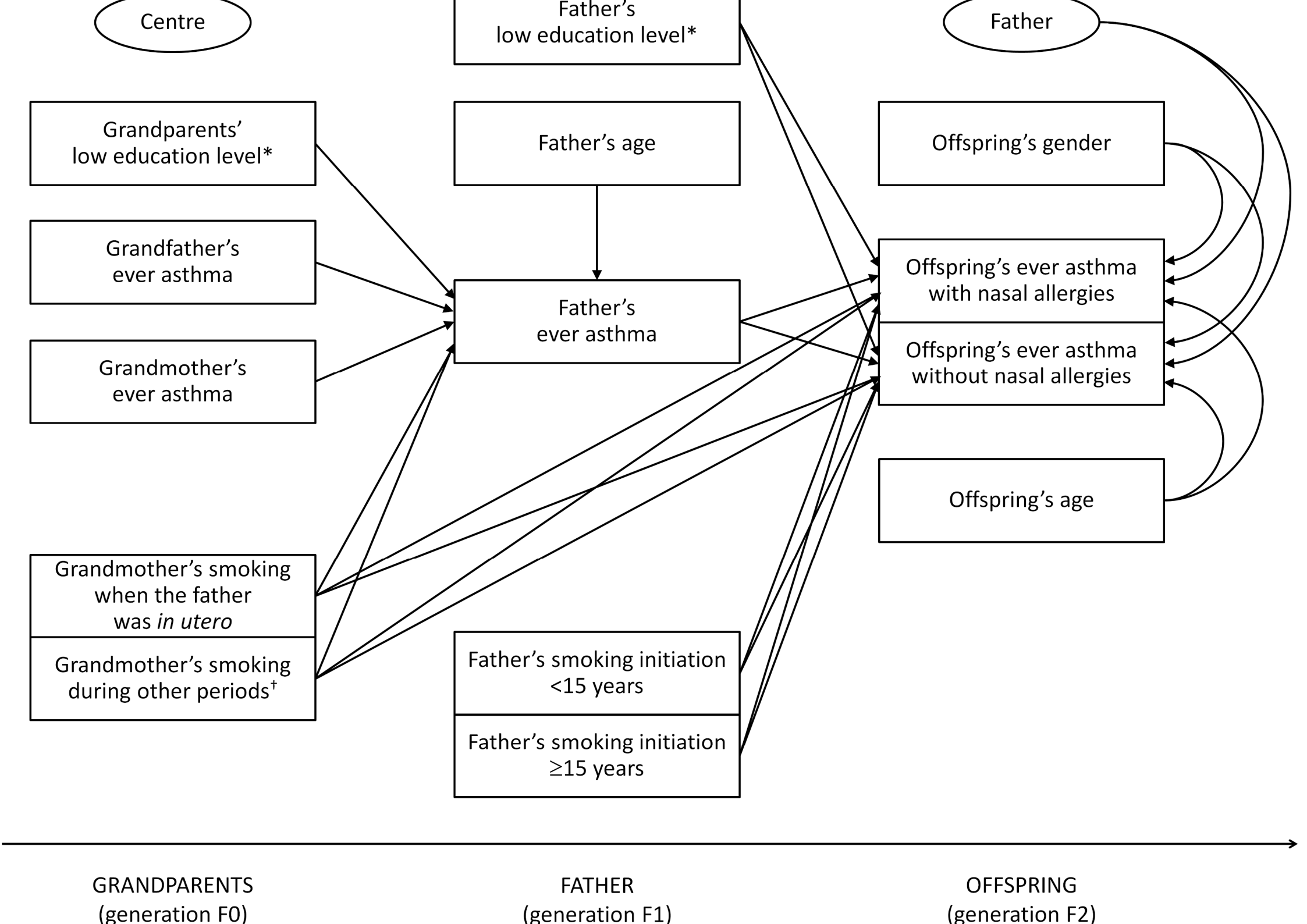
Table 4: Controlled direct effects[24] within the paternal line.

GENERATION		F1	F2	
		Father's ever asthma	Offspring's ever asthma with nasal allergies	Offspring's ever asthma without nasal allergies
		OR (95% CI)	RRR (95% CI)	RRR (95% CI)
F0	Grandmother's ever asthma (present *vs* absent)	3.08 (1.96-4.85)	-	-
	Grandfather's ever asthma (present *vs* absent)	2.38 (1.51-3.75)	-	-
	Grandparents' education level* (low *vs* high)	0.96 (0.71-1.30)	-	-
	Grandmother's smoking (*vs* not smoking)			
	when the father was *in utero*	0.82 (0.47-1.44)	1.60 (0.95-2.68)	1.08 (0.55-2.13)
	during other periods (or unknown smoking period)	1.02 (0.62-1.67)	1.24 (0.81-1.91)	1.35 (0.87-2.09)
F1	Father's age (1-year increase)	0.99 (0.96-1.02)	-	-
	Father's ever asthma (present *vs* absent)	-	2.37 (1.63-3.43)	1.70 (1.14-2.53)
	Father's education level* (low *vs* high)	-	0.47 (0.27-0.83)	0.87 (0.49-1.53)
	Father's smoking initiation (*vs* not smoking)			
	<15 years of age	-	1.19 (0.74-1.90)	1.43 (1.01-2.01)
	≥15 years of age	-	0.98 (0.71-1.36)	0.88 (0.70-1.11)
F2	Offspring's gender (female *vs* male)	-	0.71 (0.59-0.84)	0.83 (0.70-0.98)
	Offspring's age (1-year increase)	-	1.00 (0.98-1.02)	0.96 (0.94-0.99)

Comparison of the goodness-of-fit between the present mediation model and the cluster-robust (centre = cluster variable) two-level (father = level 2 unit) multinomial regression model (outcome: offspring's ever asthma with or without nasal allergies; covariates: grandmother's smoking, father's ever asthma, education level and smoking initiation, offspring's gender and age): p-value (Satorra-Bentler scaled chi-squared test for nested models[27] with 8 degrees of freedom) <0.0001. OR: odds ratio; RRR: relative risk ratio; CI: confidence interval. * The estimates for the "*unknown*" category are not shown.


MATERNAL LINE
3194 females participated in the ECRHS I and III
2354 mothers* reported they had at least one offspring at the ECRHS III
5002 offspring
2315 mothers provided complete information on their offspring†
4838 offspring
2233 mothers reported their parents' and their own asthma and smoking history
4666 offspring
PATERNAL LINE
2909 males participated in the ECRHS I and III
2095 fathers reported they had at least one offspring at the ECRHS III
4563 offspring
2038 fathers provided complete information on their offspring†
4355 offspring
1964 fathers reported their parents' and their own asthma and smoking history
4192 offspring


Centre
Mother's low education level*
Mother
Grandparents' low education level*
Mother's age
Offspring's gender
Grandfather's ever asthma
Mother's ever asthma
Offspring's ever asthma with nasal allergies
Offspring's ever asthma without nasal allergies
Grandmother's ever asthma
Offspring's age
Grandmother's smoking when the mother was in utero
Grandmother's smoking during other periods†
Mother's smoking when the offspring was in utero
Mother's smoking during other periods
GRANDPARENTS (generation F0)
MOTHER (generation F1)
OFFSPRING (generation F2)


Centre
Father's low education level*
Father
Grandparents' low education level*
Father's age
Offspring's gender
Grandfather's ever asthma
Father's ever asthma
Offspring's ever asthma with nasal allergies
Grandmother's ever asthma
Offspring's ever asthma without nasal allergies
Offspring's age
Grandmother's smoking when the father was in utero
Grandmother's smoking during other periods†
Father's smoking initiation <15 years
Father's smoking initiation ≥15 years
GRANDPARENTS (generation F0)
FATHER (generation F1)
OFFSPRING (generation F2)

ONLINE SUPPLEMENTARY APPENDIX

A three-generation study on the association of tobacco smoking with asthma

Simone Accordini,[1,*] Lucia Calciano,[1] Ane Johannessen,[2] Laura Portas,[1] Bryndis Benediktsdóttir,[3] Randi Jacobsen Bertelsen,[4,5] Lennart Bråbäck,[6] Anne-Elie Carsin,[7,8,9] Shyamali C Dharmage,[10] Julia Dratva,[11,12] Bertil Forsberg,[6] Francisco Gomez Real,[4] Joachim Heinrich,[13] John W Holloway,[14] Mathias Holm,[15] Christer Janson,[16] Rain Jögi,[17] Bénédicte Leynaert,[18] Andrei Malinovschi,[19] Alessandro Marcon,[1] Jesús Martínez-Moratalla Rovira,[20,21] Chantal Raherison,[22] José Luis Sánchez-Ramos,[23] Vivi Schlünssen,[24,25] Roberto Bono,[26] Angelo G Corsico,[27] Pascal Demoly,[28,29] Sandra Dorado Arenas,[30] Dennis Nowak,[13,31] Isabelle Pin,[32,33,34] Joost Weyler,[35] Deborah Jarvis,[36,37,†] Cecilie Svanes,[2,5,†]; on behalf of the Ageing Lungs in European Cohorts (ALEC) Study.

*Corresponding author. [†]Equally contributed.

[1]Unit of Epidemiology and Medical Statistics, Department of Diagnostics and Public Health, University of Verona, Verona, Italy; [2]Centre for International Health, University of Bergen, Bergen, Norway; [3]Faculty of Medicine, University of Iceland, Reykjavik, Iceland; [4]Department of Clinical Science, University of Bergen, Bergen, Norway; [5]Department of Occupational Medicine, Haukeland University Hospital, Bergen, Norway; [6]Division of Occupational and Environmental Medicine, Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [7]ISGlobal, Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain; [8]Universitat Pompeu Fabra (UPF), Barcelona, Spain; [9]CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain; [10]School of Population and Global Health, University of Melbourne, Melbourne, Australia; [11]ZHAW School of Health Professions, Winterthur, Switzerland; [12]Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel University, Basel, Switzerland; [13]Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, Inner City Clinic, University Hospital Munich, Ludwig-Maximilians-Universität Munich, Germany; [14]Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK; [15]Department of Occupational and Environmental Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [16]Department of Medical Sciences: Respiratory, Allergy and Sleep Research, Uppsala University, Uppsala, Sweden; [17]Lung Clinic, Tartu University Hospital, Tartu, Estonia; [18]INSERM UMR 1152, Pathophysiology and Epidemiology of Respiratory Diseases, Paris, France; [19]Department of Medical Sciences: Clinical Physiology, Uppsala University, Uppsala, Sweden; [20]Servicio de Neumología del Complejo Hospitalario Universitario de Albacete (CHUA), Servicio de Salud de Castilla-La Mancha (SESCAM), Albacete, Spain; [21]Facultad de Medicina de Albacete, Universidad de Castilla-La Mancha, Albacete, Spain; [22]INSERM U1219, Bordeaux University, Bordeaux, France; [23]Department of Nursing, University of Huelva, Huelva, Spain; [24]Department of Public Health, Aarhus University, Aarhus, Denmark; [25]National Research Center for the Working Environment, Copenhagen, Denmark; [26]Department of Public Health and Pediatrics, University of Turin, Turin, Italy; [27]Division of Respiratory Diseases, IRCCS 'San Matteo' Hospital Foundation-University of Pavia, Pavia, Italy; [28]Département de Pneumologie et Addictologie, Hôpital Arnaud de Villeneuve, University Hospital of Montpellier, Montpellier, France; [29]UMR-S 1136 INSERM, IPLESP, UPMC, Sorbonne Universités, Paris, France; [30]Unit of Pneumology, Galdakao-Usansolo Hospital, Galdakao, Spain; [31]Comprehensive Pneumology Center Munich, Member of the German Center for Lung Research, Munich, Germany; [32]Pediatrie, CHU Grenoble Alpes, Grenoble, France; [33]INSERM 1209, Institute for Advanced Biosciences, Grenoble, France; [34]Université Grenoble Alpes, Grenoble, France; [35]Epidemiology and Social Medicine and the StatUA Statistics Centre, University of Antwerp, Antwerp, Belgium; [36]Faculty of Medicine, National Heart & Lung Institute, Imperial College, London, UK; [37]MRC-PHE Centre for Environment and Health, Imperial College, London, UK.

HIERARCHICAL DATA STRUCTURE

Our data have a hierarchical (three-level) structure because we included multiple offspring from the same parent in the analyses and because many parents had been sampled from each of the different centres, that is:

i) Individual men and women (rather than men and women who were living as couples) were recruited in the ECRHS and each person provided information on all her/his offspring. The number of offspring per parent ranged from 1 to 8 (see Table 2S). Accordingly, we included one or more offspring (level 1 units) for each parent (level 2 units) in our analyses. Therefore, "*parent*" is the clustering variable for the offspring from the same parent (siblings).

ii) In the ECRHS, individual men and women (one of the two parents; level 2 units) were sampled from each of the 26 centres (level 3 units) included in the present analyses (see Table 1S). Therefore, "*centre*" is the clustering variable for the parents sampled from the same centre.

MEDIATION MODELS

The statistical evaluation of associations across multiple generations [grandparents (F0), parents (F1), offspring (F2)] raises several methodological issues. Firstly, the association of grandparental exposures with offspring's health may be mediated by factors measured in the intermediate generation (parents). These variables are called "mediators"[1] and appropriate statistical methods (mediation models)[2] must be used to quantify their role across generations. Secondly, multi-generation data have a hierarchical structure, as multiple offspring may be siblings and originate from the same parent. Therefore, the variables of interest are measured at different hierarchical levels (i.e. in parents and in their offspring), requiring complex configurations for mediation modelling. Finally, further complexity is present in data from multicentre studies, due to the correlation among subjects from the same centre, and when the outcome includes different phenotypes (e.g. allergic and non-allergic asthma) with a non-normal distribution.

In the present study, two-level 2→2→1 mediation models[2,3] were used separately within the maternal and paternal lines. Mediation analyses were carried out within a complex hierarchical framework because:

i) Our data have a hierarchical (three-level) structure, having investigated multiple offspring (siblings; level 1 units) from the same parent (level 2 units) and having sampled many parents from each of the different centres (level 3 units).

ii) The mediation pattern is 2→2→1, having measured the predictors of the mediator (e.g. parent-reported grand-maternal smoke), the mediator (parental

asthma) and the outcome (offspring's asthma with or without nasal allergies) at different hierarchical levels, that is:

- the predictors of the mediator at level 2 (parent): **2**→2→1
- the mediator at level 2 (parent): 2→**2**→1
- the outcome at level 1 (offspring): 2→2→**1**

iii) The outcome has a multinomial distribution.

At present, to the best of our knowledge, three-level 2→2→1 non-normal mediation modelling is not included in statistical software. Therefore, we used two-level models with cluster-robust standard errors to take the correlation among parents within each of the different centres (cluster variable) into account.[4] In addition, software routines for computing total, direct, indirect and interactive[5] effects under the counterfactual theory[6] are not available with a two-level 2→2→1 mediation pattern and a multinomial-distributed outcome. Accordingly, in the present study, only controlled direct effects[6] (i.e. the effects of exposures on the outcome that would be observed if the mediator were controlled uniformly at a fixed value) were computed.

Despite these limitations, mediation analyses were carried out in our study because:

i) The temporal ordering among variables can be modelled (according to the MacArthur's approach).[7]

ii) The pattern of associations among all the variables can be evaluated by fitting a single model, instead of using different regression models for the mediator and the outcome. Accordingly, we computed the Satorra-Bentler scaled chi-square test for nested models,[8] showing the higher goodness-of-fit of our mediation models as compared to the cluster-robust (centre = cluster variable) two-level (parent = level 2 unit) multinomial regression models (outcome: offspring's ever asthma with or without nasal allergies; covariates:

grandmother's smoking, parent's ever asthma, education level and smoking, offspring's gender and age; see the legend of Table 3 and Table 4 in the manuscript).

iii) The impact of unmeasured confounders (for the exposure-outcome, exposure-mediator and mediator-outcome relationships) on the estimates can be investigated[9] (see the "*Sensitivity analyses*" section).

SENSITIVITY ANALYSES

Minimal sufficient adjustment set

We checked if the covariates included in the models represent the "*minimal sufficient adjustment set*" (i.e. the group of measured covariates that needs to be included in order to eliminate confounding) through a directed acyclic graph (DAG),[10] using DAGitty (*http://dagitty.net*). This approach helps to avoid the risk of over-adjustment and to establish whether the statistical models used are the most parsimonious. In DAG, we included the same paths as in our mediation models (see Figure 1S).
This analysis supported the assumption that the minimal sufficient adjustment set contains grandparents' asthma ("*asthma_GF*" and "*asthma_GM*" in Figure 1S), grandparents' and parents' education level ("*education_GP*", "*education_P*"), parents' and offspring's age ("*age_P*", "*age_O*"), and offspring's gender ("*gender_O*"), for estimating the relationships among grandmothers' and parents' smoke ("*smoke_GM*", "*smoke_P*"), parents' asthma ("*asthma_P*") and offspring's asthma ("*asthma_O*").

Unmeasured confounding

We evaluated the impact of unmeasured confounding[9] on the estimate of the direct effects of grandmothers' smoking on offspring's asthma, using the Umediation package (*https://github.com/SharonLutz/Umediation*) in R3.4.1. This package makes it possible to simulate unmeasured confounding in mediation models (using a computer-intensive approach) in order to investigate how the results would change if unmeasured confounding were present.

In our simulations:

- We included the same paths as in the mediation models and we added one unmeasured normally-distributed confounder ("*U*" variable) for the exposure-outcome, exposure-mediator and mediator-outcome relationships, with mean 0 and variance 0.001.[9]
- As Umediation allows for normally-distributed or Bernoulli-distributed variables,[9] we carried out four simulation analyses (two for each parental line) splitting the multinomial-distributed outcome (offspring's asthma with or without nasal allergies) into two Bernoulli-distributed outcomes (reference category: no asthma), that is:

  simulation (a) - paternal line, outcome: asthma without nasal allergies

  simulation (b) - paternal line, outcome: asthma with nasal allergies

  simulation (c) - maternal line, outcome: asthma without nasal allergies

  simulation (d) - maternal line, outcome: asthma with nasal allergies
- We carried out the simulation analyses under multiple scenarios for the effects (beta regression coefficients) of the unmeasured confounder U on "*asthma_O*" (outcome; $beta_{UO}$), "*asthma_P*" (mediator; $beta_{UM}$) and "*smoke_GM*" (exposure; $beta_{UE}$), by fixing $beta_{UO} = beta_{UM} = beta_{UE}$ = 0, 1, 3, 5, 7 and 9.
- As inputs for Umediation, we used the coefficients of the cluster-robust (centre = cluster variable) logistic regression models with outcomes: "*asthma_O*" (covariates: "*smoke_GM*", "*asthma_P*", "*smoke_P*", "*education_P*", "*age_O*", "*gender_O*"); "*asthma_P*" (covariates: "*education_GP*", "*asthma_GF*", "*asthma_GM*", "*smoke_GM*", "*age_P*"); "*smoke_GM*" (null model). These coefficients were estimated from 800 bootstrap samplings of one offspring per parent (n = n° offspring = n° parents = 2233 and 1964 in the maternal and

paternal lines, respectively). This was done to avoid the 2→2→1 mediation pattern.

- We specified 1000 simulation runs and 1000 Monte Carlo draws for the nonparametric bootstrap in each of the four simulation analyses.

In these simulations (see Figure 2S), the inclusion of one unmeasured confounder U in the mediation models had a limited impact on the estimate of the direct effects of grandmothers' smoking on offspring's asthma, also when U had a very strong effect on the outcome, the mediator and the exposure ($beta_{UO} = beta_{UM} = beta_{UE} > 5$). Indeed, as the effect increased, the proportion of simulations where the results matched (whether U was included or excluded from the model) remained greater than 89% and the average absolute difference of the direct effects remained lower than 0.008.

DISCUSSION

Mechanisms of disease/exposure transfer

Three mechanisms are recognized:

i) shared environmental exposures, lifestyle or behaviour (e.g. smoking, microbiome or pollution) across generations, acting directly on the individual;
ii) genetic multi-generation inheritance;
iii) epigenetic multi-generation inheritance.

Epigenetic multi-generation inheritance postulates that the environmental exposure of the parent (F0) has direct effects on the epigenome of the developing foetus and potentially on the germline of the foetus, the former leading to altered phenotypes of the child (F1) and the latter leading to altered phenotypes of the grandchild (F2). This implies that effects of exposures in current generations are passed to future generations independently of direct environmental exposures ("*vertical epigenetic transmission model*"), i.e. exposures in the great-grandmother during pregnancy may influence disease development in her great-grandchild (F3), even in the absence of any exposure.[11] Alternatively, it has been proposed that epigenetic variations result in the presence of exposure-related diseases in later generations F1 and F2 ("*induced epigenetic transmission model*").[12] The exposure of the foetus to the disease (e.g. asthma) or the pollutant (e.g. tobacco smoking) could initiate specific differential epigenetic marks in offspring, which in turn can result in offspring experiencing diseases (e.g. asthma) or behaviour (e.g. smoking initiation).

Associations of low education level with asthma across three-generations

We found that grandparents' and fathers' low education levels were negatively associated with maternal asthma (Table 3 in the manuscript) and with asthma with nasal allergies in offspring (Table 4 in the manuscript), respectively. These results are broadly consistent with the "*hygiene hypothesis*".[13] We also observed that mothers' low education level was positively associated with asthma without nasal allergies in their offspring (Table 3 in the manuscript), which is in line with evidence on the higher risk of non-atopic asthma in the lower socio-economic classes.[14]

Information bias across generations

In our study, a potential information bias could affect measurements of asthma and smoking in both grandparents (generation F0) and parents (generation F1), and asthma in offspring (generation F2). In particular, misclassification should be more likely for the variables measured in generations F0 and F2, because this information was parent-reported.

The multicentre trans-generational database RHINESSA (*https://helse-bergen.no/fag-og-forsking/forsking/rhinessa/rhinessa-english*) has shown that:

- Offspring's report of their mother's smoking status during pregnancy (information on 679 mothers and their 807 adult offspring) was similar to the mother's own report, with a high specificity, a relatively high sensitivity and a moderate agreement (Kathrine Pape. Data presented at the European Respiratory Society International Congress 2017). In addition, there was no statistically significant difference in the risk of discrepant answers in male offspring as compared to female offspring.

- In RHINESSA, 7826 adult offspring reported whether their parents had asthma, as did their 7598 parents (Ingrid N Kuiper. Data presented at the European Respiratory Society International Congress 2016). Offspring's reports showed a high specificity, a somewhat lower sensitivity and a good agreement with parental reports. In addition, there was a statistically significant increased risk of discrepant answers in offspring with wheezing, who could be asthmatic patients, as compared to offspring without wheezing.
- In the latter analysis (Ingrid N Kuiper. Data presented at the European Respiratory Society International Congress 2016), parents' reports showed a good agreement for offspring's early onset asthma (<10 years) and a moderate agreement for offspring's late onset asthma. In addition, parents who were smokers (as compared to never- and ex-smokers) and fathers (as compared to mothers) were more likely to report their offspring's asthma incorrectly.

These results indicate that the direction of information bias is difficult to predict in our study, always being towards the null value only in very special cases, for example if only one of two binary variables (predictor and outcome) is misclassified and misclassification is non-differential.[15]

REFERENCES

1. Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. J Pers Soc Psychol 1986; 51:1173-1182.
2. Muthén BO, Muthén LK, Asparouhov T. Regression and mediation analysis using Mplus. Los Angeles: Muthén & Muthén, 2016.
3. Tofighi D, Thoemmes F. Single-level and multilevel mediation analysis. J Early Adolesc 2014; 34:93-119.
4. Muthén LK, Muthén BO. Mplus User's Guide. Eight edition. Los Angeles: Muthén & Muthén, 1998-2017.
5. VanderWeele TJ. A three-way decomposition of a total effect into direct, indirect, and interactive effects. Epidemiology 2013; 24:224–232.
6. Pearl J. Direct and indirect effects. In: Proceedings of the Seventeenth Conference on Uncertainty in Artificial Intelligence. San Francisco: Morgan Kaufmann, 2001; pp. 411-420.
7. Kraemer HC, Kiernan M, Essex M, Kupfer DJ. How and why criteria defining moderators and mediators differ between the Baron & Kenny and MacArthur approaches. Health Psychol 2008; 27(Suppl 2):S101-S108.
8. Satorra A, Bentler PM. Ensuring positiveness of the scaled difference chi-square test statistic. Psychometrika 2010; 75:243-248.
9. Lutz SM, Thwing A, Schmiege S, et al. Examining the role of unmeasured confounding in mediation analysis with genetic and genomic applications. BMC Bioinformatics 2017; 18:344.

10. Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. Epidemiology 1999; 10:37-48.
11. Skinner MK. Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. Mol Cell Endocrinol 2014; 398:4-12.
12. Arshad SH, Karmaus W, Zhang H, Holloway JW. Multigenerational cohorts in patients with asthma and allergy. J Allergy Clin Immunol 2017; 139:415-421.
13. Martinez FD, Vercelli D. Asthma. Lancet 2013; 382:1360-1372.
14. Bråbäck L, Hjern A, Rasmussen F. Social class in asthma and allergic rhinitis: a national cohort study over three decades. Eur Respir J 2005; 26:1064-1068.
15. Höfler M. The effect of misclassification on the estimation of association: a review. Int J Methods Psychiatr Res 2005; 14:92-101.

Supplementary information on the European Community Respiratory Health Survey (ECRHS)

Co-ordinating centre: P. Burney, S. Chinn, C. Luczynska†, D. Jarvis, E. Lai (London).

Project management group: P. Burney (Project leader, UK), S. Chinn (UK), C. Luczynska† (UK), D. Jarvis (UK), P. Vermeire† (Antwerp), H. Kesteloot (Leuven), J. Bousquet (Montpellier), D. Nowak (Hamburg), J. Prichard† (Dublin), R. de Marco† (Verona), B. Rijcken (Groningen), J.M. Antó (Barcelona), J. Alves (Oporto), G. Boman (Uppsala), N. Nielsen (Copenhagen), P. Paoletti (Pisa).

Steering Committee of the ECRHS II: U. Ackermann-Liebrich (University of Basel, Switzerland); N. Kuenzli (University of Basel, and University of Southern California, Los Angeles, USA); J.M. Antó and J. Sunyer [Institut Municipal d'Investigació Médica (IMIM-IMAS), Universitat Pompeu Fabra (UPF), Spain]; P. Burney (project leader), S. Chinn, D. Jarvis, J. Knox, and C. Luczynska† (King's College London, UK); I. Cerveri (University of Pavia, Italy); R. de Marco† (University of Verona, Italy); T. Gislason (Iceland University Hospital, Iceland); J. Heinrich and M. Wjst (GSF–Institute of Epidemiology, Germany); C. Janson (Uppsala University, Sweden); B. Leynaert and F. Neukirch [Institut National de la Santé et de la Recherche Medicale (INSERM), France]; J. Schouten (University of Groningen, The Netherlands); C. Svanes (University of Bergen, Norway); P. Vermeire† (University of Antwerp, Belgium).

Principal investigators and senior scientific teams of the ECRHS II: Australia: Melbourne (M. Abramson, E.H. Walters, J. Raven); Belgium: South Antwerp and Antwerp City (P. Vermeire†, J. Weyler, M. van Sprundel, V. Nelen); Estonia: Tartu (R. Jõgi, A. Soon); France: Paris (F. Neukirch, B. Leynaert, R. Liard, M. Zureik), Grenoble (I. Pin, J. Ferran-Quentin); Bordeaux (A. Taytard, C. Raherison), Montpellier (J. Bousquet, P.J. Bousquet); Germany: Erfurt (J. Heinrich, M. Wjst, C. Frye, I. Meyer); Iceland: Reykjavik (T. Gislason, E. Bjornsson, D. Gislason, K.B. Jörundsdóttir); Italy: Turin (R. Bono, M. Bugiani, P. Piccioni, E. Caria, A. Carosso, E. Migliore, G. Castiglioni), Verona (R. de Marco†, G. Verlato, E. Zanolin, S. Accordini, A. Poli, V. Lo Cascio, M. Ferrari, I. Cazzoletti), Pavia (A. Marinoni, S. Villani, M. Ponzio, F. Frigerio, M. Comelli, M. Grassi, I. Cerveri, A. Corsico); The Netherlands: Groningen and Geleen (J. Schouten, M. Kerkhof); Norway: Bergen (A. Gulsvik, E. Omenaas, C. Svanes, B. Laerum); Spain: Albacete (J. Martinez-Moratalla Rovira, E. Almar, M. Arévalo, C. Boix, G. González, J.M. Ignacio García, J. Solera, J. Damián), Galdakao (N. Muñozguren, J. Ramos, I. Urrutia, U. Aguirre), Barcelona (J.M. Antó, J. Sunyer, M. Kogevinas, J.P. Zock, X. Basagaña, A. Jaen, F. Burgos, C. Acosta), Huelva (J. Maldonado, A. Pereira, J.L. Sanchez), Oviedo (F. Payo, I. Huerta, A. de la Vega, L. Palenciano, J. Azofra, A. Cañada); Sweden: Göteborg (K. Toren, L. Lillienberg, A.C. Olin, B. Balder, A. Pfeifer-Nilsson, R. Sundberg), Umea (E. Norrman, M. Soderberg, K.A. Franklin, B. Lundback, B. Forsberg, L. Nystrom), Uppsala (C. Janson, G. Boman, D. Norback, G. Wieslander, M. Gunnbjornsdottir); Switzerland: Basel (N. Küenzli, B. Dibbert, M. Hazenkamp, M. Brutsche, U.

Ackermann-Liebrich); United Kingdom: Ipswich (D. Jarvis, R. Hall, D. Seaton), Norwich (D. Jarvis, B. Harrison).

Principal investigators and senior scientific teams of the ECRHS III: Australia: Melbourne (M. Abramson, G. Benke, S. Dharmage, B. Thompson, S. Kaushik, M. Matheson); Belgium: South Antwerp and Antwerp City (J. Weyler, H. Bentouhami, V. Nelen); Denmark: Aarhus (V. Schlunssen, T. Sigsgaard, R.Dahl); Estonia: Tartu (R. Jõgi, H.Orru); France: Bordeaux (C. Raherison, P.O. Girodet), Grenoble (I. Pin, V. Siroux, J. Ferran, J.L. Cracowski), Montpellier (P. Demoly, A. Bourdin, I. Vachier), Paris (B. Leynaert, D. Soussan, D. Courbon, C. Neukirch, L. Alavoine, X. Duval, I. Poirier); Germany: Erfurt (J. Heinrich, E. Becker, G. Woelke, O. Manuwald), Hamburg (H. Magnussen, D. Nowak, A.M. Kirsten); Iceland: Reykjavik (T. Gislason, B. Benediktsdottir, D. Gislason, E.S. Arnardottir, M. Clausen, G. Gudmundsson, L. Gudmundsdottir, H. Palsdottir, K. Olafsdottir, S. Sigmundsdottir, K. Bara-Jörundsdottir); Italy: Pavia (I. Cerveri, A. Corsico, A. Grosso, F. Albicini, E. Gini, E.M. Di Vincenzo, V. Ronzoni, S. Villani, F. Campanella, M. Gnesi, F. Manzoni, L. Rossi, O. Ferraro), Turin (M. Bugiani, R. Bono, P. Piccioni, R. Tassinari, V. Bellisario, G. Trucco), Verona (R. de Marco†, S. Accordini, L. Calciano, L. Cazzoletti, M. Ferrari, A.M. Fratta Pasini, F. Locatelli, P. Marchetti, A. Marcon, E. Montoli, G. Nguyen, M. Olivieri, C. Papadopoulou, C. Posenato, G. Pesce, P. Vallerio, G. Verlato, E. Zanolin); The Netherlands: Groningen (H.M. Boezen); Norway: Bergen (C. Svanes, E. Omenaas, A. Johannessen, T. Skorge, F. Gomez Real); Spain: Albacete (J. Martinez-Moratalla Rovira, E. Almar, A. Mateos, S. García, A. Núñez, P. López, R. Sánchez, E. Mancebo), Barcelona (J.M. Antó, J.P. Zock, J. Garcia-Aymerich, M. Kogevinas, X. Basagaña, A.E. Carsin, F. Burgos, C. Sanjuas, S. Guerra, B. Jacquemin, P. Davdand), Galdakao (N. Muñozguren, I. Urrutia, U. Aguirre, S. Pascual), Huelva (J. Antonio Maldonado, A. Pereira, J. Luis Sánchez, L. Palacios), Oviedo (F. Payo, I. Huerta, N. Sánchez, M. Fernández, B. Robles); Sweden: Göteborg (K. Torén, M. Holm, J.L. Kim, A.C. Olin, A. Dahlman-Höglund), Umea (B. Forsberg, L. Braback, L. Modig, B. Järvholm, H. Bertilsson, K.A. Franklin, C. Wahlgreen), Uppsala (B. Andersson, D. Norback, U. Spetz Nystrom, G. Wieslander, G.M. Bodinaa Lund, K. Nisser); Switzerland: Basel (N.M. Probst-Hensch, N. Künzli, D. Stolz, C. Schindler, T. Rochat, J.M. Gaspoz, E. Zemp Stutz, M. Adam, C. Autenrieth, I. Curjuric, J. Dratva, A. Di Pasquale, R. Ducret-Stich, E. Fischer, L. Grize, A. Hensel, D. Keidel, A. Kumar, M. Imboden, N. Maire, A. Mehta, H. Phuleria, M. Ragettli, M. Ritter, E. Schaffner, G.A. Thun, A. Ineichen, T. Schikowski, M. Tarantino, M. Tsai); UK: London (P. Burney, D. Jarvis, S. Kapur, R. Newson, J. Potts), Ipswich (N. Innes), Norwich (A. Wilson).

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Table 1S. The parents and their offspring who were included in the present study, according to the parental line and centre.

		Maternal line		Paternal line	
Country	**Centre**	**N° of mothers**	**N° of offspring**	**N° of fathers**	**N° of offspring**
Australia	Melbourne	98	232	87	202
Belgium	Antwerp City	62	124	52	113
	Antwerp South	74	165	70	160
Denmark	Åarhus	81	171	76	162
Estonia	Tartu	66	139	36	79
France	Bordeaux	72	140	79	173
	Grenoble	133	289	137	313
	Montpellier	71	149	58	117
	Paris	123	239	94	180
Germany	Erfurt	141	231	124	219
	Hamburg	79	135	73	135
Iceland	Reykjavik	178	435	142	365
Italy	Pavia	33	53	27	42
	Turin	21	37	17	31
	Verona	34	55	39	74
Norway	Bergen	139	321	169	401
Spain	Albacete	64	151	72	145
	Barcelona	53	98	48	100
	Galdakao	120	225	98	168
	Huelva	45	96	36	80
	Oviedo	51	90	49	88
Sweden	Goteborg	122	255	93	190
	Umea	106	251	80	191
	Uppsala	137	299	124	277
United Kingdom	Ipswich	66	159	40	90
	Norwich	64	127	44	97
	Total	2233	4666	1964	4192

Table 2S. Distribution of the number of offspring per parent, according to the parental line.

N° of offspring per parent	Mothers (%)	Fathers (%)
1	536 (24.0)	442 (22.5)
2	1114 (49.9)	990 (50.4)
3	466 (20.9)	400 (20.4)
4	94 (4.2)	98 (5.0)
5	14 (0.6)	28 (1.4)
6	7 (0.3)	4 (0.2)
7	0	2 (0.1)
8	2 (0.1)	0
Total	2233	1964

Table 3S: Controlled direct effects within the maternal line, obtained after the exclusion of grandmaternal/maternal smoking and grandparental/maternal education level from the mediation model.

GENERATION		F1	F2	
		Mother's ever asthma	**Offspring's ever asthma with nasal allergies**	**Offspring's ever asthma without nasal allergies**
		OR (95% CI)	RRR (95% CI)	RRR (95% CI)
F0	Grandmother's ever asthma (present *vs* absent)	2.27 (1.62-3.18)	-	-
	Grandfather's ever asthma (present *vs* absent)	2.46 (1.87-3.24)	-	-
F1	Mother's age (1-year increase)	1.00 (0.98-1.01)	-	-
	Mother's ever asthma (present *vs* absent)	-	2.56 (1.99-3.29)	1.71 (1.25-2.33)
F2	Offspring's gender (female *vs* male)	-	0.80 (0.66-0.96)	0.90 (0.72-1.12)
	Offspring's age (1-year increase)	-	0.99 (0.97-1.00)	0.97 (0.95-0.98)

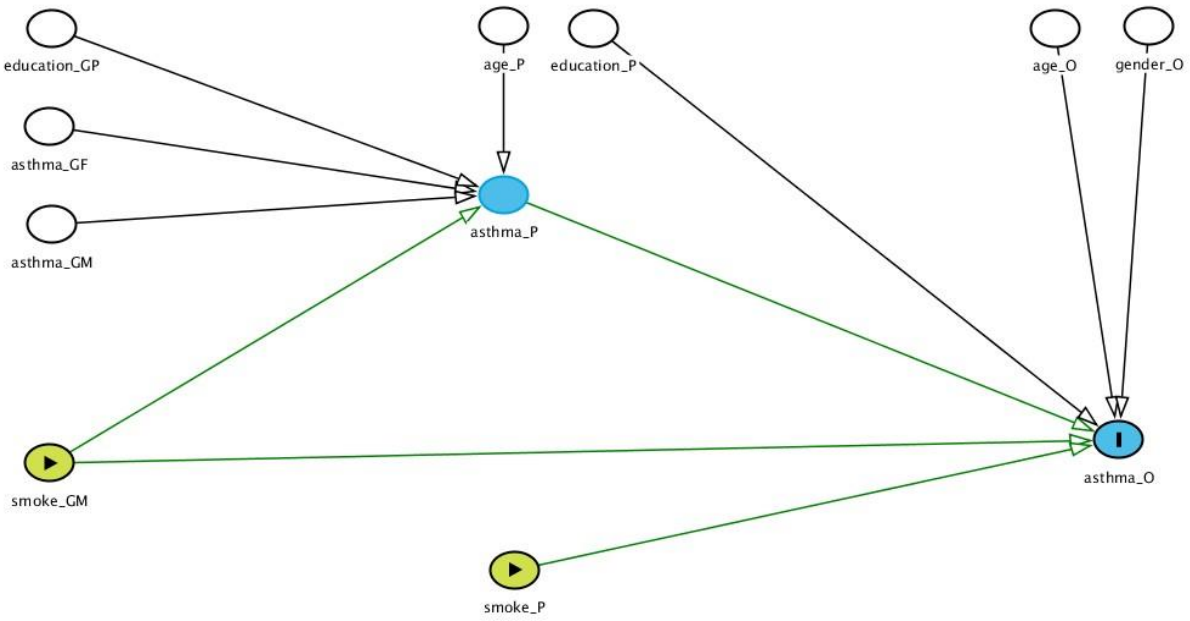
OR: odds ratio; RRR: relative risk ratio; CI: confidence interval.

Table 4S: Controlled direct effects within the paternal line, obtained after the exclusion of grandmaternal/paternal smoking and grandparental/paternal education level from the mediation model.

GENERATION		F1	F2	
		Father's ever asthma	**Offspring's ever asthma with nasal allergies**	**Offspring's ever asthma without nasal allergies**
		OR (95% CI)	RRR (95% CI)	RRR (95% CI)
F0	Grandmother's ever asthma (present *vs* absent)	3.05 (1.94-4.82)	-	-
	Grandfather's ever asthma (present *vs* absent)	2.37 (1.51-3.74)	-	-
F1	Father's age (1-year increase)	0.99 (0.96-1.02)	-	-
	Father's ever asthma (present *vs* absent)	-	2.41 (1.68-3.47)	1.71 (1.14-2.57)
F2	Offspring's gender (female *vs* male)	-	0.70 (0.59-0.84)	0.83 (0.70-0.97)
	Offspring's age (1-year increase)	-	0.99 (0.97-1.01)	0.96 (0.94-0.98)

OR: odds ratio; RRR: relative risk ratio; CI: confidence interval.

Figure 1S: Directed acyclic graph (DAG) used to check if the covariates included in the mediation models represent the "*minimal sufficient adjustment set*".



"*education_GP*": grandparents' education level; "*asthma_GF*": grandfather's asthma; "*asthma_GM*": grandmother's asthma; "*smoke_GM*": grandmother's smoke; "*age_P*": parent's age; "*education_P*": parent's education level; "*asthma_P*": parent's asthma; "*smoke_P*": parent's smoke; "*age_O*": offspring's age; "*gender_O*": offspring's gender; "*asthma_O*": offspring's asthma.

Figure 2S: The proportion of simulations where the results match and the average absolute difference for the direct effects of grandmothers' smoking on offspring's asthma (whether the unmeasured confounder U is included or excluded from the mediation models): (a) paternal line, outcome: asthma without nasal allergies *vs* no asthma; (b) paternal line, outcome: asthma with nasal allergies *vs* no asthma; (c) maternal line, outcome: asthma without nasal allergies *vs* no asthma; (d) maternal line, outcome: asthma with nasal allergies *vs* no asthma.