Prenatal and prepubertal exposures to tobacco smoke in men may cause lower lung function in future offspring: a three-generation study using a causal modelling approach

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ABSTRACT

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Mechanistic research suggests that lifestyle and environmental factors impact respiratory health across generations by epigenetic changes transmitted through male germ cells. Evidence from studies on humans is very limited.

We investigated multi-generation causal associations to estimate the causal effects of tobacco smoking on lung function within the paternal line. We analysed data from 383 adult offspring (age: 18-47; female: 52.0%) and their 274 fathers, who had participated in the ECRHS/RHINESSA generation study and had provided valid measures of pre-bronchodilator lung function. Two counterfactual-based, multi-level mediation models were developed with: paternal grandmothers’ smoking in pregnancy and fathers’ smoking initiation in prepuberty as exposures; fathers' FEV₁ and FVC, or FEV₁/FVC z-scores as potential mediators (proxies of unobserved biological mechanisms that are true mediators); offspring’s FEV₁ and FVC, or FEV₁/FVC z-scores as outcomes. All effects were summarised as differences in expected z-scores related to fathers’ and grandmothers’ smoking history.

Fathers’ smoking initiation in prepuberty had a negative direct effect on both offspring’s FEV₁ (-0.36; 95% confidence interval: -0.63, -0.10) and FVC (-0.50; -0.80, -0.20) compared to fathers’ never smoking. Paternal grandmothers’ smoking in pregnancy had a negative direct effect on fathers’ FEV₁/FVC (-0.57; -1.09, -0.05) and a negative indirect effect on offspring’s FEV₁/FVC (-0.12; -0.21, -0.03) compared to grandmothers’ not smoking before fathers’ birth nor during fathers’ childhood.

Fathers’ smoking in prepuberty and paternal grandmothers’ smoking in pregnancy may cause lower lung function in offspring. Our results support the concept that lifestyle-related exposures during these susceptibility periods influence the health of future generations.
TAKE HOME MESSAGE

Fathers’ prepuberty and paternal grandmothers’ pregnancy are vulnerable periods to the adverse effects of smoking on offspring’s lung function. Preventing smoking in these susceptibility time windows might improve the next generation’s health.

KEY WORDS: lung function; tobacco smoking; prepuberty; pregnancy; paternal line; causal inference; multi-level mediation modelling; ALEC; ECRHS; RHINESSA.
INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are major health challenges across the world [1, 2]. Lifestyle and environmental exposures are important risk factors for these diseases [3, 4], not least when exposure occurs in early life. Emerging evidence suggests that the environment before conception may impair respiratory health of the offspring [5, 6], as supported by animal research [7–9]. In line with this hypothesis, we previously found negative associations between fathers’ smoking before 15 years of age (a period compatible with prepuberty, as 15 years is the mean age of completed puberty in boys [10]) and their offspring’s asthma phenotypes [11, 12]. We also identified the same susceptibility time window for fathers’ overweight [13].

Studies on human health [14], including respiratory diseases [15–18], are currently more focused on epigenetics. Heritable modification of DNA methylation may be a key mechanism for the effects of preconception and early life exposures on disease phenotypes in subsequent generations. Foetal life and prepuberty are time windows with a higher susceptibility to environmental exposures through their impact on epigenetic programming of the male germ cell line [19, 20]. These vulnerable periods may represent windows of opportunity for preventive interventions in males aimed at improving the health of current and future generations.

Causal statistical inference [21] can contribute to shed light on the mechanisms involved in the pathways from exposures in different generations and susceptibility periods, to health outcomes in subsequent generations. A multiple-exposure, multiple-mediator, multiple-outcome (MEMMMO) framework has recently been proposed for the identification of complex multi-generation effects [22]. In line with this new perspective, we investigated causal associations to estimate the causal effects of fathers’ smoking initiation in prepuberty (generation G1) and grandmothers’ smoking in pregnancy (generation G0) on offspring’s lung function (generation G2) within the paternal line. As part of the Ageing Lungs in European Cohorts (ALEC) Study (www.alecstudy.org), we analysed...
data from an ongoing survey on respiratory health in adults (European Community Respiratory Health Survey, ECRHS; www.ecrhs.org) and its extension to the generation of their offspring (Respiratory Health in Northern Europe, Spain and Australia, RHINESSA; www.rhinessa.net).
METHODS

Study design

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) [23]. 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). An additional “symptomatic sample” of adults with asthma-like symptoms was also recruited at stage 2. The follow-up of the participants in stage 2 took place in 1998-2002 (ECRHS II) [24] and in 2010-2013 (ECRHS III) [25]. These subjects underwent a standardized clinical interview, lung function and laboratory tests on all the occasions. RHINESSA is an international study on health and disease in the offspring of the ECRHS participants in ten centres from Northern Europe, Spain, and Australia [13]. Extensive questionnaire and lung function data were collected in 2013-2016, based on protocols adapted to those used in the ECRHS. Written informed consent was obtained from each participant in the ECRHS/RHINESSA studies. Ethics approval was obtained by the appropriate ethics committee in each centre (see helse-bergen.no/sekjon/RHINESSA/Documents/Ethic%20Committees%20list.pdf and the online supplementary material).

Lung function and definitions

Maximum pre-bronchodilator forced expiratory volume in one second (FEV$_1$) and forced vital capacity (FVC) from at least two technically satisfactory manoeuvres were measured according to the American Thoracic Society (ATS) criteria for repeatability [26], as part of the ECRHS and RHINESSA clinical examinations. Post-bronchodilator spirometry was also measured in ECRHS at the last follow-up contact (ECRHS III) and in RHINESSA. In the present analyses, we used the fathers’ lung function measurements at baseline (ECRHS I) or at the first available occasion.
(ECRHS II or ECRHS III) if baseline spirometry had not been performed or had not fulfilled the ATS criteria. The Global Lung function Initiative (GLI) z-scores [27] were calculated by using the European Respiratory Society freeware software (www.ers-education.org/guidelines/global-lung-function-initiative/spirometry-tools/desktop-sheet-calculator.aspx), in order to control for the dependency of lung function on sex, age and height.

The fathers provided detailed information on their own smoking history, including when they had started smoking, during interviews at each ECRHS examination. Fathers’ smoking was categorized as “smoking initiation before 15 years of age” (i.e. during prepuberty), “smoking initiation at 15 years of age or older” or “never smoking”. Fathers’ education level was self-reported at ECRHS I and was considered “low” if less than or equal to the minimum school-leaving age in their country before the start of the ECRHS study [28]. Fathers’ occupational class was derived from the longest-held job during the follow-up period between ECRHS I and II, with categories based on the major group classification in the International Standard Classification of Occupations (ISCO) [29]. At ECRHS I, the fathers reported whether their mother had smoked before or after their birth. Consequently, grandmothers’ smoking was classified as “smoking when the father was in utero”, “smoking before pregnancy or during father’s childhood (or unknown smoking period)” or “not smoking before father’s birth nor during father’s childhood”. At ECRHS III, the fathers provided information on their parents’ education level (defined “low” if both grandparents were reported to have only studied up to the minimum school-leaving age). In RHINESSA, the offspring reported their own smoking history and education level (defined “low” if a subject had only studied up to the minimum school-leaving age), and whether their mother had smoked before or after their birth, during interviews with similarly worded questions to the ECRHS.
Study subjects

Of the 2,302 males from Estonia (Tartu), Norway (Bergen), Spain (Albacete, Huelva) and Sweden (Gothenburg, Umea, Uppsala) who had undergone a clinical examination in ECRHS I-III, 1,241 participated in ECRHS III. Of these, 913 men reported having at least one offspring and 297 of them had at least one adult offspring who participated in the RHINESSA clinical stage and were thus eligible for inclusion in the present analyses (figure 1). These fathers had valid lung function measurements and reported complete information on their own and their mother’s smoking history. Of the 420 adult offspring who had originated from these 297 fathers, 383 individuals had valid lung function measurements. These offspring and their 274 fathers (92.7% from the ECRHS “random sample”) were included in our study.

Statistical analyses

Counterfactual-based mediation analyses [30] within a hierarchical framework were carried out to investigate the pathways among grandmothers’ and fathers’ tobacco smoking in vulnerable periods and offspring’s lung function within the paternal line. Our data have a hierarchical structure because the offspring siblings (level 1 units) have the same biological father (level 2 units) and because their fathers were sampled from different centres (level 3 units). The total effect of each exposure on each outcome was decomposed into its natural direct effect (i.e. the effect of the exposure on the outcome via a pathway that does not involve the mediator) and its natural indirect (mediated) effect (i.e. the effect of the exposure on the outcome due to the effect of the exposure on the mediator) [31]. The main requirement for mediation is that the indirect effect is statistically significant [32], when the observed mediated effect is robust to potential confounding by some unmeasured variable [33]. Two multi-level mediation models were used within the paternal line. Model 1 and model 2 were aimed at estimating the multi-generation effects of smoking on pre-bronchodilator FEV₁ and FVC.
(figure 2), or FEV₁/FVC (figure 3), respectively. Model 1 included fathers' FEV₁ and FVC z-scores as the normally distributed, correlated, parallel mediators, and offspring’s FEV₁ and FVC z-scores as the normally distributed, correlated, parallel outcomes. Model 2 included fathers' FEV₁/FVC z-score as the normally distributed mediator and offspring’s FEV₁/FVC z-score as the normally distributed outcome. Each lung function variable in fathers (measured in adulthood) was considered a proxy of unobserved biological mechanisms that are true mediators. Latent mediators were not included in the models as we had a single indicator for each mediator, and using single-indicator latent variables often causes identification problems in model specification [34]. In both models, grandmothers’ and fathers’ smoking were the exposures of interest, and grandparents’ and fathers’ education level, and fathers’ occupational class were analysed as potential confounders. We verified whether these potential confounders represent the “minimal sufficient adjustment set” (i.e. the smallest set of measured covariates that needs to be included in order to eliminate confounding), by using directed acyclic graphs (DAGs) [35] in DAGitty (dagitty.net; see the online supplementary material). In addition, fathers’ age, mothers’ smoking before or after offspring’s birth, and offspring’s sex, age, education level and smoking were analysed as adjusting variables of the exposure-mediator-outcome relationships. Model 1 and model 2 had random intercept terms at level 2 (father), cluster-robust standard errors (country = cluster variable) and a “2→2→1” configuration [36] [i.e. the exposures and the mediators were measured at level 2 (father), whereas the outcomes were measured at level 1 (offspring)]. The natural (counterfactual-based) direct and indirect effects of the exposures (grandmothers’ and fathers’ smoking) on the normally distributed mediators (fathers’ z-scores) and outcomes (offspring’s z-scores) were summarised as differences (Δ) in expected z-scores. The natural direct effect is offspring’s Δz-score for the change in exposure status, keeping fathers’ z-score at its expected value when the exposure is absent. The natural indirect effect is offspring’s Δz-score when the exposure is present, but fathers’ z-score changes from its expected value when the
exposure is absent to its expected value when the exposure is present. The magnitude of the direct and indirect effects, and their 95% confidence interval (95%CI) were computed based on the maximum likelihood estimator with robust standard errors [36] and the distribution-of-the-product method [37], respectively.

STATA 15 (StataCorp, College Station, TX), Mplus 8 (Muthén & Muthén, Los Angeles, CA), and R 3.6.1 (www.R-project.org) were used for the statistical analyses.

**Sensitivity analyses**

Sensitivity analyses were performed in order to assess:

- whether the estimated effects changed after the inclusion of up to two unmeasured confounders \( (U_1 \text{ and } U_2) \) in model 1 and model 2, by using probabilistic (Monte Carlo) simulations in the Umediation package [38] (github.com/SharonLutz/Umediation; see the online supplementary material);
- which is the minimum strength of association that an unmeasured confounder would need to have with both the mediator and outcome, conditional on the measured confounders, to fully explain away the observed direct or indirect effects (mediational E-values) [39], by using the Evalue package [40] (github.com/mayamathur/evalue_package). The mediational E-values were returned on the risk ratio (RR) scale through an approximate conversion [41, 42];
- whether the estimated effects of fathers’ smoking in prepuberty changed when the analyses were repeated by excluding the 182 offspring whose father had smoked after their birth;
- whether the estimated effects changed when using offspring’s post-bronchodilator lung function measurements (available from 369 offspring).
RESULTS

Main characteristics of the study subjects

At the time of lung function assessment, the median age of the 274 fathers and their 383 offspring (female: 52.0%) was 37 and 28 years, respectively (table 1 and table 2). In our sample, 10.2%, 8.4%, and 7.7% of the fathers had FEV\textsubscript{1}, FVC or FEV\textsubscript{1}/FVC z-scores below -1.645 (fifth percentile), respectively, whereas these figures were 6.0%, 3.7% and 6.8% for their offspring.

Among the fathers, 9.1% had started smoking before 15 years of age (i.e. during prepuberty) and 7.7% reported that their mother (grandmothers in the present analyses) had smoked during their pregnancy. Among the offspring, the percentage of ever smokers was 30.3% and 50.7% reported that their mother had smoked before or after their birth.

Fathers’ smoking initiation in prepuberty

Fathers’ smoking initiation in prepuberty (generation G1) had a negative direct effect on their own FEV\textsubscript{1}/FVC [Δz-score (95%CI): -0.36 (-0.68, -0.04)] compared to fathers’ never smoking (table 3). This exposure had a negative direct effect on both offspring’s FEV\textsubscript{1} [-0.36 (-0.63, -0.10)] and FVC [-0.50 (-0.80, -0.20)] (generation G2), but we did not observe a statistically significant effect on offspring’s FEV\textsubscript{1}/FVC. No mediated effect of fathers’ smoking in prepuberty on offspring’s pulmonary values was identified (table 4) (direct-only non-mediation). Fathers’ smoking initiation at later ages had a negative direct effect on their own FEV\textsubscript{1} [-0.27 (-0.51, -0.02)] and FEV\textsubscript{1}/FVC [-0.20 (-0.37, -0.04)], but we did not find an effect on offspring’s lung function.

Grandmothers’ smoking in pregnancy

Grandmothers’ smoking when the father was in utero (generation G0) had a negative direct effect on fathers’ FEV\textsubscript{1}/FVC (generation G1) [-0.57 (-1.09, -0.05)] compared to grandmothers’ not
smoking before fathers’ birth nor during fathers’ childhood (table 3). Grandmothers’ smoking while pregnant with the father had no direct effect on grandchildren’s lung function (generation G2). This exposure had a negative mediated effect, through unobserved biological mechanisms for which fathers’ FEV$_1$/FVC in adulthood is an indicator, on grandchildren’s FEV$_1$/FVC [-0.12 (-0.21, -0.03)] (table 4) (indirect-only mediation).

**Sensitivity analyses**

Simulations suggested that unmeasured confounding could reasonably be assumed to have a low impact on the effects of fathers’ and grandmothers’ smoking (supplementary Figure S4 and Figure S5). When the effect (beta regression coefficient) of each unmeasured confounder (U$_1$ and U$_2$) on the exposure, mediator and outcome was set less than or equal to five, the proportion of simulations where the results matched (whether U$_1$ and U$_2$ were included or excluded from the models) was greater than 72.8% and the average absolute difference of the average effects was lower than 0.083. U$_1$ and U$_2$ changed the results for very strong effects only (i.e. for beta regression coefficients greater than five).

The mediational E-value for the natural direct effect of father’s smoking in prepuberty on offspring’s FEV$_1$ and FVC was 2.31 and 2.85, respectively, whereas the mediational E-value for the natural indirect effect of grandmother’s smoking in pregnancy on offspring’s FEV$_1$/FVC was 1.50. Therefore, an unmeasured confounder must be associated with both the mediator and outcome with an approximate RR at least equal to 2.31, 2.85 or 1.50 (when the outcome is FEV$_1$, FVC or FEV$_1$/FVC, respectively) to completely explain away the observed effects.

Exclusion of the offspring of fathers who had smoked after their birth provided stronger estimates of the direct effect of fathers’ smoking in prepuberty on offspring’s FEV$_1$ [-0.55 (-1.18, 0.07)] and FVC [-0.77 (-1.61, 0.07)] than that obtained in the main analysis. However, these estimates did not
reach statistical significance because of data sparseness, as only 8 offspring were born to 7 fathers who had started smoking in prepuberty and had quit smoking before their birth.

When offspring’s post-bronchodilator values were used, the direct effect of fathers’ smoking on offspring’s FVC [-0.35 (-0.62, -0.07)] and the indirect effect of grandmothers’ smoking on grandchildren’s FEV$_1$/FVC [-0.09 (-0.17, -0.01)] were reduced. However, the direct effect of fathers’ smoking on offspring’s FEV$_1$ did not reach statistical significance [-0.19 (-0.48, 0.11)].
DISCUSSION

In the present study, we found that fathers’ and paternal grandmothers’ smoking in vulnerable time windows may negatively affect lung function in the next two generations. These results were obtained using a method of causal inference with observational data and were strengthened by sensitivity analyses accounting for unmeasured confounding. Our novel observation is that the offspring of fathers who had smoked during prepuberty may have lower FEV\textsubscript{1} and FVC values (suggesting an effect on reducing the overall lung volumes), compared to the offspring of fathers who had never smoked. Another key result is the negative indirect effect of grandmothers’ smoking when the father was in utero on grandchildren’s FEV\textsubscript{1}/FVC. This finding suggests that smoking during pregnancy (generation G0) may not only increase the risk of airflow obstruction in generation G1, but it may also have a negative effect on generation G2 within the paternal line. Our results support the concept that lifestyle-related exposures during prepuberty in males and during pregnancy influence the health of future generations.

Fathers’ smoking initiation in prepuberty

In the present analyses, the effect of fathers’ prepubertal smoking on offspring’s FEV\textsubscript{1} and FVC was observed regardless of the effect of mothers’ smoking before or after offspring’s birth, and it was even present after the exclusion of the offspring of fathers who had smoked after their birth. When offspring’s post-bronchodilator values were used, the effect on FVC was reduced but it was still statistically significant. Moreover, fathers’ smoking initiation at later ages had no effect on their offspring’s lung function.

Unmeasured genetic confounding unlikely explains away the direct effect of paternal early smoking on offspring’s FEV\textsubscript{1} and FVC, as the few risk loci for both nicotine dependence and lung function identified in genetic studies (such as rs16969968 in CHRNA5 gene) [43, 44] have weaker
associations than the computed mediational E-values [39]. Therefore, we speculate that our findings could reflect epigenetic alterations (such as DNA methylation, histone modification and microRNAs) in developing germ cells [45-47] leading to reduced lung volumes in offspring. The heritable effects of smoking in young men seem to be biologically plausible, because male prepuberty represents a critical period for the germ line development [19, 20], which might give higher susceptibility to tobacco-related effects on the epigenetic profile of gametes. In addition, the observed negative impact of fathers’ prepubertal smoking on offspring’s FEV<sub>1</sub> and FVC may reflect the direct toxicogenic effects of cigarette smoke on biological processes involved in metabolic health. Prepubertal start of father’s smoking may contribute to obesity in adolescent [48] and adult sons [49], and obese adults show a spirometry pattern characterised by lower FEV<sub>1</sub>, FVC, total lung capacity and residual volume [50].

Our results are supported by findings from previous analyses of the same population, in which we observed that fathers’ smoking initiation in prepuberty was associated with an increased risk for non-allergic asthma in offspring [11, 12]. In addition, we found that fathers’ overweight onset before 15 years of age had a direct effect on non-allergic asthma in the next generation [13]. All these results strengthen the hypothesis that life-style and environmental exposures during male prepuberty may affect the respiratory health of future offspring. Our current findings may have considerable public health implications, particularly as tobacco smoking in 11- to 15-year-old boys has increased in different European regions over recent decades [51], as well as the use of moist oral tobacco and e-cigarettes among the very young. Nevertheless, we acknowledge that early smoking in men could also be a marker for other causative factors, as risky behaviours of several kinds can be associated with smoking in adolescents.
Grandmothers’ smoking in pregnancy

Our study highlights that grandmothers’ smoking in pregnancy may negatively affect FEV<sub>1</sub>/FVC in the next two generations within the paternal line. Accordingly, findings from the UK Biobank indicate an excess reduction in FEV<sub>1</sub>/FVC among sons due to maternal smoking around delivery, whereas the reduction in FVC is very modest [52]. The negative direct effect of this prenatal exposure on fathers’ lung function is supported by previous evidence in humans and animals [53], because nicotine can penetrate the placental barriers and adversely affect lung development [54]. In addition, maternal smoking during pregnancy is associated with low birth weight and preterm delivery [55, 56], which are linked to reduced lung function in adulthood [57, 58].

We speculate that the negative indirect effect of grandmothers’ smoking during pregnancy (through biological mechanisms for which fathers’ lung function in adulthood is an indicator) on grandchildren’s FEV<sub>1</sub>/FVC could be explained by epigenetic changes [59, 60], resulting in airflow obstruction in offspring [60], and this effect is unlikely fully explained away by unmeasured risk loci, as suggested by the computed mediational E-value [39]. Tobacco smoking may cause heritable changes of the germ cell epigenome, particularly in the prenatal period [60, 61]. An animal model of multi-generation nicotine-induced asthma showed that epigenetic modifications can affect lung function in second-generation offspring [62]. Furthermore, a study on humans highlighted a link between prenatal exposure to tobacco smoke, DNA methylation changes and asthma-related lung function [63].

Strengths and weaknesses

Epidemiological studies on the early life origins of diseases usually focus on mothers’ exposures immediately before conception and during pregnancy. By contrast, RHINESSA included the offspring of both male and female participants in ECRHS, and the ECRHS survey followed adult men and women through their reproductive age. Thus, the ECRHS/RHINESSA studies collected...
extensive data on exposures occurring well in advance before conception (together with objective measurements of lung function) in two generations within both the paternal and maternal lines. Another major strength of the present analyses is the statistical approach used for assessing causal associations to estimate causal effects across generations. We used mediation models [30], which have become increasingly relevant for causal inference in epidemiological studies, in order to investigate complex pathways among multiple exposures, multiple mediators and multiple outcomes (MEMMOMO framework) [22]. Finally, our results were supported by: (i) probabilistic simulations on the impact of unmeasured confounding [38]; (ii) mediational E-values [39] to quantify robustness to unmeasured confounding, e.g. due to risk loci for both nicotine dependence and lung function [43, 44]; (iii) a subgroup analysis to strengthen the causal interpretation of the observed effects of fathers’ smoking in prepuberty on lung function in the next generation; (iv) a sensitivity analysis based on offspring’s post-bronchodilator spirometry, which is required to identify irreversible airflow obstruction [2].

The main limitation of our analyses is that parental information from the ECRHS study was available for one parent only. The information on mothers and paternal grandparents was offspring- and father-reported, respectively, rather than directly assessed, generating a possible information bias. However, validation studies from our cohorts on tobacco smoking [64] and asthma [65] reports across generations suggested that, although the recall bias is present, it is likely to have a limited effect. Secondly, post-bronchodilator spirometry was not used in the main analyses because it was available for a subset of the ECRHS fathers only. Thirdly, the number of exposed subjects was small. Due to this data sparseness, we could not assess the moderating effects of offspring’s sex [66] and mothers’ smoking before or after offspring’s birth [5] (i.e. specific patterns of effects where grandmothers’ and fathers’ risk factors differently affect offspring according to their sex or their exposure to mothers’ smoking). Lastly, it is possible that unmeasured confounders (such as...
genetic, other lifestyle and environmental factors in the three generations) may be important. In particular, we could not control for the potential confounding effect of genetic factors in our models because polymorphisms were available for a subset of the fathers and for offspring only, whereas genotypes on grandmothers, grandfathers and mothers are also needed to separate the influence of genetic inheritance from the effect of lifestyle/environmental exposures [67]. However, we estimated that unmeasured confounding had a limited impact on the effects identified in our analyses.

**Conclusions**

Men who initiated smoking before 15 years of age may have offspring with lower lung function compared to men who had never smoked. Grandmothers’ smoking in pregnancy may have a negative impact on their sons’ lung function, an effect that could be carried over to their grandchildren. These results support the concept that lifestyle-related exposures in male prepuberty and in pregnancy influence the health of future generations. Preventing smoking in these susceptibility time windows might have potential benefits for several generations.
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CONFLICT OF INTEREST

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Table 1. Main characteristics of the fathers and father-reported information on their parents (grandparents).

<table>
<thead>
<tr>
<th>Generation</th>
<th>N° of fathers = 274</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grandparents (G0)</strong></td>
<td></td>
</tr>
<tr>
<td>Education level, %</td>
<td></td>
</tr>
<tr>
<td>low*</td>
<td>31.0</td>
</tr>
<tr>
<td>high</td>
<td>36.9</td>
</tr>
<tr>
<td>unknown</td>
<td>32.1</td>
</tr>
<tr>
<td>Grandmother’s smoking, %</td>
<td></td>
</tr>
<tr>
<td>when the father was <em>in utero</em></td>
<td>7.7</td>
</tr>
<tr>
<td>before pregnancy or during father’s childhood (or unknown smoking period)</td>
<td>18.6</td>
</tr>
<tr>
<td>not smoking before father’s birth</td>
<td></td>
</tr>
<tr>
<td>nor during father’s childhood</td>
<td>73.7</td>
</tr>
<tr>
<td><strong>Father (G1)</strong></td>
<td></td>
</tr>
<tr>
<td>Age (years), median (range)</td>
<td>37 (21-63)</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV₁ z-score, mean (sd)</td>
<td>-0.35 (1.09)</td>
</tr>
<tr>
<td>Pre-bronchodilator FVC z-score, mean (sd)</td>
<td>-0.27 (0.97)</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV₁/FVC z-score, mean (sd)</td>
<td>-0.16 (1.01)</td>
</tr>
<tr>
<td>Low education level†, %</td>
<td>7.7</td>
</tr>
<tr>
<td><strong>Occupational class‡, %</strong></td>
<td></td>
</tr>
<tr>
<td>managers and professionals (non-manual)</td>
<td>37.2</td>
</tr>
<tr>
<td>technicians and associate professionals</td>
<td>15.3</td>
</tr>
<tr>
<td>other non-manual workers</td>
<td>8.4</td>
</tr>
<tr>
<td>skilled manual workers</td>
<td>14.6</td>
</tr>
<tr>
<td>semi-skilled or unskilled manual workers</td>
<td>11.3</td>
</tr>
<tr>
<td>not occupationally active, unclassifiable or unknown</td>
<td>13.2</td>
</tr>
<tr>
<td><strong>Smoking initiation, %</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;15 years of age</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>----</td>
</tr>
<tr>
<td>≥15 years of age</td>
<td>52.6</td>
</tr>
<tr>
<td>never smoking</td>
<td>38.3</td>
</tr>
</tbody>
</table>

* Both grandparents were reported to have only studied up to the minimum school-leaving age.

† Less than or equal to the minimum school-leaving age in their country before the start of the ECRHS [28].

‡ Derived from the longest-held job during the follow-up period between ECRHS I and II, using the International Standard Classification of Occupations (ISCO) [29].
Table 2. Main characteristics of the offspring and offspring-reported information on their mothers.

<table>
<thead>
<tr>
<th>Generation</th>
<th>N° of offspring = 383</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother (G1)</td>
<td></td>
</tr>
<tr>
<td>Smoking before or after offspring’s birth, %</td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>50.7</td>
</tr>
<tr>
<td>absent</td>
<td>25.3</td>
</tr>
<tr>
<td>unknown</td>
<td>24.0</td>
</tr>
<tr>
<td>Offspring (G2)</td>
<td></td>
</tr>
<tr>
<td>Female, %</td>
<td>52.0</td>
</tr>
<tr>
<td>Age (years), median (range)</td>
<td>28 (18-47)</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV(_1) z-score, mean (sd)</td>
<td>-0.38 (0.75)</td>
</tr>
<tr>
<td>Pre-bronchodilator FVC z-score, mean (sd)</td>
<td>-0.23 (0.83)</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV(_1)/FVC z-score, mean (sd)</td>
<td>-0.26 (0.91)</td>
</tr>
<tr>
<td>Ever smoking, %</td>
<td>30.3</td>
</tr>
<tr>
<td>Education level, %</td>
<td></td>
</tr>
<tr>
<td>low*</td>
<td>37.9</td>
</tr>
<tr>
<td>high</td>
<td>57.4</td>
</tr>
<tr>
<td>unknown</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Offspring had only studied up to the minimum school-leaving age.
Table 3. Natural direct effects* on fathers’ and offspring’s pre-bronchodilator FEV$_1$ and FVC (model 1), or FEV$_1$/FVC (model 2) z-scores within the paternal line.

<table>
<thead>
<tr>
<th>GENERATION</th>
<th>Father (G1)</th>
<th>Offspring (G2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔFEV$_1$ (95%CI)</td>
<td>ΔFVC (95%CI)</td>
</tr>
<tr>
<td>Grandparents (G0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grandmother’s smoking†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>when the father was in utero</td>
<td>-0.33 (-0.89, 0.23)</td>
<td>-0.05 (-0.54, 0.44)</td>
</tr>
<tr>
<td>before pregnancy or during father’s childhood (or unknown smoking period)</td>
<td>-0.27 (-0.70, 0.17)</td>
<td>-0.11 (-0.35, 0.13)</td>
</tr>
<tr>
<td>Father (G1)</td>
<td>Smoking initiation (vs never smoking)</td>
<td></td>
</tr>
<tr>
<td>&lt;15 years of age</td>
<td>-0.22 (-0.78, 0.34)</td>
<td>-0.05 (-0.64, 0.53)</td>
</tr>
<tr>
<td>≥15 years of age</td>
<td>-0.27 (-0.51, -0.02)</td>
<td>-0.11 (-0.32, 0.11)</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV$_1$ z-score (1-unit increase)</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>Pre-bronchodilator FVC z-score (1-unit increase)</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>Pre-bronchodilator FEV$_1$/FVC z-score (1-unit increase)</td>
<td>- - -</td>
<td>- - -</td>
</tr>
</tbody>
</table>

* Difference (Δ) in offspring’s expected z-score for the change in exposure status, keeping fathers’ z-score at its expected value when the exposure is absent. Models 1 and 2 also include the potential confounders and adjusting variables of the mediators (grandparents’ education level, and fathers’ age, education level and occupational class) and of the outcomes (fathers’ education level and occupational class, mother’s smoking before or after offspring’s birth, and offspring’s age, sex, education level and smoking).
† Vs not smoking before father’s birth nor during father’s childhood.

The estimates in bold are statistically significant (p-value <0.05).
**Table 4.** Natural indirect effects* on offspring’s pre-bronchodilator FEV<sub>1</sub> and FVC (model 1), or FEV<sub>1</sub>/FVC (model 2) z-scores within the paternal line.

<table>
<thead>
<tr>
<th>GENERATION</th>
<th>Offspring (G2)</th>
<th>ΔFEV&lt;sub&gt;1&lt;/sub&gt;</th>
<th>ΔFVC</th>
<th>ΔFEV&lt;sub&gt;1&lt;/sub&gt;/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
</tr>
<tr>
<td>Grandparents (G0)</td>
<td>Grandmother’s smoking†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>when the father was <em>in utero</em></td>
<td>-0.06</td>
<td>-0.01</td>
<td>-0.12 (0.21, -0.03)</td>
</tr>
<tr>
<td></td>
<td>before pregnancy or during father’s childhood</td>
<td>-0.05</td>
<td>-0.02</td>
<td>-0.07 (-0.17, 0.02)</td>
</tr>
<tr>
<td></td>
<td>(or unknown smoking period)</td>
<td>(-0.13, 0.03)</td>
<td>(-0.06, 0.02)</td>
<td></td>
</tr>
<tr>
<td>Father (G1)</td>
<td>Smoking initiation (<em>vs</em> never smoking)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;15 years of age</td>
<td>-0.04</td>
<td>-0.01</td>
<td>-0.07 (-0.18, 0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-0.14, 0.06)</td>
<td>(-0.10, 0.09)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥15 years of age</td>
<td>-0.05</td>
<td>-0.02</td>
<td>-0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-0.10, 0.004)</td>
<td>(-0.06, 0.02)</td>
<td></td>
</tr>
</tbody>
</table>

* Difference (Δ) in offspring’s expected z-score when the exposure is present, but fathers’ z-score changes from its expected value when the exposure is absent to its expected value when the exposure is present. Models 1 and 2 also include the potential confounders and adjusting variables of the mediators (grandparents’ education level, and fathers’ age, education level and occupational class) and of the outcomes (fathers’ education level and occupational class, mother’s smoking before or after offspring’s birth, and offspring’s age, sex, education level and smoking).

† *Vs* not smoking before father’s birth nor during father’s childhood.

The estimates in bold are statistically significant (p-value <0.05).
FIGURE LEGENDS

Figure 1. Selection of the study subjects (fathers and their offspring).

Figure 2. Mediation model for FEV$_1$ and FVC within the paternal line (model 1).

The green boxes represent the exposures of interest, the yellow boxes the mediators and the blue boxes the outcomes.

The dotted boxes represent the set of potential confounders and adjusting variables of the mediators ($X_1$: grandparents’ education level, and fathers’ age, education level and occupational class) and of the outcomes ($X_2$: fathers’ education level and occupational class, mother’s smoking before or after offspring’s birth, and offspring’s age, sex, education level and smoking). The two ellipses represent the level 2 unit (father; the arrows indicate the random intercept terms at level 2) and the cluster variable (country; no arrow indicates that cluster-robust standard errors were computed in order to take the correlation among fathers within countries into account).

Figure 3. Mediation model for FEV$_1$/FVC within the paternal line (model 2).

The green boxes represent the exposures of interest, the yellow box the mediator and the blue box the outcome. The dotted boxes represent the set of potential confounders and adjusting variables of the mediator ($X_1$: grandparents’ education level, and fathers’ age, education level and occupational class) and of the outcome ($X_2$: fathers’ education level and occupational class, mother’s smoking before or after offspring’s birth, and offspring’s age, sex, education level and smoking). The two ellipses represent the level 2 unit (father; the arrow indicates the random intercept term at level 2) and the cluster variable (country; no arrow indicates that cluster-robust standard errors were computed in order to take the correlation among fathers within countries into account).
Figure 1. Selection of the study subjects (fathers and their offspring).

2,302 males from Estonia, Norway, Spain and Sweden underwent a clinical examination in ECRHS I-III

1,241 males participated in ECRHS III

931 fathers reported to have at least one offspring

297 fathers had
- at least one adult offspring who participated in the RHINESSA clinical stage
- valid lung function measurements
- complete information on their own and their mother's smoking history

420 adult offspring

383 adult offspring had valid lung function measurements

820x755mm (96 x 96 DPI)
Figure 2. Mediation model for FEV1 and FVC within the paternal line (model 1).

275x189mm (96 x 96 DPI)
Figure 3. Mediation model for FEV1/FVC within the paternal line (model 2).

275x129mm (96 x 96 DPI)
MINIMAL SUFFICIENT ADJUSTMENT SET

We explored whether the potential confounders included in model 1 and model 2 represent the “minimal sufficient adjustment set” to estimate the total effect of each exposure on the outcomes (i.e. the smallest group of measured covariates that needs to be included in order to eliminate confounding). We used directed acyclic graphs (DAGs) [1] in DAGitty (dagitty.net) (figure S1 and figure S2). DAGs help to minimize the magnitude of the bias in the estimates, to avoid the risk of over-adjustment and to establish whether the statistical models used are the most parsimonious.

The DAG analysis supported the assumption that the minimal sufficient adjustment set contains grandparents’ education level, fathers’ age, education level and occupational class, mother’s smoking before or after offspring’s birth, and offspring’s age, education level, sex and smoking (“education_GP”, “age_F”, “education_F”, “occupation_F”, “smoke_M”, “age_O”, “education_O”, “sex_O” and “smoke_O” in figure S1 and figure S2).

UNMEASURED CONFOUNDING

We evaluated the impact of unmeasured confounding [2] on the estimate of the natural direct and indirect effects of fathers’ and grandmothers’ smoking on offspring’s lung function, using the Umediation package (github.com/SharonLutz/Umediation) in R3.6.1. Umediation makes it possible to simulate unmeasured confounding of the exposure-outcome, exposure-mediator and mediator-outcome relationships in order to investigate how the results would change if up to two unmeasured confounders were included in the mediation models.

We carried out the simulation analyses as follows:

- In model 1 and model 2, we subdivided the paths in order to simulate unmeasured confounding within a single-exposure, single-mediator, single-outcome framework. Then, we added up to two unmeasured normally distributed confounders with mean 0 and variance 0.001 to the models (“U_I” and “U_J” variables). Figure S3 shows how the data were simulated for “smoke_GM” (exposure), “FEV1/FVC_F” (mediator) and “FEV1/O” (outcome).
- As inputs for the simulations, we used the beta regression coefficients obtained from the cluster-robust (cluster variable: centre) linear models, defined as follows:
  - “FEV1_F” as the outcome and “education_GP”, “smoke_GM”, “age_F”, “education_F”, “occupation_F”, “smoke_F” as covariates;
  - “FVC_F” as the outcome and “education_GP”, “smoke_GM”, “age_F”, “education_F”, “occupation_F”, “smoke_F” as covariates;
  - “FEV1/FVC_F” as the outcome and “education_GP”, “smoke_GM”, “age_F”, “education_F”, “occupation_F”, “smoke_F” as covariates;
  - “smoke_GM” as the outcome and no covariates (null model);
  - “smoke_F” as the outcome and no covariates (null model).

The beta regression coefficients were estimated from 800 bootstrap samplings of one offspring per parent (n = n° offspring = n° fathers = 274). This was done to avoid the “2→2→I” mediation pattern.

- We carried out the simulations under multiple scenarios for the effects (beta regression coefficients) of the unmeasured confounder “U_I” on “smoke_GM” and “smoke_F” (exposure; beta_I→smoke_GM), on “FEV1_F”, “FVC_F” and “FEV1/FVC_F” (mediator; beta_I→med), and on “FEV1/O”, “FVC_O” and “FEV1/FVC_O” (outcome; beta_I→obs), by fixing beta_I→smoke_GM = beta_I→med = beta_I→obs = 0, 1, 3, 5, 7 and 9. We repeated the simulations by adding the second unmeasured confounder “U_J” to the models under the same assumptions.
- We selected 1,000 simulation runs and 1,000 Monte Carlo draws for the nonparametric bootstrap in each of the simulation analyses.
REFERENCES

Figure S1. Directed acyclic graph used to check if the potential confounders included in model 1 represent the “minimal sufficient adjustment set”. Grandparents (generation G0): “education_GP”, grandparents’ education level; “smoke_GM”, grandmother’s smoke. Father/mother (generation G1): “age_F”, father’s age; “education_F”, father’s education level; “FEV1_F”, father’s FEV1 z-score; “FVC_F”, father’s FVC z-score; “occupation_F”, father’s occupational class; “smoke_F”, father’s smoke; “smoke_M”, mother’s smoke. Offspring (generation G2): “age_O”, offspring’s age; “education_O”, offspring’s education level; “FEV1_O”, offspring’s FEV1 z-score; “FVC_O”, offspring’s FVC z-score; “sex_O”, offspring’s sex; “smoke_O”, offspring’s smoke.
Figure S2. Directed acyclic graph used to check if the potential confounders included in model 2 represent the “minimal sufficient adjustment set”. Grandparents (generation G0): “education_GP”, grandparents’ education level; “smoke_GM”, grandmother’s smoke. Father/mother (generation G1): “age_F”, father’s age; “education_F”, father’s education level; “FEV1/FVC_F”, father’s FEV1/FVC z-score; “occupation_F”, father’s occupational class; “smoke_F”, father’s smoke; “smoke_M”, mother’s smoke. Offspring (generation G2): “age_O”, offspring’s age; “education_O”, offspring’s education level; “FEV1/FVC_O”, offspring’s FEV1/FVC z-score; “sex_O”, offspring’s sex; “smoke_O”, offspring’s smoke.
Figure S3. Directed acyclic graph used to simulate the impact of two unmeasured confounders (“U1” and “U2”) on the direct and indirect effects of grandmothers’ smoking in pregnancy on offspring’s FEV1 z-score. Grandparents (generation G0): “education_GP”, grandparents’ education level; “smoke_GM”, grandmother’s smoke. Father/mother (generation G1): “age_F”, father’s age; “education_F”, father’s education level; “FEV1_F”, father’s FEV1 z-score; “occupation_F”, father’s occupational class; “smoke_F”, father’s smoke; “smoke_M”, mother’s smoke. Offspring (generation G2): “age_O”, offspring’s age; “education_O”, offspring’s education level; “FEV1_O”, offspring’s FEV1 z-score; “sex_O”, offspring’s sex; “smoke_O”, offspring’s smoke.
Figure S4. Proportion of Monte Carlo simulations where results match (solid line) and average absolute difference (dashed line) in the average direct (black line) and indirect (red line) effects of fathers’ smoking initiation in prepuberty on offspring’s lung function (whether one or two unmeasured confounders are included or excluded from the models). Outcomes: (a) FEV$_1$, (b) FVC and (c) FEV$_1$/FVC z-scores.
Figure S5. Proportion of Monte Carlo simulations where results match (solid line) and average absolute difference (dashed line) in the average direct (black line) and indirect (red line) effects of grandmothers’ smoking in pregnancy on offspring’s lung function (whether one or two unmeasured confounders are included or excluded from the models). Outcomes: (a) FEV\textsubscript{1}, (b) FVC and (c) FEV\textsubscript{1}/FVC z-scores.
SUPPLEMENTARY INFORMATION ON THE EUROPEAN COMMUNITY RESPIRATORY HEALTH SURVEY (ECRHS)

ECRHS I


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. Paololetti (Pisa).

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Coordination: The co-ordination of this work was supported by the European Commission; the authors and participants are grateful to the late C. Baya and M. Hallen for their help during the study, and to K. Vuylsteek and the members of the COMAC for their support.

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Coordination: The coordination of this work was supported by the European Commission, as part of their Quality of Life programme (QLK4-CT-1999-01237).

ECRHS III


Financial Support: Australia: National Health & Medical Research Council; Belgium: Antwerp South, Antwerp City: Research Foundation Flanders (FWO) (G.0.410.08.N.10); Estonia: Tartu: Estonian Ministry of Education (SF0180060s09); France: (All centres) Ministère de la Santé, Programme Hospitalier de Recherche Clinique (PHRC) national 2010; Bordeaux: INSERM U897 Université Bordeaux segalen; Grenoble: Comité Scientifique AGRAdom 2011; Paris: Agence Nationale de la Santé, Région Ile de France, domaine d’intérêt majeur (DIM); Germany: Erfurt: German Research Foundation (HE 3294/10-1); Hamburg: German Research Foundation (MA 711/6-1, NO 262/7-1); Iceland: Reykjavik: The Landspitali University Hospital Research Fund, University of Iceland Research Fund, ResMed Foundation, California, USA, Orkuveita Reykjavikur (Geothermal plant), Vegagerðin (The Icelandic Road Administration (ICERA). Italy: (All centres) Italian Ministry of Health, Chiesi Farmaceutici SpA; Verona: Cariverona foundation, Education Ministry (MIUR). Norway: Norwegian research council (214123), Western Norway Regional Health Authorities (911631), Bergen Medical Research Foundation. Spain: Fondo de Investigación Sanitaria (PS09/02457, PS09/00716, 09/01511, PS09/02185, PS09/03190), Servicio Andaluz de Salud, Sociedad Española de Neumología y Cirugía Torácica (SEPAR 2010/068), Fondo de Investigación Sanitaria (PS09/02457), Barcelona: Fondo de Investigación Sanitaria (FIS PS09/00716); Galdakao: Fondo de Investigación Sanitaria (FIS 09/01511); Huelva: Fondo de Investigación Sanitaria (FIS PS09/02185), Servicio Andaluz de Salud; Oviedo: Fondo de Investigación Sanitaria (FIS PS09/03190). Sweden: (All centres) The Swedish Heart and Lung Foundation, The Swedish Asthma and Allergy Association, The Swedish Association against Lung and Heart Disease, Swedish Research Council for health, working life and welfare (FORTE), Göteborg: Swedish Council for Working life and Social Research, Umeå: Vasterbotten Country Council ALF grant. Switzerland: The Swiss National Science Foundation (33CSCH-134276/1, 33CSCH-108796, 3247BO-104283, 3247BO-104288, 3247BO-104284, 3247-065896, 3100-059302, 3200-052720, 3200-042532, 4026-028099), The Federal office for forest, environment and landscape, The Federal Office of Public Health, The Federal Office of Roads and Transport, the canton’s government of Aargau, Basel-Stadt, Basel-Land, Geneva, Luzern, Ticino, Valais, and Zürich, the Swiss Lung League, the canton's Lung League of Basel Stadt/Basel, Landschaft, Geneva, Ticino, Valais, and Zurich, SUVA, Freiwillige Akademische Gesellschaft, UBS Wealth Foundation, Talecris Biotherapeutics GmbH, Abbott Diagnostics, European Commission (018996) (GABRIEL), Wellcome Trust (WT 084703MA). UK: Medical Research Council (92091). Support also provided by the National Institute for Health Research through the Primary Care Research Network.

Coordination: The coordination was funded through the Medical Research Council (92091).

† deceased

Ethics approval: Ethics approval was obtained by all centres from the appropriate ethics committees: Antwerp City and Antwerp South: Adviesscommissie Medische Ethiek UZA-UA (CME); Tartu: Research Ethics Committee of the University of Tartu, Estland (N° 2007-1-); French centres: Comité de protection des personnes, Sud V Est (N° 2011-A00013-38); German centres: Ethik-Kommission der Bayerischen Landesarztekammer (N° 10015); Reykjavik: National Biotec Committe of Iceland (NBCI) (N° VSNb2011090016/03.11); Sweden: (All centres) The coordination was funded through the Medical Research Council (92091). Support also provided by the National Institute for Health Research through the Primary Care Research Network.