Regular smoking of male ancestors in adolescence and fat mass in young adult grandchildren and great-grandchildren [version 2; peer review: 2 approved, 2 approved with reservations]

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Abstract

Background: Previous studies using the Avon Longitudinal Study of Parents and Children (ALSPAC) have shown that if men commenced smoking prior to the onset of puberty their sons, their granddaughters and great-granddaughters were more likely to have excess fat (but not lean) mass during childhood, adolescence and early adulthood. In this study we assess associations between ancestral smoking during adolescence (ages 11–16 years) with fat and lean mass of subsequent generations at two ages.

Methods: We analysed data on exposures of grandparents and great-grandparents collected by ALSPAC. The outcomes were the fat masses of their grandchildren and great-grandchildren measured at ages 17 and 24. Measures of lean mass were used as controls. Adjustment was made for 8–10 demographic factors using multiple regression.

Results: We found associations between adolescent smoking of the paternal grandfathers and the adjusted fat mass of their grandchildren, but no associations with the grandchildren’s lean mass. Grandchildren at age 17 had an average excess fat mass of +1.65 [95% CI +0.04, +3.26] Kg, and at age 24 an average excess of +1.55 [95% CI -0.27, +3.38] Kg. Adolescent smoking by the maternal grandfather showed similar, but weaker, associations: at 17 an average excess fat mass of +1.02 Kg [95% CI -0.20, +2.25] Kg, and at 24 an average excess of +1.28 [95% CI -0.11, +2.66] Kg. There were no pronounced differences between the sexes of the children. For the great-grandparents there were few convincing results, although numbers were small.

Conclusions: We have shown associations between grandfathers’ smoking in adolescence and increased fat (but not lean) mass in their children. Confirmation of these associations is required, either in a
further data set or by demonstrating the presence of supportive biomarkers.

**Keywords**
ALSPAC, cigarette smoking, adolescence, grandparents, fat mass, lean mass, intergenerational effects, great-grandparents

This article is included in the **Avon Longitudinal Study of Parents and Children (ALSPAC)** gateway.

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Amendments from Version 1

Changes made to the manuscript.

(i) The number of tables inserted into the previous text has been halved, and the omitted tables are now available as Supplementary Tables in Extended data (OSF). Thus, previous Table 2b, Table 2d, Table 3b, Table 3c, Table 4b, Table 5a, Table 5b and Table 5c have become Supplementary Tables 1-8. Table 2c, Table 3a, Table 4c, and Table 4d have become Table 2b, Table 3, Table 4a, Table 4b and Table 4c respectively. Relevant changes have been made to the text. In Table 2a an error was noted and the odds ratios and 95% CIs have been corrected for maternal grandparents.

(ii) In light of the reviewers’ comments, the meaning of lean mass has been clarified in the Introduction and in the first paragraph of the Discussion. Additions have also been made to the Discussion in light of the reviewers’ comments comprising, in paragraphs 4-5, suggestions as to possible explanations of the effects shown; in addition, an extra weakness is acknowledged by item (c) of the 6th paragraph.

Any further responses from the reviewers can be found at the end of the article.

Introduction

The major spur towards the initiation of recent studies examining associations between human ancestral exposures and their descendants was a detailed analysis comparing the survival of individuals born in Sweden on the edge of the Arctic Circle between 1880 and 1915. Three cohorts of individuals were identified, based on year of birth occurring in the village of Örkalix. Exposures to harvest glut and/or famine during the childhood of grandparents was identified and details linked to their grandchildren’s health indices. Detailed analyses highlighted the following effects on the grandchild: (i) there were strong relationships which were sex-specific, both in regard to the sex of the exposed grandparent and of the affected grandchild’s sex; and (ii) the exposure effects were specific to particular ages of exposure – the most susceptible being the years prior to puberty.

This study prompted a number of projects assessing associations between exposures during the pre-pubertal period and health and development of the grandchildren. For example, van den Berg and Pinger studied the children and grandchildren of individuals who were exposed to the Berlin famine at ages 8–12 years. They demonstrated that those whose mothers had been exposed during these ages had worse health outcomes, particularly if they were male. Subsequently in the next generation, those granddaughters had higher (better) mental health scores if their maternal grandmothers were exposed to the famine pre-puberty, and those grandsons whose paternal grandfathers had pre-pubertal famine exposure had higher mental health scores.

Among major cohort studies, information on environmental exposures during the childhood of parents has been collected occasionally, and rarely in the grandparents. The Avon Longitudinal Study of Parents and Children (ALSPAC) was one pre-birth cohort which collected information on the ages at which the parents of the index children had started smoking regularly. These data were used to ascertain whether index children whose parents had a history of starting to smoke regularly pre-puberty were likely to have differing growth patterns than those who started smoking later. We showed that if fathers had commenced regular smoking prior to the age of 11, their sons (but not their daughters) were more at risk of an increased body mass index (BMI), largely associated with excess fat mass at ages 13, 15 and 17. Subsequently, a detailed study of antecedents associated with fat mass at age 24 indicated that the association remained with paternal smoking <11, and increased in size on adjustment. However, this study also showed an adjusted association between fat mass of the offspring and maternal onset of smoking during adolescence (i.e. at ages 11–16).

We have subsequently determined whether the pre-pubertal ages at commencement of regular smoking of grandparents and or great-grandparents was also associated with fat mass of the grandchildren and great-grandchildren. We compared the fat mass measurements of the different generations according to whether their ancestors had started smoking pre-puberty with those who started smoking in adolescence (11–16). We hypothesised that any effects would differ according to the sex of both the ancestral smoker, and that of the grandchild and great-grandchild. In order to provide a comparison with the results for fat mass, we analysed the results for lean mass (which measures muscle and other tissue excluding bone), and specifically looked at the outcomes of early onset smoking of the great-grandparents, grandparents and parents on the body composition of the index offspring in late adolescence and early adulthood. The results showed that granddaughters, but not grandsons, whose paternal grandfather commenced smoking pre-puberty (<13) were significantly fatter than those whose paternal grandfathers commenced smoking between the ages of 13 and 16. There were similar associations with the great-granddaughters (but not great-grandsons) of fathers of maternal grandfathers who had started pre-puberty. The analyses did not compare grandchildren and great-grandchildren of those ancestors who smoked during adolescence with those who did not. This is the aim of the present study.

Here we hypothesise: (i) that there are likely to be differences between the subsequent generations of children who started smoking before age 17 and those who either never smoked or who started smoking after age 16; (ii) that these are likely to vary with sex of the grandchildren and or great-grandchildren, as well as with (iii) the mode of inheritance (i.e. whether down the maternal or paternal line).

Methods

The ALSPAC population

ALSPAC was designed to assess the ways in which aspects of the environment and genes of individuals interact to result in disadvantages or benefits to health and development. Pregnant women who were residents in a predefined area of Avon with an expected date of delivery between April 1991 and December 1992 inclusive were recruited. Eligible women were contacted as early in pregnancy as feasible. Initial numbers
enrolled were 14,541 pregnancies (and at least one questionnaire had been returned or at least one attendance by mid-September 1999 at a “Children in Focus” clinic). These initial 14,541 pregnancies resulted in a total of 14,676 fetuses, culminating in 14,062 live births. 13,988 of these children were alive at 1 year of age. These participants were followed throughout pregnancy and they, their partners and their offspring throughout subsequent years. The collection of information continued with bolstering of the initial sample, with those who were eligible but who had not enrolled during pregnancy, taking place from the age of 7 years. The total sample size, therefore, for analyses using any data collected after age 7 is 15,454 pregnancies, resulting in 15,589 fetuses, of which 14,901 were alive at 1 year of age. Data were collected using a variety of methods including questionnaires completed by mothers, their partners and offspring; analyses of biological samples; linkage to standard data sets, and hands-on examinations including anthropometrical measures.

From the age of 22, study data were collected and managed using REDCap electronic data capture tools hosted at the University of Bristol. REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies.

The study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool: http://www.bristol.ac.uk/alspac/researchers/our-data/.

Ethical approval
Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee (ALEC; IRB00003312) and the Local Research Ethics Committees. Detailed information on the ways in which confidentiality of the cohort is maintained may be found in the book by Birmingham and on the study website: http://www.bristol.ac.uk/alspac/researchers/research-ethics/

All methods were performed in accordance with the relevant guidelines and regulations. Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the relevant time.

Nomenclature used
The ways in which we refer to the ancestors are shown in Figure 1. The four ancestors on the maternal side of generation F0 are referred to as MGMM (maternal grandmother’s mother), MGMF (maternal grandmother’s father), MGFM (maternal grandfather’s mother) and MGFF (maternal grandfather’s father). The paternal side of generation F0 are labelled PGMM, PGMF, PGFM and PGFF, where P = paternal. For the F1 generation, the labels are MGM and MGF on the maternal side and PGM and PGF on the paternal side. F2 is represented by M (mother) and F (father). F3 is the proband who is referred to as the great-grandchild, grandchild, or child depending on which generation is under consideration.

The Exposures
Questionnaires administered to the study mother and her partner (usually the father of the study child) elicited details of their childhood and adolescence, including the age at which they had commenced smoking regularly, together with other information on their smoking habits, and those of their parents (i.e. the study child’s grandparents (F1s). Unfortunately, smoking habits of the F1s did not include the ages at which they

Figure 1. Family structure with nomenclature used (see text in Methods section). This figure has been reproduced with permission from [Golding et al. 2022] (Creative Commons Attribution 4.0 International license (CC-BY 4.0)).
had started smoking. Consequently, more recently a new questionnaire was administered to those biological parents (F2) with whom the study was still in contact, to obtain further information on their parents (F1s) and grandparents (F0s), including whether they had started smoking regularly during childhood and at what age (defined as < 17 years). Questionnaires were administered online or a paper version posted for those who preferred it. Full details of the methodology and the questions asked can be found elsewhere. In brief, for each ancestor the question asked was: ‘During his/her childhood, up to age 16, did he/she start smoking regularly?’ If yes, the age at which the smoking was started (in years) was requested, with the option ‘yes but don’t know what age’. For the analyses presented here, we have included all who started smoking prior to age 17 and who were therefore smoking in adolescence.

The Outcomes
Total fat mass was estimated with the use of a Lunar Prodigy DXA scanner (GE Medical Systems Lunar, Madison, WI). In this analysis we have concentrated on the measurements of fat mass at ages 17 (approximating to the end of puberty) and 24 years (early adulthood). Measurements of lean mass were measured at the same times using the same equipment as a control. Both were measured on a continuous scale.

Confounders considered
For each ancestor studied, the following were considered as potential confounders: (i) their year of birth; (ii) ethnic group (white/non-white for F1s); (iii) social class based on occupation (manual/non-manual); (iv) no. of older siblings (0/1/2+); (v) no. of younger siblings; (vi) age of ancestor at the birth of the next generation; (vii) level of education (for F1s, not F0s and coded as equivalent to O-level+/ < O-level (examinations taken at the age of 16)); (viii) whether born in England (yes/no for F0s); (ix) trend in gross domestic product (GDP) of year of birth (F1s only); (x) business cycle of year of birth (F1s only).

Statistical analyses
The analyses were structured to take account of the different numbers of ancestors available for study. As shown in Table 1a, the numbers available for analysis ranged from 276 (for the PGFFs) to 2462 (for the MGMs). In general, the

| Table 1a. Numbers (proportions) of grandchildren/great-grandchildren given a DXA scan at ages 17 and/or 24 and whose ancestors were reported to have been smoking in their adolescence (SIA). |
| --- |
| **Ancestor concerned** | DXA scan at 17 |  | DXA scan at 24 |  |
|  | N | no. SIA | %SIA | N | no. SIA | %SIA |
| **Maternal ancestors** |  |  |  |  |  |  |
| MGMM | 1076 | 211 | 20% | 1104 | 178 | 16% |
| MGF | 967 | 542 | 62% | 768 | 471 | 61% |
| MGFM | 913 | 152 | 17% | 765 | 117 | 15% |
| MGFF | 639 | 415 | 65% | 539 | 338 | 63% |
| **Paternal ancestors** |  |  |  |  |  |  |
| PGMM | 520 | 81 | 16% | 472 | 77 | 16% |
| PGMF | 401 | 242 | 60% | 379 | 225 | 59% |
| PGFM | 405 | 61 | 15% | 357 | 49 | 14% |
| PGFF | 297 | 183 | 62% | 276 | 166 | 60% |
| **Maternal grandparents** |  |  |  |  |  |  |
| MGM | 2462 | 415 | 17% | 2089 | 351 | 17% |
| MGF | 2148 | 975 | 45% | 1818 | 820 | 45% |
| **Paternal grandparents** |  |  |  |  |  |  |
| PGM | 1017 | 206 | 20% | 1065 | 181 | 17% |
| PGF | 1048 | 437 | 42% | 924 | 394 | 43% |

MGMM = maternal grandmother; MGF = maternal grandfather; MGMM = maternal grandmother’s mother; MGFM = maternal grandmother’s father; MGFF = maternal grandfather’s mother; MGFM = maternal grandfather’s father; PGM = paternal grandmother; PGF = paternal grandfather; PGMM = paternal grandmother’s mother; PGFM = paternal grandmother’s father; PGFF = paternal grandfather’s father.
The unadjusted associations between each of the four grandparents (F1s) and the eight great-grandparents (F0s) in regard to the grandchild’s (F3) outcomes separately for (i) all grandchildren, (ii) grandsons only and (iii) granddaughters only.

For all outcomes, adjustments were made for potential confounders that contributed 0.1% or more to $R^2$ for the relevant outcome using multiple regression. The analyses were run for all grandchildren and great-grandchildren as appropriate. The analyses were then repeated with a term for the interaction between the sex of the F3 individual and whether or not the relevant ancestor had commenced smoking prior to 17 years of age.

**Results**

**Grandparents’ smoking in adolescence**

**Fat mass.** The unadjusted associations between each of the grandparents who smoked regularly in adolescence and the fat mass of their grandchildren are shown in Table 2a. There were marked associations for increased fat mass for the grandchildren if a maternal or paternal grandparent had smoked regularly in adolescence; the associations at age 17 tended to be more likely to be significant at the P values we have used than those at age 24. There were no significant differences between the sexes.

The demographic variables associated with grandchild’s fat mass are depicted for each grandparent in Supplementary Table 1 (Extended data\(^{12}\)). Those with $R^2 >0.1\%$ were included as covariates. The consequent adjusted associations are shown in Table 2b. The numbers involved in the adjusted analyses were only approximately half of the numbers in the unadjusted analyses due to missing data in the confounders. There were no significant adjusted associations with either of the grandmothers smoking in adolescence, but associations with the grandfather smoking in adolescence remained, especially for the paternal grandfather. There were no indications of differences in effect sizes between the sexes of the grandchildren (Supplementary Table 2, Extended data\(^{12}\)).

**Lean mass.** In complete contrast with Table 2a: (i) whereas 23 of 24 associations showed an increase in fat mass (i.e. greater fat mass if the grandparent had started smoking by 16 years of age), only 16 of the 24 associations with lean mass showed an increase; (ii) whereas 15 out of 24 unadjusted mean differences in fat mass were highlighted as reaching our defined P value cut-points, only 3 of the 24 unadjusted statistics for lean mass did so (Table 3).
### Table 2a. Unadjusted associations between regular smoking during the adolescence of the grandparents and fat mass in their grandchildren at ages 17 and 24. (In bold are results where the P value was <0.10 for maternal ancestors and <0.20 for paternal ancestors).

| Individual | Age | All grandchildren | Grandsons | Granddaughters |
|------------|-----|-------------------|-----------|----------------|
|            |     | MD [95%CI]Kg     | P         | MD [95%CI]Kg   | P     | MD [95%CI]Kg | P |
| **Maternal grandparents** |     |                   |           |                |       |               |   |
| MGM        | 17  | 1.62 [0.56, 2.67] | 0.003     | 1.94 [0.38, 3.51] | 0.015 | 1.01 [-0.24, 2.27] | 0.114 |
|            | 24  | 1.21 [0.02, 2.40] | 0.046     | 1.16 [-0.65, 2.97] | 0.209 | 0.88 [-0.64, 2.40] | 0.257 |
| MGF        | 17  | 1.67 [0.82, 2.52] | <0.001    | 1.34 [0.13, 2.55] | 0.030 | 1.50 [0.46, 2.54] | 0.005 |
|            | 24  | 1.54 [0.59, 2.49] | 0.001     | 0.77 [-0.59, 2.13] | 0.267 | 1.77 [0.52, 3.02] | 0.005 |
| **Paternal grandparents** |     |                   |           |                |       |               |   |
| PGM        | 17  | 1.38 [-0.01, 2.76] | 0.052     | 1.67 [-0.67, 3.27] | 0.196 | 1.19 [-0.48, 2.87] | 0.163 |
|            | 24  | 0.23 [-1.37, 1.82] | 0.781     | 1.49 [-0.90, 3.87] | 0.222 | -0.63 [-2.70, 1.45] | 0.552 |
| PGF        | 17  | 1.69 [0.51, 2.87] | 0.005     | 1.30 [-0.67, 3.27] | 0.031 | 1.21 [-0.24, 2.67] | 0.102 |
|            | 24  | 0.95 [-0.37, 2.28] | 0.158     | 0.45 [-1.42, 2.32] | 0.636 | 1.16 [-0.62, 2.93] | 0.203 |

CI = confidence interval; MD = mean difference in Kg fat mass; MGM = maternal grandmother; MGF = maternal grandfather; PGM = paternal grandmother; PGF = paternal grandfather

### Table 2b. Adjusted associations between regular smoking during adolescence (<17) of grandparents and fat mass in their grandchildren (F3) at ages 17 and 24.

| Ancestor F1 | Age of F3 | N    | MD [95%CI] Kg | P    | R²   | P int |
|-------------|-----------|------|--------------|------|------|-------|
| **Maternal grandparents** | | | | | | |
| MGM         | 17        | 1340 | +0.88 [-0.63, 2.38] | 0.254 | 2.11 | 0.660 |
|            | 24        | 1184 | +1.03 [-0.57, 2.64] | 0.208 | 2.94 | 0.984 |
| MGF         | 17        | 1080 | +1.02 [-0.20, 2.25] | 0.100 | 2.31 | 0.814 |
|            | 24        | 905  | +1.28 [-0.11, 2.66] | 0.071 | 3.01 | 0.718 |
| **Paternal grandparents** | | | | | | |
| PGM         | 17        | 509  | -0.18 [-2.27, 1.90] | 0.863 | 1.03 | 0.233 |
|            | 24        | 449  | -0.73 [-3.27, 1.82] | 0.575 | 1.16 | 0.591 |
| PGF         | 17        | 563  | +1.65 [0.04, 3.26]  | 0.045 | 1.93 | 0.793 |
|            | 24        | 423  | +1.55 [-0.27, 3.38] | 0.095 | 1.85 | 0.483 |

CI = confidence interval; MD = mean difference in Kg fat mass; MGM = maternal grandmother; MGF = maternal grandfather; PGM = paternal grandmother; PGF = paternal grandfather

P int = P value for interaction between the sexes

Interestingly, very few of the socioeconomic and demographic variables were associated with lean mass, compared with fat mass (Supplementary Table 2, Extended data12). For example, the social class and education levels of each of the grandparents contributed to the grandchild’s fat mass, whereas this only occurred rarely for lean mass. Adjustment for these potential confounders showed little of interest (Supplementary Table 3, Extended data12) apart from an interaction with the sex of...
Table 3. Unadjusted associations between regular smoking in adolescence of grandparents and lean mass in their grandchildren at ages 17 and 24.

| Individual ancestor | Age | All grandchildren | | Grandsons | | Granddaughters | |
|---------------------|-----|-------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                     |     | MD [95%CI] Kg | P | MD [95%CI] Kg | P | MD [95%CI] Kg | P |
| Maternal grandparents |     |             |     |             |     |             |     |
| MGM                 | 17  | -0.01 [-1.05, 1.04] | .991 | 0.68 [-0.32, 1.69] | .182 | 0.32 [-0.26, 0.90] | .274 |
|                     | 24  | -0.83 [-1.95, 0.29] | .147 | 0.13 [-1.26, 1.51] | .856 | -0.08 [-0.85, 0.69] | .835 |
| MGF                 | 17  | -0.47 [-1.31, 0.37] | .274 | 0.40 [-0.38, 1.17] | .313 | -0.17 [-0.65, 0.31] | .483 |
|                     | 24  | -0.40 [-1.30, 0.49] | .380 | 0.59 [-0.45, 1.63] | .264 | -0.13 [-0.76, 0.50] | .677 |
| Paternal grandparents |     |             |     |             |     |             |     |
| PGM                 | 17  | 0.43 [-1.04, 1.90] | .569 | 1.13 [-0.31, 2.57] | .125 | 0.47 [-0.33, 1.28] | .250 |
|                     | 24  | 0.49 [-1.06, 2.94] | .536 | 1.55 [-0.31, 3.41] | .101 | 0.05 [-1.01, 1.10] | .931 |
| PGF                 | 17  | -0.23 [-1.43, 0.97] | .707 | 0.04 [-1.16, 1.23] | .953 | 0.45 [-2.1, 1.12] | .179 |
|                     | 24  | 0.24 [-1.02, 1.49] | .712 | 0.79 [-0.71, 2.30] | .301 | 0.40 [-4.7, 1.27] | .365 |

CI = confidence interval; MD = mean difference in Kg fat mass; MGM = maternal grandmother; MGF = maternal grandfather; PGM = paternal grandmother; PGF = paternal grandfather

the grandchild if the PGM had smoked in adolescence (with increased effect size among 24-year-old grandsons compared to granddaughters) (Supplementary Table 4, Extended data).

Great-grandparents’ smoking in adolescence

Fat mass. The unadjusted associations between the great-grandparents’ age <17 at smoking regularly and fat mass of the great-grandchildren is shown in Table 4a. When the maternal great-grandparents had smoked in adolescence, their great-grandchildren tended to have more fat mass on average, with the exception of the great-grandchildren of the MGFF’s, where the associations were negative. The only associations at P<0.10 concerned an excess of fat mass at age 17 if the MGMMM had smoked regularly in adolescence; there was no such association at age 24. For paternal grandparents, there were four associations at P<0.20, each involving the 24-year-olds.

On adjustment for the demographic variables (Supplementary Table 5, Extended data), two of the 16 associations reached the P value stipulated in advance (P<0.10); both associations were negative and were related to the 24-year-olds (involving the MGFM and MGFF). This number of significant adjusted associations was no greater than would have been expected by chance. Similarly, examination of the 32 associations considering the sexes separately, revealed only five below the P value cut-off, and none exhibited consistency between the two age groups (Table 4b and Table 4c).

Lean mass. Of the 48 unadjusted associations between adolescent smoking of great-grandparents and fat mass in their great-grandchildren, only five reached an appropriate P value – i.e. no more than would be expected by chance. On adjustment, two of the 16 comparisons reached a relevant P value, again no more than expected (Supplementary Tables 6–8, Extended data).

Discussion

Our research aim has been to ascertain whether exposure to an environmental insult such as regular smoking in the adolescence of ancestors had any discernible consequences on fat mass in the grandchildren and/or great-grandchildren. We used lean mass effects as a contrast, to ensure that any effect of fat mass was not true of the other anthropometric measures that contributes to body mass index (BMI). Body mass index (BMI) has limitations as a measure of adiposity as it uses a combination of both fat and lean mass. A recent study using the ALSPAC cohort has shown that lean mass predicts markers of pre-clinical atherosclerosis in 24-year-olds, quite different from those predicted by fat mass.

Based on both the Överkalix studies, and our earlier findings of an association between pre-pubertal onset of paternal smoking and increased fat mass in sons, but not daughters, we showed in a previous study that there were sex-specific effects on grand-children and great-grandchildren if their ancestor had commenced regular smoking pre-puberty. Despite small numbers and wide confidence intervals, we found that there was evidence of increased fat mass in granddaughters and great-granddaughters at ages 17 and 24, associated with ancestors who commenced smoking pre-puberty (<13 years) compared with those who commenced in adolescence (aged 13–16). No such associations were noted with lean mass.

In this set of analyses, we have assessed whether there were associations between the amount of fat mass in the grandchildren and great-grandchildren of men and women who...
### Table 4a. Unadjusted associations between regular smoking in adolescence (<17) of great-grandparents and fat mass in their great-grandchildren at ages 17 and 24. Data shown comprise the mean differences (MD) between the fat mass of the great-grandchildren of those great-grandparents who smoked <17 compared with the rest of the population.

| Individual | age | MD [95%CI]Kg | P   | MD [95%CI]Kg | P   | MD [95%CI]Kg | P   |
|------------|-----|---------------|-----|---------------|-----|---------------|-----|
| **Maternal great-grandparents** |     |               |     |               |     |               |     |
| MGMM       | 17  | 2.06 [0.60, 3.52] | .006 | 2.72 [0.70, 4.73] | .008 | 1.62 [-0.15, 3.39] | .073 |
|            | 24  | 1.16 [-0.48, 2.80] | .164 | 1.42 [-1.17, 4.00] | .281 | 0.63 [-1.44, 2.69] | .550 |
| MGMF       | 17  | 0.57 [-0.72, 1.86] | .386 | 0.71 [-1.03, 2.46] | .423 | 0.53 [-1.05, 2.10] | .512 |
|            | 24  | 1.08 [-0.32, 2.48] | .130 | 1.15 [-0.92, 3.21] | .276 | 1.04 [-0.80, 2.88] | .267 |
| MGFM       | 17  | 0.57 [-1.13, 2.28] | .509 | 1.68 [-0.62, 3.98] | .151 | -0.46 [-2.58, 1.65] | .667 |
|            | 24  | 0.39 [-1.62, 2.40] | .700 | 0.12 [-2.65, 2.89] | .934 | 0.67 [-0.21, 3.38] | .630 |
| MGFF       | 17  | -0.17 [-1.69, 1.35] | .831 | -0.16 [-2.38, 2.07] | .889 | -0.30 [-2.15, 1.56] | .754 |
|            | 24  | -1.15 [-2.88, 0.58] | .192 | -0.26 [-2.89, 2.37] | .845 | -1.50 [-3.74, 0.73] | .186 |
| **Paternal great-grandparents** |     |               |     |               |     |               |     |
| PGMM       | 17  | 0.36 [-1.89, 2.60] | .754 | 1.33 [-1.94, 4.60] | .423 | -0.33 [-3.00, 2.34] | .808 |
|            | 24  | -0.96 [-3.36, 1.44] | .432 | 0.81 [-3.07, 4.68] | .682 | -2.01 [-5.03, 1.02] | .192 |
| PGMF       | 17  | 1.86 [-0.96, 4.68] | .209 | -0.01 [-2.84, 2.82] | .994 | 1.21 [-0.87, 3.29] | .254 |
|            | 24  | 0.33 [-1.60, 2.26] | .734 | -2.23 [-5.29, 0.84] | .153 | 1.74 [-0.71, 4.19] | .162 |
| PGFM       | 17  | 0.55 [-1.95, 3.04] | .608 | 2.18 [-1.49,5.84] | .243 | -0.96 [-3.95, 2.03] | .529 |
|            | 24  | 0.31 [-2.53, 3.16] | .828 | 2.42 [-2.83,7.66] | .363 | -0.94 [-4.27, 2.39] | .578 |
| PGFF       | 17  | 1.60 [-1.68,4.88] | .337 | -0.85 [-3.76, 5.45] | .717 | 1.39 [-2.59, 5.38] | .491 |
|            | 24  | 1.94 [-1.65,5.54] | .289 | 5.47 [-5.52, 1.15] | .073 | 0.10 [-4.57, 4.37] | .966 |

CI = confidence interval; MD = mean difference in Kg fat mass MGMM = maternal grandmother’s mother; MGMF = maternal grandmother’s father. MGFM = maternal grandfather’s mother; MGFF = maternal grandfather’s father. PGMM = paternal grandmother’s mother; PGMF = paternal grandmother’s father. PGFM = paternal grandfather’s mother; PGFF = paternal grandfather’s father.

### Table 4b. Adjusted associations between regular smoking in adolescence (<17) of great-grandparents (F0) and fat mass in their great-grandchildren (F3) at ages 17 and 24. Data shown comprise the mean differences (MD) between the fat mass of the great-grandchildren of those great-grandparents who smoked <17 compared with the rest of the population.

| Ancestor F0 | Age of F3 | N    | MD [95%CI]Kg | P   | R² | P_int |
|-------------|-----------|------|--------------|-----|----|-------|
| **Maternal great-grandparents** |     |     |               |     |    |       |
| MGMM        | 17        | 634  | 1.81 [-0.42, 4.04] | .111 | 3.12 | 0.558 |
|            | 24        | 563  | 0.66 [-1.88, 3.19] | .611 | 3.41 | 0.927 |
| MGMF        | 17        | 386  | -0.41 [-2.36, 1.54] | 0.679 | 3.71 | 0.763 |
|            | 24        | 317  | -0.88 [-3.05, 1.29] | 0.425 | 4.89 | 0.439 |
| MGFM        | 17        | 229  | -0.54 [-3.86, 2.77] | 0.748 | 6.56 | 0.509 |
|            | 24        | 467  | -3.48 [-6.21, -0.74] | 0.013 | 2.49 | 0.547 |
| MGFF        | 17        | 372  | 0.43 [-1.54, 2.40] | 0.667 | 1.04 | 0.763 |
|            | 24        | 282  | -2.00 [-4.26, 0.26] | 0.082 | 2.30 | 0.934 |
| Ancestor F0 | Age of F3 | N  | MD [95%CI]Kg     | P   | R²  | P\_int |
|------------|-----------|----|-----------------|-----|-----|--------|
| PGMM       | 17        | 186| -2.74 [-0.63, 2.38] | 0.254 | 2.11 | 0.660  |
|            | 24        | 123| -2.45 [-8.96, 4.05] | 0.457 | 3.45 | 0.853  |
| PGMF       | 17        | 232| 1.35 [-1.13, 3.84]  | 0.285 | 3.02 | 0.480  |
|            | 24        | 139| 0.17 [-3.30, 3.64]  | 0.924 | 4.91 | 0.140  |
| PGFM       | 17        | 86 | -1.23 [-6.60, 4.13] | 0.649 | 1.93 | 0.823  |
|            | 24        | 82 | -2.38 [-8.83, 4.08] | 0.466 | 2.90 | 0.596  |
| PGFF       | 17        | 102| 1.75 [-1.82, 5.32]  | 0.333 | 17.1 | 0.948  |
|            | 24        | 102| 0.05 [-3.60, 3.70]  | 0.980 | 13.4 | 0.886  |

MD = mean difference in Kg fat mass; MGMM = maternal grandmother’s mother; MGMF = maternal grandmother’s father. MGFM = maternal grandfather’s mother; MGFF = maternal grandfather’s father. PGMM = paternal grandmother’s mother; PGMF = paternal grandmother’s father. PGFM = paternal grandfather’s mother; PGFF = paternal grandfather’s father.

P\_int = P value for interaction between the sexes

**Table 4c.** Adjusted associations between regular smoking during adolescence of the great-grandparents (F1) and fat mass in their great-grandchildren (F3) at ages 17 and 24. (In bold are results where the P value was <0.10 for maternal ancestors and <0.20 for paternal ancestors).

| Great-Grandparent | GREAT GRANDSONS F3s | GREAT GRANDDAUGHTERS F3s |
|-------------------|----------------------|--------------------------|
|                   | n     | MD [95%CI] | P     | n     | MD [95%CI] | P     |
| Fat mass at 17    |       |           |       |       |           |       |
| MGMM              | 287   | **3.43 [0.59, 6.27]** | **0.018** | 347   | 1.68 [-1.15, 4.50] | 0.244 |
| MGMF              | 181   | 0.37 [-1.91, 2.64] | 0.751 | 205   | 0.94 [-1.59, 3.46] | 0.466 |
| MGFN              | 110   | 0.64 [-3.86, 5.14] | 0.779 | 119   | -1.70 [-5.60, 2.20] | 0.389 |
| MGFF              | 174   | 1.07 [-1.70, 3.84] | 0.447 | 198   | 0.03 [-2.38, 2.44] | 0.980 |
| PGMM              | 81    | -0.94 [-7.02, 5.14] | 0.760 | 105   | **-4.23 [-8.64, 0.19]** | **0.060** |
| PGMF              | 58    | 1.76 [-1.93, 5.45] | 0.343 | 86    | 0.43 [-1.33, 2.18] | 0.629 |
| PGFM              | 30    | -0.05 [-6.22, 6.12] | 0.986 | 56    | **2.45 [-0.35, 5.25]** | **0.086** |
| PGFF              | 39    | -0.96 [-5.47, 3.55] | 0.668 | 62    | -0.05 [-1.92, 1.81] | 0.953 |
| Fat mass at 24    |       |           |       |       |           |       |
| MGMM              | 232   | 0.11 [-3.71, 3.93] | 0.956 | 331   | 0.71 [-2.56, 3.97] | 0.671 |
| MGMF              | 141   | 0.49 [-3.04, 4.02] | 0.784 | 176   | -1.30 [-3.98, 1.39] | 0.342 |
| MGFM              | 197   | **-4.44 [-8.29, -0.59]** | **0.024** | 270   | -2.92 [-6.65, 0.80] | 0.123 |
| MGFF              | 126   | -1.61 [-4.70, 1.49] | 0.307 | 156   | -2.51 [-5.62, 0.61] | 0.114 |
| PGMM              | 48    | -3.14 [-14.8, 8.51] | 0.589 | 75    | -4.78 [-12.9, 3.31] | 0.242 |
| PGMF              | 55    | -2.36 [-7.57, 2.86] | 0.369 | 84    | 1.65 [-3.21, 6.51] | 0.501 |
| PGFM              | 27    | -3.16 [-31.3, 25.0] | 0.817 | 55    | **-4.17 [-9.53, 1.19]** | **0.025** |
| PGFF              | 38    | 0.78 [-6.55, 8.11] | 0.829 | 64    | -0.33 [-4.71, 4.05] | 0.880 |

CI = confidence interval; MD = mean difference in Kg fat mass; MGMM = maternal grandmother’s mother; MGMF = maternal grandmother’s father. MGFN = maternal grandfather’s mother; MGFF = maternal grandfather’s father. PGMM = paternal grandmother’s mother; PGMF = paternal grandmother’s father. PGFM = paternal grandfather’s mother; PGFF = paternal grandfather’s father.
had smoked regularly in adolescence compared with the rest of their peers. Here we have shown associations between the grandfathers smoking in adolescence and the fat mass of their grandchildren, and that this was apparent for the grandchildren in both their late teens and early adulthood (ages 17 and 24), and for both the maternal and paternal lines, contrary to our hypothesis. There were no such associations if either of the grandmothers had smoked in adolescence. There were no convincing associations between the great-grandparents smoking in adolescence and the fat or lean mass of their great-grandchildren.

Previous analyses have stressed the importance of the timing of exposures in regard to outcomes in succeeding generations. We have shown this in regard to exposures in utero as well as in the pre-puberty period, with apparent effects on outcomes as diverse as autistic traits, myopia, obesity and IQ\textsuperscript{11}. Here we have demonstrated an association with an exposure to cigarette smoking in the adolescent period and suggest that this period of time should also be considered in further multi-generational studies. However, it should be noted that, unlike the associations with pre-pubertal smoking, there was no indication of any consistent associations in the great-grandchildren. This may be a consequence of relatively small numbers, or it may suggest that transgenerational inheritance is an unlikely mechanism.

It should be noted that although the effects of cigarette smoke and of nicotine exposures across generations have been shown down both the male and female lines in rodent models\textsuperscript{18–21} there is still confusion as to possible mechanisms for such epigenetic effects. As Donkin and Barres\textsuperscript{20} have said: ‘the mechanism of how epigenetic factors are established and altered in the germline, as well as in somatic cells, are not well understood. Causality is yet to be explained, and it is still highly debated to what extent genetic and epigenetic factors interplay in the environmentally influenced manipulation of gene expression and phenotype’. However, there are possible clues from the methylation literature. Grandmaternal smoking during pregnancy has been associated with some differences in DNA methylation\textsuperscript{23}; as has paternal smoking in adolescence (Kitaba et al. 2023, preprint: https://www.biorxiv.org/content/10.1101/2023.01.13.523912v1). A couple of the sites associated with paternal smoking in adolescence were associated with offspring weight and BMI. Together we think this suggests that DNA methylation is an interesting candidate mechanism for the associations found here.

This study has a number of weaknesses: (a) the data on age at onset of regular smoking of their ancestors was obtained retrospectively from their children and grandchildren. Although there is anecdotal evidence that ancestors who started smoking pre-adolescence are prone to remember and even boast about this, it is unclear as to whether those starting smoking at later ages (i.e. age 13–16) were as likely to recall such detail. (b) There was a large amount of information missing on age at onset of smoking; we did not try to impute these data since we were unsure whether they were missing at random. Consequently, the adjusted analyses were carried out with complete data only, with obvious reduction in statistical power, particularly for the paternal line. To compensate for this, and to ensure that we did not ignore relevant associations, we considered P values <0.10 for the maternal line and <0.20 for the paternal line. (c) We did not allow for instances where both grandparents or great-grandparents smoked, because that would have reduced the statistical power even further. However, our results do suggest that a history of adolescent smoking in only one member of each pair of grandparents or great-grandparents was particularly associated with fat mass in the (great) grandchildren. Examples pinpoint MGF but not MGM; PGF but not PGM (Table 2b), and PGFM but not PGFF among others (Table 4c). (d) We were not able to replicate our findings as we are not aware of any other studies with similar relevant data.

The strengths of the study lie in: (i) its longitudinal nature; (ii) the fact that outcomes used the DXA measures of fat and lean mass, which are considerably more accurate than indicators such as BMI (body mass index) which do not distinguish between fat, lean or bone mass\textsuperscript{23}, and (iii) the associations we demonstrated were apparent for the two ages tested.

In conclusion, our research question concerned whether exposures to cigarette smoking in the age group 13–16 years compared with not starting smoking until age 17 or later, or not at all, was associated with outcomes in the grandchildren or great-grandchildren. We have shown here that exposures to cigarette smoking at this age by the grandfather, but not the grandmother, were associated with fat, but not lean, body mass. The fact that no such effects were found among the great-grandchildren may indicate that the associations are intergenerational rather than transgenerational. Alternatively, it may indicate weakening of effects across generations possibly obscured by a multitude of other factors. Clearly further longitudinal family studies are important in order to assess whether these results are generalisable.

Data availability
ALSPAC data access is through a system of managed open access. The steps below highlight how to apply for access to the data included in this Data Note and all other ALSPAC data:

1. Please read the ALSPAC access policy which describes the process of accessing the data and samples in detail, and outlines the costs associated with doing so.

2. You may also find it useful to browse our fully searchable research proposal database which lists all research projects that have been approved since April 2011.

3. Please submit your research proposal for consideration by the ALSPAC Executive Committee. You will receive a response within 10 working days to advise you whether your proposal has been approved.

If you have any questions about accessing data, please email alspac-data@bristol.ac.uk.
This project contains the following extended data:

- Data file 1. (Supplementary Tables)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Acknowledgements
We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses.

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Open Peer Review

Current Peer Review Status:  ✔  ✔  ?  ?

Version 2

Reviewer Report 27 March 2023

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Anthony P. Monaco
Tufts University, Medford, MA, USA

The authors have revised the manuscript and addressed the reviewers’ concerns.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Human genetics, epigenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 09 February 2023

https://doi.org/10.21956/wellcomeopenres.19893.r54462

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Gemma Lewis
Division of Psychiatry, Faculty of Brain Sciences, University College London, London, UK

This study uses the large ALSPAC cohort to investigate associations between smoking among ancestors and ‘fat mass’ in their grandchildren. The main finding was a positive association between adolescent smoking by grandfathers and increased ‘fat mass’ among their young adult grandchildren. The authors explained the complex design clearly and the ALSPAC cohort is large,
with a rich amount of novel data. The long follow-up into young adulthood is also a strength. I have several suggestions to improve the manuscript:

The hypotheses were clearly stated. However, the introduction would benefit from a summary of the theoretical (not just empirical) justification of the hypotheses proposed: what is the theory underlying/leading to these hypotheses, and why are they plausible in terms of potential mechanisms/explanations?

Throughout the manuscript, the authors could tone down strongly causal language which seems inconsistent with the observational design (e.g. effects, determined, consequences, ascertain etc).

In the introduction, the authors could consider replacing the term ‘fatter’ with a more medical objective term.

In the introduction there was a good critical appraisal of existing evidence but I didn't get a strong sense of the broader context/bigger picture: why is this research question important; where would it lead in terms of advancements / implications?

The discussion would benefit from a section on potential mechanisms that underlie these associations. Why do the authors think that this pattern of associations was observed in terms of underlying mechanisms?

The discussion would also benefit from consideration of the potential practical implications of these findings.

The limitations section could be expanded upon. I wasn't convinced by the anecdotal evidence that people boast about smoking pre-puberty and thought that could be removed. The authors could justify their assumption/uncertainty about whether data were MAR. The large amount of systematic attrition across follow-up is a limitation of the ALSPAC cohort, and strategies to replace missing data (such as MI and FIML) are commonly used and recommended. I think that lack of a strategy to address missing data (particularly in the outcomes due to attrition to adulthood) is one of the main limitations of the study. If not addressed analytically, this should be discussed in more detail. It isn't just the reduction in power that is a potential problem – the risk of bias due to missing data should also be addressed/discussed. The limitations section should also cover the possibility of residual confounding, which can never be ruled out in observational studies. Some of the confounders – mainly SES – were measured quite crudely (manual, non-manual) and this could be discussed. Genetic confounding of these associations also seems possible.

There are a lot of Tables and it would the manuscript easier to digest if some of these were moved to the supplement (perhaps the ones not containing main findings).

The paper takes a binary approach to interpreting p values, which are instead a continuous construct. Could the authors reconsider this? I thought ALSPAC guidance was to avoid the binary terms ‘significant’ and ‘non-significant’ which encourage over reliance on an arbitrary binary threshold. I would reconsider the related strategy of emboldening ‘significant’ findings.

The authors select confounders based on the R squared value of the confounder. This is an unusual approach in epidemiology. I think the authors should either reconsider this strategy or
justify their approach in more detail, with supporting evidence/references etc. Usually, a confounder would be included if it altered the association between exposure and outcome. Reliance upon R squared is a strategy more suitable for predictive modelling. It also does not explore whether the confounder is associated with the exposure (as well as the outcome).

The regression modelling could be explained more clearly

In the section on data availability, I think the authors should mention the financial costs required to access ALSPAC. Definitions of open access vary but one is: Open access is a set of principles and a range of practices through which research outputs are distributed online, free of access charges or other barriers. Could the authors consider whether the ALSPAC data are in fact open access to researchers outside of Bristol University (because of the financial costs)?

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Psychiatric epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 25 October 2022
https://doi.org/10.21956/wellcomeopenres.19893.r52588

© 2022 Holloway A. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
The strength of this work is that it is based on the ALSPAC longitudinal study which has collected information regarding smoking across multiple generations. The authors have looked at the timing of smoking initiation in great-grandparents and grandparents and the association with fat mass in the study child (F3). They have attempted to control for a number of confounding factors; whether or not there are more than is justified by the sample size, is a question for a statistical expert.

While the authors make conclusions about the impact of ancestral smoking it is somewhat unclear how they have dealt with the fact that there seems to be more than one observation per F0 ancestor. For example, according to table 1 there are a total of 389 MGMM subjects but 1289 grandchildren/great grandchildren. How did the authors deal with multiple family members in the study? It is also not clear how the authors dealt with smoking by both ancestors. Did the analysis consider if both great-grandparents or grandparents were smokers? Based on other available literature which suggests an additive effect of parental smoking (more than just maternal or paternal alone) this is an important consideration and if it was not considered it should be clearly identified as a limitation of this study. It is not clear how the lack of change in lean mass is "a contrast to ensure that any effect of fat mass was not true of any other anthropometric measure". Finally there is a limited and vague discussion of why the associations in the great-grandchildren were not observed--this should be strengthened with some insights from other cohort studies or animal experiments.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Early life influences on metabolic health, parental smoking

I confirm that I have read this submission and believe that I have an appropriate level of
expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Feb 2023

Yasmin Iles-Caven

Thank you very much for your constructive criticisms. These have been very useful in our rewriting of the paper. You will see that we have made the following changes in response to your review:

1. You were right to point out the confusion over numbers created by a reading of Table 1. We have revised this table and trust that it is now much clearer. In response to your query, there are no more than one grandchild/ great-grandchild per ancestor studied.

2. You questioned how we dealt with instances where both grandparents or great-grandparents smoked. We did not investigate this question due the small number of individuals with the required information. However, our results do suggest that only one member of each pair was particularly associated with fat mass in the (great) grandchildren. Examples pinpoint MGF but not MGM; PGF but not PGM (Table 2b), and PGFM but not PGFF (Table 4c). We have added this to the limitations.

3. We agree that it was unclear how the lack of change in lean mass is "a contrast to ensure that any effect of fat mass was not true of any other anthropometric measure". We have now clarified this in the first paragraph of the Discussion.

4. In response to your query about "why the associations in the great-grandchildren were not observed", we have noted that these kinds of questions have arisen in rodent studies of smoke and nicotine exposure and remain unresolved, even though investigation of mechanism is relatively easier than in human studies.

Competing Interests: None

Author Response 15 Mar 2023

Yasmin Iles-Caven

Thank you very much for your constructive criticisms. These have been very useful in our rewriting of the paper. You will see that we have made the following changes in response to your review:

1. You were right to point out the confusion over numbers created by a reading of Table 1. We have revised this table and trust that it is now much clearer. In response to your query, there are no more than one grandchild/ great-grandchild per ancestor studied.

2. You questioned how we dealt with instances where both grandparents or great-grandparents smoked. We did not investigate this question due the small number of individuals with the required information. However, our results do suggest that only one member of each pair was particularly associated with fat mass in the (great) grandchildren. Examples pinpoint MGF but not MGM; PGF but not PGM (Table 2b), and PGFM but not PGFF (Table 4c). We have added this to the limitations.
individuals with the required information. However, our results do suggest that only one member of each pair was particularly associated with fat mass in the (great) grandchildren. Examples pinpoint MGF but not MGM; PGF but not PGM (Table 2b), and PGFM but not PGFF (Table 4c). We have added this to the limitations.

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4. In response to your query about “why the associations in the great-grandchildren were not observed”, we have noted that these kinds of questions have arisen in rodent studies of smoke and nicotine exposure and remain unresolved, even though investigation of mechanism is relatively easier than in human studies.

** Competing Interests:** None

**Anthony P. Monaco**
Tufts University, Medford, MA, USA

This manuscript derives from a unique longitudinal study (ALSPAC) which has been utilized by this group in prior work to show associations between grand-paternal early onset nicotine smoking with fat mass in granddaughters and great granddaughters. In this manuscript they used fat mass, compared to lean mass, and took into account a number of possible associated factors with reduced cut-off for statistical significance.

Although the approach seems sound, it needs to confirmed by a statistical expert. However, they do not define what ‘lean mass' is and why it is an appropriate control. In addition, the data correlations and statistical analysis is presented in numerous tables. It is hard for the reader to discern on which of the many tables they should focus. May I suggest they consider moving some of tables to a supplement, and keep the most salient tables in the article. Is there any other way they can present the data which is more visual than tables full of numbers?

Lastly, they make no effort to discuss possible mechanisms. Given the large volume of literature of substances of misuse in rodent models with offspring epigenetic effects, it seems like a paragraph in the discussion is warranted to point the reader to relevant animal models. It would also confirm that the transgenerational effects they are studying are not unique to humans.
Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Human genetics, epigenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Feb 2023
*Yasmin Iles-Caven*

Thank you for your helpful comments. In light of these we have made the following changes.

1. We have removed about half of the tables; They are now published in a Supplement.

2. We have described the lean mass measure and its possible importance in both the Introduction and the Discussion.

3. We have added more references in the Discussion in regard to the animal literature concerning transgenerational associations between maternal and paternal exposures to nicotine and to cigarette exposures. We have also speculated as to possible involvement of methylation changes.

**Competing Interests:** None

Author Response 15 Mar 2023
Yasmin Iles-Caven

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**Competing Interests:** None

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**Reviewer Report 26 July 2022**

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**Mathew Smith-Raska**

Division of Newborn Medicine, Department of Pediatrics, New York-Presbyterian Hospital, Weill Cornell Medicine, New York, USA

This is an important study using the ALSPAC dataset, examining the relationship between ancestral smoking and descendants' fat mass. The authors have previously demonstrated that male smoking prior to puberty was associated with increased fat mass in their sons as well as granddaughters and great-granddaughters. In this study, they examine the relationship between ancestral smoking during adolescence with fat mass in subsequent generations at ages 17 and 24. As a control, they also examined lean mass. They report that adolescent smoking of the paternal grandfather is associated with increased fat mass of their grandchildren; there was a similar but weaker association with the maternal grandfather.

This study benefits from the enormous ALSPAC dataset, which has potential to reveal many novel relationships between ancestral exposures and ancestral risk of disease. The authors very carefully and thoroughly consider multiple confounders that can potentially skew the results.

The authors do loosen the traditional p-value cutoff of < 0.05 for reasons explained in text, relating to being able to detect associations. Because I am not a statistician, I cannot comment on the validity of this approach. However, it should be considered that they are able to detect multiple
associations using this loosened statistical stringency which supports the conclusion that these associations are true, however I am not qualified to state whether this is a valid approach.

There are a lot of tables, and this manuscript may benefit from moving some to Supplemental Tables. I had some difficulty finding the important information among all of the tables. For example, under “Lean mass” on page 6: it implies that there are 23 of 24 positive associations in Table 2a, however there are only 14 of 25 positive associations in this table. Overall it was a bit difficult to find the most important information among all of the Tables. Examples that come to mind include Table 2b and Table 4b. I would recommend moving some of the Tables to a Supplemental Information section.

The manuscript would also benefit from a better description of what “lean mass” is and how it differs from “fat mass,” including relevance to health/disease. Along similar lines, in the second-to-last paragraph it is stated that DXA measures of fat and lean mass are more accurate indicators than BMI; if mentioned there should be evidence or references to support this.

**Minor point:**
- Need to add a space between “ages” and “17” on Page 12 (currently reads as “ages17”)

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
I cannot comment. A qualified statistician is required.

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Epigenetic Inheritance

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
Yasmin Iles-Caven

Thank you very much for your constructive criticisms. These have been very useful in our rewriting of the paper. You will see that we have made the following changes in response to your review:

1. We have taken your advice and moved 8 of the tables to a Supplementary file. Hopefully this will make the paper easier to read.

2. You were puzzled at the numbers of positive associations – this was meant to refer to the numbers of associations where the mean difference was +ve as opposed to -ve. This has been reworded to make it clearer that we were talking about an increase in mass, rather than a decrease.

3. You ask for a description of the differences between fat and lean mass, and why fat mass is a more relevant statistic than BMI. We have added a couple of sentences to the first paragraph of the Discussion and added relevant references.

4. We have corrected the typo.

Competing Interests: None

Author Response 15 Mar 2023

Yasmin Iles-Caven

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Competing Interests: None