The association of DNA methylation at birth with adolescent asthma is mediated by atopy

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To the Editor,

Asthma is a common chronic respiratory condition and a major public health concern worldwide. A sex reversal in asthma prevalence from pre- to post-adolescence has been observed; more boys remit asthma than girls during adolescence whereas more girls acquire asthma than boys[1]. Atopy affects 10-30% of the general population in developed countries[2] and is a risk factor for asthma[3].

DNA methylation (DNAm) or longitudinal change in DNAm at specific cytosine-phosphate-guanine (CpG) sites is associated with the risk of atopy[4] and asthma acquisition[5]. The role of atopy in the association of DNAm in newborns with asthma acquisition at a later age is unknown. Utilizing data in the Isle of Wight Birth Cohort (IOWBC), we examined whether pre-adolescence atopy mediates the association of newborn DNAm with asthma acquisition from pre- to post-adolescence.

The IOWBC was established on the Isle of Wight, UK, to prospectively study the natural history of allergic diseases among children. Of the 1,536 born between January 1, 1989, and February 28, 1990, parents of all infants born over this period were contacted, and subsequently, 1,456 infants were enrolled following informed consent and exclusion. Follow-ups were conducted at ages 1, 2, 4, 10, and 18 years via International Study of Asthma and Allergy in Childhood (ISAAC) questionnaires and clinic visits. Details on study design, enrollment, and follow-up procedures for the IOWBC are described elsewhere[6,7] and in AF1.

Asthma acquisition from 10 to 18 years was defined as not having asthma at 10 years and having asthma at 18 years (no → yes). Participants without asthma at 10 and 18 years were included as a reference group (no → no). Atopy status at age 10 was determined by skin prick test (SPT) results (≥3mm) against 11 common allergens (house dust mite, cat dander, dog

dander, grass pollen mix, tree pollen mix, *Alternaria alternate, Cladosporium herbarium*, cow's milk, hen's egg, peanut, and cod).

Selected covariates were sex, breastfeeding duration in weeks and age at specific pubertal events (i.e., age at puberty defined as age at onset of voice deepening in males and age at onset of menarche in females), socio-economic status (SES), and smoke exposure. SES included household income, number of rooms and maternal education. Smoke exposure involved combining active and second-hand smoke exposure at 18 years, the former in which a participant recorded 'yes' as a current smoker and the latter defined as tobacco exposure from mother, father, others, or outside home.

Genome-wide DNAm in DNA extracted from Guthrie cards was assessed using the Infinium MethylationEPIC BeadChip (Illumina, Inc, San Diego, CA, USA). Cell-type-adjusted DNAm (via regression of DNAm M-values on cell type proportions[8]) for each sex were used in subsequent analyses. Gene expression levels from peripheral blood samples collected at 26 years were measured using paired-end (2×75 bp) RNA sequencing using the Illumina Tru-Seq Stranded mRNA Library Preparation Kit with IDT for Illumina Unique Dual Index (UDI) barcode primers. Additional information on DNAm and RNA sequencing are available in Additional File (AF) 1.

Genome-wide screening of CpGs in newborns for their potential association with atopic status at 10 years and asthma acquisition from 10 to 18 years was conducted separately with the R package, *ttScreening* (described in AF1). Screenings were performed separately for each sex. CpGs in differentially methylated regions (DMRs) with respect to asthma acquisition identified using R package, *dmrff*, were also included as candidate CpGs. A false discovery rate (FDR) of 0.2 was selected in DMR detection to reduce the probability of losing informative CpGs. An

overview of the screening process and subsequent analyses (details in AF1) is presented in Figure 1.

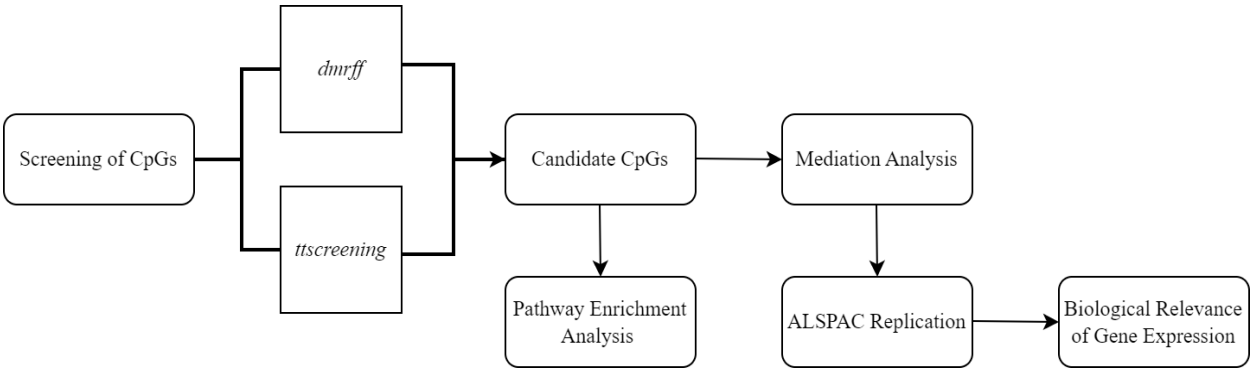

Figure 1. Diagram of screening for candidate CpGs in pathway enrichment analysis and mediation analysis.

Based on the main variables included in the analyses, the subsample (subjects with asthma acquisition status and DNA available) represented the complete cohort (subjects with asthma acquisition status available) indicated by p-value > 0.05 in AF2 Table 1.

The *ttScreening* was applied to 551,710 CpGs to identify candidate CpGs potentially associated with atopy at 10 years or asthma acquisition from 10 to 18 years, stratified by sex. In males, 126 CpGs were identified for atopy and 93 CpGs for asthma acquisition, and in females, 196 and 182 CpGs, respectively (AF3 ST 1 and 2). No common CpGs were found between atopy and asthma in both sexes, and there were no overlapping CpGs between males and females. DNAm at the genome-scale was analyzed to identify DMRs corresponding to asthma acquisition (AF4 for details). Altogether, 223 CpGs in males and 378 CpGs in females (in total, 601 CpGs) were analyzed for their enrichment in pathways or biological processes. Using *gometh*, we identified 153 biological processes in males and 215 in females (p-value < 0.05; (AF2 Tables 3a and 3b for top 10 GO terms and AF3 ST3 for all the terms). No common biological processes were identified between sexes.

After controlling for confounders, DNAm in newborns at 68 CpGs in males and 41 CpGs in females indirectly associated with asthma acquisition via atopy. At 60 of the 68 CpG in males and 37 of the 41 CpGs in females, only indirect effects were statistically significant. Of these, at 21 CpGs in males and 18 CpGs in females, a higher DNAm was indirectly associated with an increased risk of asthma acquisition via an increased risk of atopy (AF3 ST4). DNAm at some CpGs only showed direct effects on asthma acquisition; in particular, DNAm in newborns at 99 CpGs in males and 192 CpGs in females was directly associated with asthma acquisition from 10 to 18 years age. Of these, for 50 (51% of 99) CpGs in males and 72 (38%) CpGs in females, an increase in DNAm was directly associated with an increase in the risk of asthma acquisition (AF3 ST5).

Indirect associations of CpGs in IOWBC were further tested in the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) study. DNAm data in newborns were available for 41 CpGs (of the 68 in IOWBC) in males and 18 CpGs (of the 41 in IOWBC) in females. Of these, the direction of indirect effects was consistent with those in IOWBC at 20 (49%) CpGs in males and 10 (56%) CpGs in females, and none of these CpGs were statistically significant in ALSPAC. Importantly, of the 20 and 10 CpGs in males and females, respectively, all CpGs showed only indirect effects in IOWBC (AF3 ST3). For CpGs showing only direct effects in IOWBC (99 in males and 192 in females), DNAm in ALSPAC was available for 54 and 113 CpGs in males and females, respectively. Of these, at 25 (46.3%) CpGs in males and 78 (69.0%) CpGs in females (103 CpGs in total), directions of association were the same as those in IOWBC with one CpG (cg12938020) in males and 11 in females as being statistically significant (AF3 ST4).

We evaluated the association of DNAm at the identified 133 CpGs (45 in males) with the expression of neighboring genes (measured at age 26 years). Significant effects of DNAm were observed at 35 CpGs on their association with expression of 213 neighboring genes in males, and at 61 CpGs with expression of 357 such genes in females (AF2 Table 4 and AF3 ST6). More than 40% of the 96 CpGs are located in the promoter region of their mapped genes. For 289 (130 in males) of these 570 neighboring genes (213 in males), increased DNAm was associated with increased gene expression, with the top 10 most significant associations for each sex illustrated in Figure 2.

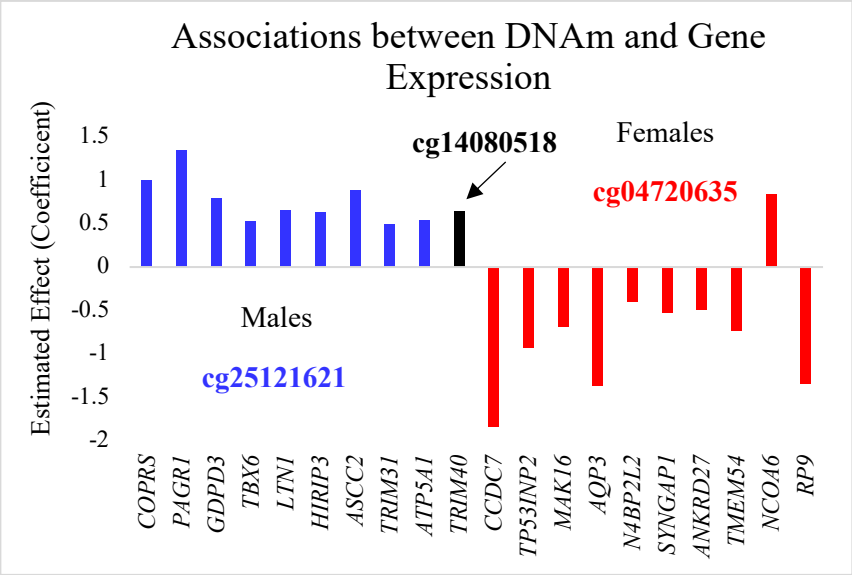

Figure 2. Estimated effects (regression coefficients) of DNAm on gene expression levels of neighboring mapped genes in each sex.

To our knowledge, this is the first study to examine the direct and indirect effects of DNAm in newborns on asthma acquisition from pre- to post-adolescence via pre-adolescence atopy status. We discovered consistent mediation effects (with indirect effects only) between the two cohorts at 20 and 10 CpG sites in males and females, respectively, although the effects were not statistically significant in ALSPAC. Statistical insignificance may be due to the relatively

small number of asthmatic subjects in ALSPAC and the differences in the ages of assessment between the cohorts. In ALSPAC, atopy at age 7 years and asthma acquisition from 7 to 17 years was included, while in IOWBC, atopy was at age 10 years and asthma acquisition from 10 to 18 years. Additionally, DNAm in IOWBC was measured in Guthrie cards while DNAm in ALSPAC was measured in cord blood. Our earlier study demonstrated an agreement of 70% in DNAm between these two sources (Guthrie cards and cord blood)[9]. This discrepancy between the DNA sources may also have affected the statistical power in the replication analyses.

Asthma can be atopic or non-atopic. Atopic asthma is IgE mediated while non-atopic asthma is not. We postulate that the 30 CpGs showing only indirect effects might be markers linked to atopic asthma development due to atopy mediation. Conversely, the sole direct effects of the 103 CpGs may contribute to non-atopic asthma occurrence since an individual's atopic state lacked influence on the association of DNAm in newborns with asthma acquisition. In addition, although statistically insignificant after adjusting for multiple testing, pathways and biological processes detected through the enrichment analysis have been suggested to play an important role in the asthma and allergy pathogenesis. Future studies are encouraged to investigate the role of DNAm at these CpGs as a mediator in the association of exposures during fetal development with asthma and allergic conditions. A complete discussion of our work is presented in AF4.

Author Contributions: H.Z. designed the study; A.R. analyzed the data; M.R. helped with *ttScreening* package application. A.R. and H.Z. interpreted findings and drafted the manuscript; W.K., H.A., C.L.R. and J.W.H. supervised the study; S.E. and J.W.H. provided DNA methylation data, R.M. performed additional analyses and revised the manuscript, and all authors

reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: For IOWBC, the study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of The Isle of Wight Local Research Ethics Committee (protocol code 18/98 and date of approval 07/20/1998), and the Isle of Wight, Portsmouth, and SE Hampshire Local Research Ethics Committee (protocol code 06/Q1701/34 and date of approval 06/16/2006)" for studies involving humans. The Internal Review Board (IRB) at the University of Memphis

(FWA00006815, IRB ID: #3917) gave approval for data analysis at the University of Memphis. For ALSPAC, ethical approval for the study was obtained from the ALSPAC Law and Ethics Committee and the Local Research Ethics Committees (NHS Haydock REC: 10/H1010/70).

Conflicts of Interest: The authors declare no conflict of interest.

Declarations: Ethics approval and consent to participate. The Isle of Wight Local Research Ethics Committee approved the 10-years assessment as well as the collection of blood for genetic studies into asthma and allergy (No. 18/98, dated 07/20/1998). For the 18-year follow-up, ethical approval was given by the Isle of Wight, Portsmouth, and SE Hampshire Local Research Ethics Committee (No. 06/Q1701/34, dated 06/16/2006). The Internal Review Board (IRB) at the University of Memphis (FWA00006815, IRB ID: #3917) gave approval for data analysis at the University of Memphis. Written informed consent was obtained from parents during in-person visits. For participants assessed by phone interview, consent was documented on the consent form with the name of the person giving consent, and the name and signature of the person taking the form were recorded. For ALSPAC, ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for collection of biological samples was provided in accordance with the Human Tissue Act (2004). 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 time. For the age of seven years, the United Bristol Healthcare Trust: E4168 (ALSPAC Hands on Assessments at Age Seven), Southmead Health Services: 67/98 (Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC)—Hands on Assessments at Age Seven), and the Frenchay Healthcare Trust: 98/52 (Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) provided approval. For the age of 15 years, the Central and South Bristol

Research Ethics Committee (UBHT): 06/Q2006/53 Avon Longitudinal Study of Parents and Children (ALSPAC) Hands on Assessments: Teen Focus 3 (Focus 15+) were used, and for the age of 17 years, North Somerset and South Bristol Research Ethics Committee: 08/H0106/9 Avon Longitudinal Study of Parents and Children (ALSPAC) Hands on Assessments: Teen Focus 4 (Focus 17+) were used. Full details of ethical approvals (local committees and approval numbers) are available at http://www.bristol.ac.uk/media-library/sites/alspac/documents/governance/Research%20Ethics%20Committee%20approval%20references.pdf (accessed on 12 August 2019).

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