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# Association of Season of Birth with DNA Methylation and Allergic Disease

**Short title:** Season of birth, DNA methylation and allergy

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## Key words

- 450K array
- DNA methylation
- Epigenetics
- Epigenome-wide association study
- Season of birth

## Abstract

**Background:** Season of birth influences allergy risk, however the biological mechanisms underlying this observation are unclear. The environment affects DNA methylation, with

potentially long-lasting effects on gene expression and disease. This study examined whether DNA methylation could underlie the association between season of birth and allergy.

**Methods:** In a subset of 18-year-old participants from the Isle of Wight (IoW) birth cohort (*n*=367), the risks of birth season on allergic outcomes were estimated. Whole blood epigenome-wide DNA methylation was measured, and season-associated CpGs detected using a training-and-testing-based technique. Validation examined the 8-year-old Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort. The relationships between DNA methylation, season of birth and allergy were examined. CpGs were analysed in IoW third generation cohort newborns.

**Results:** Autumn birth increased risk of eczema, relative to spring birth. Methylation at 92 CpGs showed association with season of birth in the epigenome-wide association study. In validation significantly more CpGs had the same directionality than expected by chance, and four were statistically significant. Season-associated methylation was enriched among networks relating to development, the cell cycle, and apoptosis. Twenty CpGs were nominally associated with allergic outcomes. Two CpGs were marginally on the causal pathway to allergy. Season-associated methylation was largely absent in newborns, suggesting it arises postnatally.

**Conclusions:** This study demonstrates that DNA methylation in adulthood is associated with season of birth, supporting the hypothesis that DNA methylation could mechanistically underlie the effect of season of birth on allergy, though other mechanisms are also likely to be involved.

## Introduction

The environment experienced during key *in utero* and perinatal developmental periods can have long-lasting and profound effects on disease risk throughout life (1). Season of birth has been associated with allergic disease risk in numerous studies. In general, children born in autumn or winter are more likely to develop a wide range of allergic diseases including asthma (2), rhinitis (3), hayfever (4), eczema (5), food sensitisation and atopic disease (6), and food allergy (7). At birth, children born in autumn or winter already have increased serum immunoglobulin (Ig)E levels (8), and birth season has been shown to affect neonatal immune development and lead to altered levels of inflammatory mediators in airway mucosa lining fluid (9). Season of birth also influences other traits and diseases such as adult height (10) and schizophrenia (11), suggesting long-lasting effects on a broad range of characteristics, however the mechanisms for such effects remain unknown.

Several hypotheses have been put forward to explain the link between season of birth and allergy, not necessarily exclusively. One hypothesis is that lower UV-B levels during autumn and winter reduce vitamin D synthesis, which increases allergy risk (12). Other hypotheses are based on seasonal fluctuation in levels of allergens such as pollen (13), and seasonality of early life respiratory viral infections (14). A fourth possible mechanism is seasonal variation in maternal nutrition (15, 16). These mechanisms, acting at key times during early development, could produce long-lasting differences in disease risk associated with season of birth.

Regardless of the ultimate environmental stimulus, the molecular mechanisms responsible for regulating and maintaining long-lasting responses to season of birth remain unknown. Gene transcription has been shown to exhibit seasonal periodicity: the immune system has a profoundly pro-inflammatory transcriptomic profile during winter, with increased levels of soluble IL-6 receptor and C-reactive protein – risk biomarkers for cardiovascular, psychiatric

and autoimmune diseases that also have higher incidence among winter-borns (17). The epigenome is one way in which transcriptional responses to changing environments can be established and maintained over long periods of time. A number of diseases are now recognised as having an epigenetic component (18), including allergic diseases (19, 20). While genetic variation contributes to allergic disease risk (21), asthma and allergy susceptibility genes also interact with epigenetic marks to influence allergic outcomes (22-24).

Given the known effect of season of birth on immune gene expression, neonatal immune function and allergic disease risk, together with the established role of epigenetics in allergic disease, we investigated whether DNA methylation could be a molecular mechanism for the effect of season of birth on allergic disease risk in adulthood. To test this hypothesis we assessed the association of DNA methylation in individuals from an unselected population birth cohort, with season of birth and allergic outcomes. First, we examined the effect of season of birth on allergic disease risk up to age 18. Second, we conducted an epigenome-wide association study (EWAS) to test whether season of birth is associated with differential DNA methylation. Third, we validated season-associated methylation in the Dutch Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort. Fourth, we investigated biological networks enriched among differentially methylated genes. Fifth, we modelled the effect of season-associated methylation on allergic outcomes, and used path analyses to test whether DNA methylation causally mediates this effect. Lastly we examined whether season-associated methylation is already detectable at birth.

## Methods

Date of birth data were collected in the Isle of Wight (IoW) birth cohort (*n*=1456), born from January 1989 to February 1990. Total serum IgE was measured at ages 10 and 18, atopy was assessed by skin prick tests at ages 4, 10 and 18, and eczema, rhinitis and asthma

were assessed at ages 1-or-2, 4, 10 and 18, as previously described (25-28). Generalised estimating equations (GEEs) were used to model the effects of season of birth on repeated measures of allergy over time in the whole cohort, corrected for multiple testing using Bonferroni-adjustment.

DNA methylation was profiled using the Illumina Infinium HumanMethylation450 beadchip in peripheral blood samples collected at age 18 from a subset of participants (*n*=367). Methylation data were extracted using GenomeStudio software (Illumina), pre-processed using the R package Illumina Methylation Analyzer (IMA) (29) and batch-corrected using ComBat (30). Estimated proportions of leukocyte subtypes were calculated from methylation data using the Minfi R package (31).

The ttScreening R package (v1.5, http://cran.r-project.org/web/packages/ttScreening/) (32) was utilised to conduct an EWAS identifying CpGs associated with season of birth (training-testing $p<0.05$ in ≥60/100 iterations). Analyses controlled for potential confounding factors (season of sample collection, maternal socioeconomic status (SES) and sex), and were conducted for each of the birth seasons relative to the other three combined. ttScreening's default settings were used, except that the iteration threshold was raised from ≥50 to ≥60/100 iterations to increase selection reliability. The ttScreening package conducts surrogate variable analysis to remove unexplained variation in the data, then analyses the data using an iterative training-testing procedure. The training-testing method performs better than the method controlling false discovery rate (FDR) and the Bonferroni method in reducing false positive and false negative results (32). In addition to providing internal validation, the use of training-testing builds a more generalised model than the models constructed by traditional methods, and can detect additional loci undetectable using traditional methods (32). Effects of the top CpG on gene expression were analysed in a subset of the IoW third generation cohort.

Season of birth-associated CpGs were validated in the PIAMA cohort (33) at age 8 (*n*=207) using generalised linear models (GLMs) controlling for season of sample collection, maternal education and sex.

Biological functions and networks enriched among genes containing season-associated methylation were characterised using Ingenuity pathway analysis (IPA; Qiagen).

Log-linear modelling was used to test the association of season-associated CpG methylation levels (methylation β values) with allergic outcomes at 18 years, controlling for season of sample collection, maternal SES and sex. Path analyses were applied to test whether any season-associated CpGs lie on the causal pathway for the effect of season of birth on allergic outcomes (Mplus v7.31). Paths were analysed from spring and autumn birth, which were both significantly associated with allergic outcomes, via the 45 CpGs associated with autumn or spring birth, to outcomes at age 18 (high serum IgE, eczema, rhinitis atopy and asthma), controlling for sex.

Season-associated CpGs were examined at birth (*n*=175) in cord blood and Guthrie card blood of the IoW third generation cohort (born 2006-2013), who are the children of the IoW birth cohort. GLMs used the same model as the EWAS, except season of sample collection was not included because participants are newborns, plus sample type (cord/Guthrie) and arraying batch. For this analysis 14 CpGs missing in some newborns were imputed using the R package MissForest; imputed values comprised a total of 3.03% of DNA methylation data points in newborn analyses. In all analyses autumn was the reference season, except for GLM analyses of CpGs associated with autumn birth, where spring was used as the reference season.

Figure 1 summarises the analyses performed. For additional details see Supplementary Methods.

## Results

### Cohort characteristics

The subset of IoW birth cohort subjects for whom DNA methylation data at age 18 was collected (*n*=367) were generally representative of the whole cohort, though had a higher proportion of females due to an initial research focus on future pregnancies, and consequently a lower prevalence of atopy at age 18 (Supplementary Table 1), due to lower prevalence of atopy in females at age 18.

### Season of birth influences allergic disease risk

The relative risk (RR) of repeated eczema was significantly lower for participants born in spring than autumn, the reference season ($RR_{spring}$=0.61, 95%CI=0.45-0.82; Table 1). Participants born in summer had reduced risk of repeated high serum IgE ($RR_{summer}$=0.72, 95%CI=0.53-0.98), but increased risk of repeated rhinitis ($RR_{summer}$=1.19, 95%CI=1.01-1.42) relative to autumn-borns. No significant effects of season of birth were observed on repeated atopy or asthma. After correction for testing five outcomes, only the difference in eczema risk between spring and autumn remained statistically significant (Bonferroni $p$<0.05).

### Season of birth is associated with DNA methylation

To examine whether DNA methylation is associated with season of birth, an EWAS was conducted. First, factors that could potentially confound a link between season of birth and DNA methylation were identified (Supplementary Table 2). The season in which each 18-year blood sample was collected (34) was not independent from season of birth ($p$<0.0001, $\chi^2$) because most were collected 3-9 months after the subject's 18$^{th}$ birthday as part of the study design. Maternal SES is known to be associated with season of birth (35) and was in our cohort ($p$=0.030, $\chi^2$). Sex did not differ between seasons of birth, though was adjusted for due to its well-established effects on allergy and methylation. Height at 18 years (10),

which is associated with lung function, along with estimated proportions of monocytes, B cells, NK cells, $CD8^+$T cells, $CD4^+$T cells, granulocytes and eosinophils, did not differ significantly between seasons of birth (Supplementary Table 2). The model for epigenome-wide analyses of season of birth therefore controlled for season of sample collection, maternal SES and sex, in addition to the surrogate variables identified by the ttScreening package.

The training-testing method detected 92 CpGs significantly associated with season of birth after ttScreening internal cross-validation (Table 2, Supplementary Table 3). The most significant CpG was cg07175945, located within a CpG island in the body of *Zinc finger RNA-binding* (*ZFR*), which had significantly higher methylation levels among autumn-born participants ($p$=4.72×$10^{-8}$; Figure 2, left). In cord blood from the IoW third generation, cg07175945 methylation was significantly associated with the expression level of *ZFR*'s exon 15 ($p$=0.040) but not 3'UTR ($p$=0.726; Supplementary Table 4), suggesting potential effects on gene expression and alternative splicing.

As a comparison, additional EWAS analyses were conducted using traditional methods (single regressions over the entire sample-set with FDR or Bonferroni correction; FDR α<0.05; Bonferroni $p$<0.05), utilising the same model as for the training-testing EWAS. With FDR and Bonferroni methods only cg07175945-*ZFR* was detected as significantly differentially methylated. In single sample-set analyses, genomic inflation λ approached 1 for all birth seasons ($\lambda_{winter}$=1.083, $\lambda_{spring}$=1.050, $\lambda_{summer}$=1.105, $\lambda_{autumn}$=1.103).

**Validation of season of birth-associated DNA methylation in PIAMA cohort**

Validation of the 85/92 available season-associated CpGs in the PIAMA cohort at age 8 ($n$=207) found four CpGs significantly associated with the same birth season ($p$<0.05) in PIAMA as in IoW, all with the same direction of effect (Table 3). This is double the number of

significant same-direction associations expected by chance. Furthermore, 55/85 CpGs (64.7%) had the same direction of effect in PIAMA, including cg07175945-*ZFR* (Figure 2, right) – significantly more CpGs than would be expected to differ in the same direction by chance alone ($p$=0.0067, $\chi^2$).

**Biological functions enriched within season of birth-associated genes**

Season-associated CpGs were annotated to 79 mapped genes, including allergy-related genes such as *ATPAF1*. Ingenuity pathway analysis (IPA) network analysis identified three significantly enriched networks: 15, 14 and 12 seasonally differentially methylated genes are in networks related to embryonic development, the cell cycle, and cell death respectively (Supplementary Table 5). Upstream analysis identified six seasonally differentially methylated genes regulated by the transcription factor NUPR1 (cg25382472-*STIL*, cg17679246-*PDK1*, cg22630160-*HIST1H2AB*, *HIST1H3B*, cg11155697-*HOXB5*, and cg22584335-*ZNF512*).

**Season of birth-associated CpGs may be on the causal pathway to allergic disease**

High serum IgE, eczema, rhinitis, atopy and asthma at 18 years were nominally significantly associated with the methylation levels of 0, 6, 4, 6 and 5 CpGs, respectively ($p_{CpG}$<0.05; Supplementary Table 3), however these became non-significant after Bonferroni-correction. To test whether any season-associated CpGs lie on the causal pathway between season of birth and allergic disease (i.e. as intervening variables), path analyses were performed. Two CpGs had marginally significant roles mediating the effect of season of birth on allergic outcomes at age 18 (Figure 3). The indirect effect via cg00787537-*KCNH1* comprised almost all of the total effect of spring birth on high serum IgE ($RR_{indirect}$=0.45, $p$=0.11; $RR_{direct}$=1.01, $p$=0.98; $RR_{total}$=0.49, $p$=0.86). The indirect effect of spring birth on atopy via cg24577417-*HGC6.3* also trended towards significance ($RR_{indirect}$=0.94, $p$=0.14), though the direct effect was stronger ($RR_{direct}$=0.64, $p$=0.07; $RR_{total}$=0.61, $p$=0.049).

**Season of birth-associated DNA methylation arises postnatally**

In newborns, blood DNA methylation levels of cg07175945-*ZFR* were significantly lower in winter-born babies ($p$=0.009) but not significantly higher in autumn-borns. 40/92 CpGs (43.5%) had the same direction of effect at birth and 18 years. Only one statistically significant association was already present at birth and in the same direction of effect: cg10063512-*ERLIN2* was more methylated in autumn-born subjects, at birth ($p$=0.046) and age 18 (Table 2).

## Discussion

In this study we describe season of birth-associated DNA methylation present in adulthood, constituting a potential mechanism for the sustained effect of season of birth on allergic disease risk. Autumn birth increased the risk of eczema up to adulthood. Methylation at 92 CpG sites was associated with season of birth at age 18. In the PIAMA validation cohort at age 8, two thirds of season-associated CpGs were directionally consistent, and four were statistically significant. Genes containing season-associated CpGs were enriched among networks related to development and apoptosis. Some methylation was nominally associated with allergic outcomes at age 18, and path analyses found two CpGs marginally on the causal path to allergic disease. Season-associated methylation was largely absent at birth, suggesting it arises postnatally.

Individuals born in autumn were significantly more likely than those born in spring to have eczema (Table 1; equivalent to $RR_{autumn}$=1.64, 95%CI=1.22-2.22), similar to previous reports of increased risk of eczema in autumn-borns at 4 years (5). Associations with high serum IgE and rhinitis did not remain significant after correction for five outcomes tested. Associations of season of birth with atopy and asthma were not detected, supporting previous findings that seasonal effects on allergy are not ubiquitous and vary geographically (36). It is possible that only specific season-associated exposures alter allergic disease risk

for each cohort, or season of birth interacts with other environmental factors to influence allergy.

This study detected 92 CpGs significantly associated with season of birth (Table 2; Supplementary Table 3) – the first report, to our knowledge, of specific DNA methylation associated with season of birth. That season of birth-associated DNA methylation exists at age 18 suggests that season-associated DNA methylation is sufficiently long-lasting it could contribute to allergy in adulthood, and other long-term outcomes influenced by season of birth. While total levels of 5-methylcytosine in placental DNA have been shown to be higher in children conceived in spring relative to autumn (37), ours is the first report of specific loci differentially methylated by season of birth. Five genes are differentially methylated with conceptional season famine in the Gambia in peripheral blood at age 9 (16), though these were not detected here, suggesting that those observed differences may relate more to famine than to season of birth, or to other inter-population differences.

The most significant CpG was in *ZFR*, whose protein functions in RNA transport and localisation and regulates apoptosis and mitosis (38). This CpG could functionally affect gene expression: ENCODE data show H3K4me3 marks are enriched at cg07175945, suggesting a potential role in activating *ZFR* expression. Moreover, cg07175945 was significantly associated with *ZFR* gene expression (Supplementary Table 4). Given the role of apoptosis in bronchial remodelling in asthma and keratinocyte death in eczema, seasonal regulation of this gene's expression provides a plausible link to allergy. While we describe the 92 CpG sites reported herein as being associated with season of birth specifically, it could be that this methylation is truly associated with other exposures temporally correlated with season of birth, such as vitamin D levels (39), season of conception, or postnatal early developmental periods such as first pollen or winter respiratory virus season.

Although only four season-associated CpGs were statistically significant in the PIAMA

validation cohort, a significant excess of CpGs showed consistent directionality ($p$=0.0067). The four validated CpGs represent the most robust findings, indicating a consistent but subtle effect of season of birth on DNA methylation. Genes containing these validated CpGs function in processes including insulin sensitivity and apoptosis, which can be responsive to vitamin D (40, 41), and vitamin K synthesis and carbohydrate processing, which could also conceivably be seasonal (42, 43) (Table 3). The remaining directionally consistent sites may represent true but weak effects that were not statistically significant with our relatively small sample sizes, or effects that changed with age. Our findings from independent validation support the method we implemented to detect these informative CpG sites using training-testing-based internal cross-validation. Cross-validation has better type I error control than FDR, and training-testing-based methods have the potential to control both type I and II errors (32), nonetheless false positives cannot be formally excluded. Bonferroni and FDR single sample-set methods would have detected only one CpG, yet ttScreening detected 92 CpGs (with significantly consistent directionality in validation), almost all of which would have been missed using single sample-set methods.

Genes containing season-associated methylation were enriched for networks related to development, the cell cycle and apoptosis. Early post-natal developmental differences in gene expression could set disease-bound trajectories in early life (1). Regulation of apoptosis and the cell cycle could relate to airway remodelling in asthma and keratinocyte death in eczema. Six of 79 genes containing differentially methylated CpGs are regulated by the transcription factor NUPR1, whose expression in the asthmatic mouse lung is regulated by a PPARγ ligand (44), suggesting this as a potential regulatory link from season-associated methylation to allergy.

Methylation at 20 season-associated CpGs was significantly associated with at least one allergic outcome (Supplementary Table 3), though after correction for multiple testing this becomes non-significant. Path analyses detected two CpGs marginally on the causal path

from spring birth to allergy at age 18: cg00787537-*KCNH1* and cg24577417-*HGC6.3*. In particular the indirect effect via cg00787537-*KCNH1* comprised almost all of the effect of spring birth on serum IgE, suggesting the potential for season of birth-associated DNA methylation to mediate allergic disease risk. Future studies should examine whether season-associated methylation is linked to other seasonal outcomes, such as schizophrenia.

Only one season of birth-associated CpG was already associated with season of birth in newborns, suggesting that most season of birth-associated DNA methylation arises postnatally. This is mechanistically consistent with postnatal exposures such as pollen, sunlight levels and viral infections contributing to season of birth effects. Although some season of birth-associated differences in immunology exist at birth, our results suggest that these are not associated with differences in DNA methylation at birth. There could also be season-associated methylation arising shortly after birth that disappears before age 18. Our sample size was small ($n$=175) so we cannot exclude the possibility that prenatal effects of season of birth on DNA methylation might be visible with a larger sample. While variation in cell type proportions was not associated with season of birth at age 18, cell type proportions could differ between cord blood and adult peripheral blood, and this could introduce bias to the newborn analysis. Environmental differences between the birth cohort born 1989-1990 and the subsequent generation born 2006-2013 also cannot be excluded.

There are several factors that could confound the association between season of birth and allergic disease, including adult height (10), proportions of different leukocyte subsets, and maternal SES (35), however these were excluded as potential confounders (Supplementary Table 2) or adjusted for in the EWAS and other analyses. Differences in DNA methylation (34) and gene expression (17) in peripheral blood have been reported in association with season of sample collection: by controlling for season of sample collection in our analyses we aimed to avoid detection of CpG sites associated with the collection season rather than the birth season. ProbeSNPs and ambiguous probe binding can bias methylation

measurement, and methQTLs could affect CpGs in accordance with the two-stage model (19) – however these are unlikely to bias season-associated methylation as genotypes should be randomly distributed across birth seasons. The imputation of methylation values for 14 CpGs in a minority of third generation newborns increased sample sizes for modelling, but also imposes limitations, as these are not biologically measured values.

Our data measure the average DNA methylation level in heterogeneous populations of leukocytes. In our cohort the estimated proportions of leukocyte subsets (including eosinophils, which are especially relevant to an analysis of allergic disease) did not differ with season, suggesting that the season-associated methylation reported here is not due to season of birth-related differences in leukocyte proportions. Profiling subsets of leukocytes improves the precision with which methylation differences can be observed (45), and may reveal larger methylation differences within some cell types. Yet small average methylation differences in heterogeneous cell populations could potentially result in biologically meaningful effects on gene expression, such as the significant effect of cg07175945 on *ZFR* expression observed here. Future studies could improve accuracy by analysing season of birth-associated DNA methylation in leukocyte subsets, particularly given that neonatal immune responses seem to be modified by season of birth (9).

Here we report that season of birth has long-lasting effects on DNA methylation, supporting the hypothesis that DNA methylation could mechanistically contribute to the persistent effect of season of birth on allergic disease risk. Season of birth influences the risk of repeated eczema, and produces significant but modest effects on DNA methylation in peripheral blood at age 18, that were directionally consistent in a validation cohort. Our discovery of season of birth-associated DNA methylation has clinical implications for observations of allergic disease and other outcomes influenced by season of birth, but advising altering pregnancy timing solely to reduce allergy seems excessive. Season-associated CpGs were enriched among developmental, cell cycle and apoptosis-related networks. Season-associated CpGs

may influence long-term allergic outcomes, and two CpGs trended towards being on the causal pathway to allergy. Methylation profiles in newborns suggest season of birth-associated methylation arises postnatally. Together these results suggest DNA methylation could be a molecular mechanism through which transcriptional responses to season of birth are established and maintained into adulthood.

## Author contributions

GAL and JWH conceived and designed the study. GAL and NSR analysed and interpreted the data. MAR, TME, VKP, WT, AK and FIR assisted with analyses or obtaining samples. CJX, GHK, DSP and UG provided PIAMA cohort data. SLE, SHA, HZ, WK and JWH obtained funding and supervised analyses. GAL wrote the manuscript. All authors commented on the manuscript and approved the final version.

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## Conflicts of interest

None of the authors have any conflicts of interest to declare.

## Supplementary Materials

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|---|---|
| Supplementary Methods | Detailed description of methods |
| Supplementary Table 1 | Comparison between cohort subset selected for DNA methylation analysis and whole cohort |
| Supplementary Table 2 | Comparison of potential confounding factors across seasons of birth |
| Supplementary Table 3 | Complete list of 92 CpGs significantly associated with season of birth |
| Supplementary Table 4 | Association of cg07175945 with *ZFR* gene expression |
| Supplementary Table 5 | Pathway analysis results |

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## Tables

**Table 1: Effect of season of birth on the relative risks of repeated high serum IgE, eczema, rhinitis, atopy and asthma**

| | Season of birth | | |
|---|---|---|---|
| | Winter | Spring | Summer |
| Repeated high serum IgE (≥200 kU/L) | 0.91 (0.70 - 1.18)<br>$p = 0.475$ | 1.00 (0.76 - 1.32)<br>$p = 0.983$ | **0.72 (0.53 - 0.98)**<br>$\mathbf{p = 0.039}$ |
| Repeated occurrence of eczema | 0.92 (0.71 - 1.20)<br>$p = 0.547$ | **0.61 (0.45 - 0.82)**<br>$\mathbf{p = 1.24\times10^{-3}}$ | 0.89 (0.67 - 1.17)<br>$p = 0.398$ |
| Repeated occurrence of rhinitis | 1.01 (0.85 - 1.20)<br>$p = 0.901$ | 1.12 (0.94 - 1.34)<br>$p = 0.220$ | **1.19 (1.01 - 1.42)**<br>$\mathbf{p = 0.044}$ |
| Repeated occurrence of atopy | 0.90 (0.73 - 1.11)<br>$p = 0.309$ | 1.11 (0.90 - 1.36)<br>$p = 0.343$ | 0.99 (0.80 - 1.22)<br>$p = 0.900$ |
| Repeated occurrence of asthma | 0.97 (0.76 - 1.24)<br>$p = 0.822$ | 1.14 (0.88 - 1.47)<br>$p = 0.331$ | 1.24 (0.97 - 1.60)<br>$p = 0.089$ |

For each birth season, the relative risk (RR), 95% confidence interval and associated *p* value for associations with repeated measures of allergy in the whole IoW birth cohort ($n \leq$ 1456 subjects) are shown. GEEs were used to estimate the effects on repeated measures over time. Significant effects of season of birth on relative risks ($p < 0.05$) are shown in bold font. Autumn was the reference season. Serum IgE was measured at ages 10 and 18, atopy was assessed at ages 4, 10 and 18, and eczema, rhinitis and asthma were assessed at ages 1-or-2, 4, 10 and 18.

**Table 2: The twenty CpGs with methylation levels most significantly associated with season of birth**

| CpG ID | Season of birth | Coeff. | *P* value | Ave. β | β diff. | PIAMA dir. | Gene/s | Putative gene function | Location |
|---|---|---|---|---|---|---|---|---|---|
| cg07175945 | Autumn | 0.119 | $5.05\times10^{-8}$ | 0.239 | 0.015 | Yes | *ZFR* | RNA transport in apoptosis and mitosis | Body |
| cg25719132 | Autumn | 0.144 | $3.82\times10^{-7}$ | 0.047 | 0.005 | Yes | *SNRPG* | Nuclear riboprotein, alternative splicing | Body |
| cg20095398 | Spring | -0.131 | $8.48\times10^{-7}$ | 0.618 | -0.013 | Yes | DHS. Intergenic: *FAM53A – NKX1-1* | *FAM53A*: neural tube development. *NKX1-1*: homeobox gene, embryonic development | Intergenic |
| cg25382472 | Autumn | 0.139 | $9.57\times10^{-7}$ | 0.073 | 0.006 | Yes | *STIL* | Mitotic spindle checkpoint | TSS200 |
| cg24883586 | Winter | -0.175 | $1.02\times10^{-6}$ | 0.163 | -0.014 | Yes | *RAB11FIP1* | Vesicle recycling | TSS200 |
| cg06365303* | Winter | 0.193 | $1.06\times10^{-6}$ | 0.229 | 0.018 | Yes | DHS, TF. Intergenic: *PCED1A - TMEM239* | *PCED1A*: esterase and lipase. *TMEM239*: brain white matter integrity | Intergenic |
| cg14136781 | Winter | -0.203 | $1.10\times10^{-6}$ | 0.904 | -0.009 | Yes | *GSN* | Actin filament assembly/disassembly, apoptosis, wound healing | Body |
| cg08089851 | Summer | 0.132 | $1.10\times10^{-6}$ | 0.049 | 0.004 | Yes | *MKRN2* | Ribonucleoprotein, possible ubiquitin ligase | TSS200 |
| cg10063512 | Autumn | 0.095 | $1.93\times10^{-6}$ | 0.101 | 0.008 | Yes | *ERLIN2* | IP3 signalling in endoplasmic reticulum | TSS200, 1stExon, 5'UTR |
| cg12209881* | Spring | -0.605 | $1.98\times10^{-6}$ | 0.943 | -0.049 | Yes | Intergenic: *HLF – STXBP4* | *HLF*: transcription factor, resistance to cell death. *STXBP4*: insulin-stimulated glucose transport | Intergenic |
| cg02294108 | Summer | -0.198 | $3.86\times10^{-6}$ | 0.028 | -0.002 | No | *C9orf80* | DNA repair, genomic stability checkpoint in cell cycle | 5'UTR |
| cg06577708 | Summer | 0.113 | $4.40\times10^{-6}$ | 0.126 | 0.007 | No | *PSMD6* | Degrades ubiquitinated proteins at DNA damage foci | Body |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| cg25730142 | Autumn | 0.111 | $4.51\times10^{-6}$ | 0.138 | 0.009 | No | *SNTA1* | Syntrophin, alters ion channel activity in cardiac tissue causing longQT syndrome | TSS200 |
| cg22062239* | Autumn | 0.122 | $5.10\times10^{-6}$ | 0.029 | 0.002 | Yes* | *VKORC1* | Vitamin K, blood clotting | 1stExon |
| cg24677732 | Autumn | -0.181 | $5.30\times10^{-6}$ | 0.033 | -0.004 | Yes | *IL15RA* | Cytokine IL15 receptor, splicing regulated by DNA methylation | 1stExon, 5'UTR |
| cg08962590 | Winter | -0.090 | $5.69\times10^{-6}$ | 0.094 | -0.003 | Yes | *FXR1* | RNA-binding protein | 5'UTR, Body |
| cg24799448 | Summer | 0.136 | $5.77\times10^{-6}$ | 0.033 | 0.003 | Yes* | DHS, TF. Intergenic: *BRSK2 – TOLLIP* | *BRSK2*: Stress-induced apoptosis. *TOLLIP*: IL1R trafficking; associated with eczema | Intergenic |
| cg23922755 | Autumn | -0.093 | $5.95\times10^{-6}$ | 0.867 | -0.006 | No | *HGFAC* | Activates hepatocyte growth factor | TSS200 |
| cg05137146 | Spring | -0.126 | $5.97\times10^{-6}$ | 0.066 | -0.007 | Yes | *TBX6* | T-box transcription factor, developmental | TSS1500 |
| cg16286281 | Summer | -0.257 | $6.05\times10^{-6}$ | 0.931 | -0.012 | No | *EXT1* | Heparan sulfate biosynthesis | Body |

The top 20 CpGs significantly associated with season of birth in the EWAS (full list of 92 CpGs associated with season of birth is in Supplementary Table 3). Associations with DNA methylation were analysed in each birth season, relative to the other three birth seasons combined; controlling for season of sample collection, maternal SES and sex. * with CpG ID indicates CpGs whose methylation levels are also significantly associated with an allergic disease phenotype: cg06365303 with eczema ($p = 0.028$), cg12209881 with atopy ($p = 0.009$), and cg22062239 with eczema ($p = 0.039$). Columns: CpG ID; the significantly associated season of birth, the regression coefficient (evaluates the difference in DNA methylation between children born in a specific season and those born in other seasons) and *p* value for the association with the significantly associated birth season/s (from training-testing); the average methylation level (β value); the difference between average methylation β value for the significant season of birth and average of the other three; whether the direction of effect was validated in the PIAMA cohort (* = association with the significant season of birth was also statistically significant in PIAMA); the gene/s annotated to the CpG, putative function of each gene (identified by literature search); type of location for each CpG (extracted from the Illumina 450K array manifest file). For

intergenic CpGs, locations within DNaseI hypersensitivity sites ("DHS") and transcription factor-binding sites ("TF"; both from ENCODE data) are shown in addition to the nearest RefSeq gene on each side of the CpG.

**Table 3: CpGs significantly differentially methylated by season of birth in both discovery and validation cohorts.** Four season-associated CpGs detected in the EWAS in the IoW discovery cohort were also significantly associated with the same season of birth (GLM $p < 0.05$) and all in the same direction of effect in the PIAMA validation cohort.

| CpG ID | Season of birth | IoW cohort | | PIAMA cohort | | Gene/s | Function and putative link to season of birth or allergy |
|---|---|---|---|---|---|---|---|
| | | Coeff. | *P* value | Coeff. | *P* value | | |
| cg22062239 | Autumn | 0.122 | $5.10\times10^{-6}$ | 0.168 | 0.010 | *VKORC1* | Vitamin K synthesis: infant vitamin K deficiency is seasonal (43). |
| cg24799448 | Summer | 0.136 | $5.77\times10^{-6}$ | 0.154 | 0.005 | *BRSK2* | Stress-induced apoptosis: vitamin D, which is sunlight-related, can induce apoptosis (reviewed by 40). |
| | | | | | | *TOLLIP* | IL1R trafficking: SNPs in this gene associated with eczema (46). |
| cg02054964 | Autumn | 0.092 | $1.99\times10^{-5}$ | 0.131 | 0.003 | *PTPRN2* | Insulin secretion: vitamin D levels influence insulin secretion (41). |
| cg17679246 | Spring | -0.160 | $7.89\times10^{-5}$ | -0.124 | 0.036 | *PDK1* | Carbohydrate fuel processing: maternal periconceptional diet alters expression of this gene in offspring (42). Could be linked to seasonal variation in maternal diet. |

Coeff. = regression coefficient: from ttScreening EWAS for the IoW cohort, and from GLMs for the PIAMA cohort. Both models controlled for season of sample collection, maternal SES and sex. In the EWAS each season of birth was compared against the other three seasons; in validation modelling the reference season was autumn, except where the season of interest was autumn, where spring was the reference. cg24799448 is intergenic between *BRSK2* and *TOLLIP*, and is located in a DNaseI hypersensitivity site and transcription factor-binding site (from ENCODE data).

## Figure legends

**Figure 1: Summary of analyses.** First, in the whole IoW birth cohort, the effects of season of birth on long-term allergic disease risk were estimated. In the subset of the cohort with DNA methylation data, an EWAS was conducted for season of birth, controlling for potential confounders. The results were compared against those obtained using Bonferroni and FDR methods. The season of birth-associated CpGs detected by the EWAS were then analysed further: they were validated in the independent PIAMA cohort, examined for enrichment among biological pathways, modelled for their association with allergic outcomes at age 18 and whether they were on the causal pathway for allergic outcomes, and finally in an exploratory analysis were examined at birth in newborns from the IoW third generation cohort.

**Figure 2: DNA methylation levels of cg07175945 (*ZFR*) are higher among people born in autumn in two cohorts.** Methylation levels at age 18 were significantly higher in IoW 1989 birth cohort participants born in autumn ($p = 4.72\times10^{-8}$, training-testing; left). Methylation of this CpG was also higher in autumn-born PIAMA cohort participants at age 8 (right), but this was not statistically significant ($p = 0.118$, GLM). cg07175945's significant association was with autumn birth, therefore spring was the reference season in the PIAMA GLM. Error bars show 95% confidence intervals; ***: $p < 0.001$.

**Figure 3: Two season of birth-associated CpGs were marginally significantly on the causal pathway with allergic disease at age 18.** Top: cg00787537 (*KCNH1*) trended towards causally mediating an effect of spring birth on high serum IgE. Bottom: cg24577417 (*HGC6.3*) trended towards causally mediating an effect of spring birth on atopy. Path analyses were done using Mplus, and controlled for sex. Solid arrows show direct effects; dashed lines show indirect effects; dashed and dotted lines show total effects.