HHS Public Access

Author manuscript

Clin Exp Allergy. Author manuscript; available in PMC 2019 January 24.

Published in final edited form as:

Clin Exp Allergy. 2013 March ; 43(3): 304–311. doi:10.1111/cea.12046.

Methylation of *IL-2* promoter at birth alters the risk of asthma exacerbations during childhood

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Summary

Background—Epigenetic modifications may have a role in asthma susceptibility.

Objective—To investigate whether epigenetic modification at birth of a CpG site necessary for the regulation of *IL-2* transcription (*IL-2 Site1*) is associated with the development of asthma during childhood.

Methods—Methylation of *IL-2 Site1* was assessed in cord blood from 303 children (225 with atopic mothers); as controls, we measured methylation of a site not important in the transcription of *IL-2* (*IL-2 Site7*) and methylation of the *LINE-1* repetitive element. Children were followed to the age of 8 years. Information on severe asthma exacerbations and hospital admissions was collected from child's primary care medical record. To account for potential confounding by bronchiolitis, we used exacerbations/hospitalizations after age 1 year as primary outcomes.

Results—There were 49 severe exacerbations amongst 33 children, and 22 hospital admissions amongst 11 children. The risk of asthma exacerbation increased 1.07-fold (95% CI 1.01–1.14, $P = 0.03$) and the risk of hospital admission increased 1.12-fold (95% CI 1.04–1.20, $P = 0.002$) for each one per cent increase in *IL-2 Site1* methylation. Children who were admitted to hospital at any time-point had significantly higher *IL-2 Site1* methylation than children not admitted to hospital ($P = 0.007$). There was a significant interaction between age at exacerbation ($P = 0.03$) or hospital admission ($P = 0.02$) and methylation, with the effect of methylation increasing with increasing age. Methylation of the control *IL-2 Site7* or *LINE-1* was not a significant predictor of asthma exacerbations/hospital admission, and we found no association between *IL-2 Site1* methylation and hospital admissions for other reasons (0.99 [0.92–1.06]). Cord blood mononuclear cell phytohemagglutinin-stimulated lymphoproliferative responses decreased significantly with increasing *IL-2 Site1* methylation ($P < 0.001$).

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Conflict of interest: We declare that we have no conflict of interest in relation to this manuscript.

Conclusions—Increasing methylation in cord blood of a functional CpG site in the *IL-2* promoter is associated with increased likelihood of severe asthma exacerbations and hospital admissions for asthma/wheeze between ages of 2 and 8 years.

Keywords

asthma; childhood; epigenetics; exacerbation; interleukin 2; methylation

Introduction

The natural history of asthma is complex and its aetiology poorly understood. Although twin studies provide evidence for a heritable component of asthma [1], genetic studies have produced heterogeneous results explaining a small proportion of the risk [2]. Heritable changes in gene expression that occur in the absence of alterations in the DNA sequence (epigenetic modifications) may explain some of the inherited component of asthma [3]. There is increasing evidence that environmentally driven epigenetic modifications may have an important role in disease susceptibility [4]. Environmental exposures *in utero* may reprogram gene expression and influence the development of disease in offspring through epigenetic alterations [5]. However, most of the evidence for the role of epigenetics comes from the animal experiments, with very few studies to date investigating the role of epigenetic factors in the development of human asthma. An increased risk of asthma amongst children whose grandmothers smoked during their mother's fetal period may be an example of an environmental exposure affecting the epigenome and altering the risk of asthma [6]. Methylation of *ACSL3* in DNA isolated from cord blood was shown to be associated with exposure to airborne polycyclic aromatic hydrocarbons and with asthma symptoms prior to age 5 years [7].

It is generally accepted that respiratory virus infection is a major cause of asthma exacerbation [8, 9]. Deficits in immune responses to viruses are observed in adult asthmatics [10], and it has been suggested that such deficiencies (either congenital or acquired) may already be present in early life, increasing the likelihood of subsequent recurrent wheezing [11]. Immune function of cord blood mononuclear cells (CBMC) at birth differs between children who go on to develop persistent wheezing compared with those who do not [12]. We propose that some of these differences in immune responses may be due to epigenetic factors.

Virus infection induces systemic cellular immune response, including a marked increase in phytohemagglutinin (PHA)-stimulated interleukin-2 (IL-2) production [13]. A change in the methylation status in the promoter of the human *IL-2* gene is necessary for the regulation of gene transcription and may act as a memory of the regulatory event: *IL-2* transcription can only occur when a single CpG site (*IL-2 Site1*) in its promoter-enhancer is demethylated [14].

On the basis of [*1*] the evidence that viruses have fundamental role in asthma exacerbations [8, 11]; [2] the relevance of IL-2 in response to virus infection [13]; and [*3*] the existence of the well-described single CpG site essential for regulation of *IL-2* transcription [14], we propose that *IL-2* may be an ideal candidate gene for a proof-of-concept study on the role of

epigenetic factors in asthma in humans. We hypothesized that epigenetic modification of a specific functional CpG site (*IL-2 Site1)* [14] at birth is associated with acute episodes of wheezing during childhood, and that prenatal environmental exposures may have an effect on epigenetic modifications.

Methods

Study design, setting and participants

The Manchester Asthma and Allergy Study is a population-based birth cohort [15–17] (Appendix S1). Subjects were recruited prenatally, when both parents provided a detailed medical history and were skin-prick tested [18]. Approval was given by the South Manchester Local Research Ethics Committee in 2003 for the Manchester Asthma & Allergy Study (MAAS): LREC ref. 03/SM/400 and parents gave written informed consent. The study is registered as ISRCTN72673620 (www.controlled-trials.com/isrctn/pf/72673620).

Collection of cord blood and assessment of methylation at birth

Mothers of children at high risk (both parents atopic) and low risk (both parents non-atopic) of atopy provided cord blood samples [19]. Samples with 1% heparin were diluted with AIM-V serum-free medium and twice separated by density gradient centrifugation; the resulting CBMC were stored or stimulated (triplicate) with PHA and purified Der p 1 (10^5 cells/well in primary culture) [19]. Proliferative responses were assessed by 3H-Thymidine (1 μCi/well) incorporation after 3 (PHA) or 6 (Der p 1) days stimulation and expressed as stimulation indices (SI) [19].

We bisulphite-converted 400 ng of DNA isolated from the stored unstimulated CBMCs (EZ-96 DNA Methylation-Gold Kit; Zymo Research, Irvine, CA, USA) and assessed the methylation status of the functional *IL-2 Site1* [14]. As controls, we measured the methylation levels of *IL-2 Site7*, which remains hypermethylated after stimulation and is not important in the transcriptional control of *IL-2* [14] (Figure S1), and of the long interspersed nucleotide element-1 (*LINE-1*) repetitive element (to assess global DNA methylation levels) [20]. The pyrosequencing assays measure the amount of cytosine (C) relative to the sum of the amounts of C and thymine (T) at each CpG and the data are presented as percentage (% methylation). All measurements were carried out in triplicate and data points > 2SD discarded. For primers, amplification conditions and pyrosequencing details, see Appendix S1.

Data sources and definition of outcomes

Primary care data extraction.—A trained paediatrician extracted data from electronic and paper-based primary care medical records, including all prescriptions, unscheduled visits and hospital admissions during the first 8 years of life. The following outcomes were defined from these data:

Severe asthma exacerbations: Either receipt of oral steroids for at least 3 days or admission to hospital or an emergency department visit because of asthma requiring oral steroid use [21].

Hospital admission: Admission for asthma/wheeze and for reasons other than asthma/wheeze. To account for potential confounding by Respiratory Syncytial Virus (RSV) bronchiolitis, we used exacerbations and hospitalizations after age of 1 year as primary outcomes [22].

Clinical follow-up.—Children attended follow-ups at ages 1, 3, 5 and 8 years [23]. Parentally reported symptoms were ascertained using interviewer-administered validated questionnaires; sensitization was determined by skin-prick testing [24]. We defined the following outcomes:

Current wheeze: Positive answer to the question 'Has your child had wheezing or whistling in the chest in the last 12 months?'

Wheeze phenotypes: *Transient early wheezing* (wheezing only during the first 3 years), *Intermittent wheezing* (wheezing at one time-point only during first 5 years or at age 8 only), *Late-onset wheezing* (wheezing started after age 3 years) and *Persistent wheezing* (wheezing during the first 3 years, current wheezing at ages 5 and 8). Children with no wheezing at any follow-up were used as a reference.

Atopic sensitization: Weal 3 mm or greater than the negative control to at least one allergen.

Prenatal environmental exposures.—During pregnancy, a home visit was made and dust sampling performed; Der p 1 was measured by ELISA [19]. We collected information on pet ownership and maternal smoking using a questionnaire [18]. Cotinine was measured in cord blood using capillary column gas-liquid chromatography [25] to objectively measure prenatal environmental tobacco smoke (ETS) exposure.

Statistical analysis

Statistical analysis was performed using Stata 11.2 (StataCorp LP; Stata Statistical Software: Release 11; College Station, TX, USA) and SPSS 15.0 (SPSS Inc; SPSS for Windows (Released 2006), Version 15.0. Chicago, USA). Methylation data followed a normal distribution. We used Levene's *F*-test for equality of variance to compare variability in methylation levels between the three sites. We used logistic regression analysis to assess if methylation of the analysed sites was associated with outcomes of interest at various ages. Longitudinal analyses were performed by Generalized Estimating Equations (GEE) using the exchangeable correlation structure and the logit link function. To take into account relatively low numbers, we used a modified robust variance estimator for generalized estimating equations with improved small-sample performance [26]. Analyses were adjusted for gender, contemporaneous maternal smoking and maternal asthma.

Results

Participant flow and descriptive data

Cord blood was available from 303 of 631 eligible participants (225 high risk, 78 low risk); children in whom cord blood was available did not differ from 328 in whom a sample was not collected in demographic characteristic or clinical outcome (Table S1). Demographics of the study population are presented in Table 1 and Table S1.

Methylation data was obtained where sufficient biological material was available in all 303 participants for *IL-2 Site1*, 294 for *LINE-1* and 228 for *IL-2 Site7*. Methylation of *IL-2 Site1* was significantly more variable (mean 53.5%, interquartile range [49.5–57.1%]) than *IL-2 Site7* (91.6% [90.4–93.0%]; $P < 0.001$) or *LINE-1* (79.6% [78.3–80.7%]; $P < 0.001$).

Epigenetic markers at birth and clinical outcomes

Severe asthma exacerbations and hospital admissions during childhood.— There were 49 severe exacerbations amongst 33 children, and 22 hospital admissions amongst 11 children (Table S2). In the multiple logistic regression models, we found that increasing methylation of *IL-2 Site1* was a significant and independent associate of severe asthma exacerbations and hospital admissions after the first year of life (Table S3). The risk of severe asthma exacerbation increased 1.07-fold (95% CI 1.01–1.14, $P = 0.03$) and the risk of hospital admission increased 1.12-fold (95% CI 1.04–1.20, $P = 0.002$) for each one per cent increase in *IL-2 Site1* methylation. Children who were admitted to hospital at any time-point ($n = 11$) had significantly higher *IL-2 Site1* methylation than children not admitted to hospital ($P = 0.007$; Table S4).

In the longitudinal GEE models, increasing methylation of *IL-2 Site1* significantly increased the likelihood of severe asthma exacerbations and hospitalization after the first year of life (RR [95% CI] 1.09 [1.01–1.17], $P = 0.02$ and 1.10 [1.10–1.21], $P = 0.02$ respectively; Table S3). There was no significant association between exacerbations or hospital admissions and methylation of the *IL-2 Site7* or *LINE-1* (Table S3).

Tables S5 and S6 show the conditional ORs for the change in risk of severe asthma exacerbations and hospitalizations with a unit (1%) increase in methylation for each age. Inspection of the data suggested that the odds of severe exacerbations and hospital admissions during the first 2 years of life decreased with increasing *IL-2 Site1* methylation; however, the apparent early-life decrease in risk was not statistically significant. Adding an interaction term to models demonstrated a significant interaction between child's age and *IL-2 Site1* methylation, with the increased effect of methylation on severe asthma exacerbations and hospital admissions with increasing age ($P = 0.03$ and $P = 0.02$, Fig. 1).

To confirm that any findings are specific to asthma/wheeze, we evaluated the association between epigenetic markers and hospital admissions for other reasons; we found no such association for *IL-2 Site1* (0.99 [0.92–1.06], $P = 0.7$), no association with methylation of *IL-2 Site7* (0.919 [0.709–1.191], $P = 0.52$) or *LINE-1* (1.293 [0.960–1.743], $P = 0.09$) and no interaction with age (0.99 [0.98–1.01], $P = 0.4$; Figure S2).

Wheeze.—In a GEE model, there was no association between the likelihood of current wheezing from the first to the 8th year of life and methylation of *IL-2*. Similarly, in the multinomial logistic regression analysis assessing the temporal pattern of wheeze, there was no association between wheeze phenotypes and methylation of any site (Table S7).

Atopic sensitization.—In a GEE model adjusted for gender, there was no association between the development of sensitization from the first to the 8th year of life and methylation of *IL-2 Site1* or *Site7* (1.02 [0.97–1.08], $P = 0.41$; 0.85 [0.70–1.03], $P = 0.11$ respectively). However, *LINE-1* methylation was significantly associated with sensitization (1.24 [1.00–1.52], $P = 0.05$). Similar results were obtained when methylation data were expressed as quartiles (Figure S3).

Epigenetic markers and lymphoproliferative responses at birth

We observed a significant inverse correlation between *IL-2 Site1* methylation and CBMC immunoproliferative responses after PHA stimulation ($r = -0.29$, $P < 0.001$; Figure S4a); no such association was observed for *IL-2 Site7 or LINE-1* ($P > 0.2$; Figure S4b and c). When methylation data were expressed as quartiles, there was a highly significant difference between the groups, with CBMC PHA-stimulated lymphoproliferative response decreasing significantly with increasing *IL-2 Site1* methylation ($P < 0.001$, Kruskal–Wallis Test, Fig. 2 and Figure S5). There was no association between methylation of any site and immunoproliferative response to mite allergen (Figure S6).

Antenatal factors and methylation at birth

Methylation of *IL-2 Site1* was significantly lower and *LINE1* significantly higher in children with atopic compared with those with non-atopic mothers (Table 2); there was no association between maternal atopy and methylation at *IL-2 Site7*. Methylation at *IL-2 Site1* was significantly lower in children of cat owners (Table 2). We observed a significant association between *IL-2 Site7* methylation and socio-economic status (higher methylation in children with higher paternal income, Table 2) and cord blood cotinine level (decrease in methylation with increasing cotinine levels, Table S8). Birth weight, gestational age, maternal age, maternal asthma, dog ownership and prenatal mite allergen exposure were not associated with methylation at any site (Table 2 and Table S8).

Discussion

We have demonstrated an increased likelihood of severe asthma exacerbations and hospital admissions for asthma/wheeze during childhood with increasing methylation in cord blood of a CpG site in the *IL-2* promoter, which is crucial for regulation of *IL-2* transcription [14]. There was a significant interaction between the age at which exacerbation/hospitalization occurred and DNA methylation, with an increasing effect of this early-life epigenetic marker with increasing age. This finding was specific for asthma/wheeze, and was not observed for hospital admissions for other reasons. PHA-stimulated CBMC immunoproliferative responses decreased significantly with increasing methylation of the functional *IL-2* CpG site. Maternal atopy and some prenatal environmental exposures were associated with methylation at birth, and these effects differed between different sites.

Limitations

Although set within the population-based birth cohort, for practical reasons relevant to collection and processing of cord blood, we had to focus on polar groups of high and low-risk children. Furthermore, as the processing could not be carried out for children born during the late-night hours (cord blood was drawn within 10 min of birth and cell separation had to commence within 45 min of collection, immediately followed by CBMC stimulation), the sample was available for ~ 50% of eligible participants. Although there were no differences in demographics or clinical outcomes between children with or without cord blood sample, we acknowledge that the possibility of selection bias cannot be excluded.

The substantial time lag between collecting CBMCs and this study has the advantage that longitudinal clinical data are available for 8 years following the cord blood collection. However, as we only had access to stored CBMCs, we could not carry out cell separation. Other disadvantages include the lack of RNA or supernatant to allow us to determine if methylation of the functional site altered expression of *IL-2*. However, the functional role of this CpG site on *IL-2* transcription has already been demonstrated [14].

We do not have the data to explain the mechanisms of the observed associations. Given the roles of IL-2, we propose that epigenetic modification of the functional CpG site and the subsequent alteration of *IL-2* expression may affect asthma exacerbation via an alteration of the response to rhinovirus. For practical reasons, we were unable to confirm the presence of viruses at the time of hospital admission. However, the role of virus infections in exacerbations of asthma/wheeze in children is clearly established [8], and it is likely that rhinovirus was responsible for the majority of hospital admissions/exacerbations of wheezing/asthma after age 2 years. We recognize that ideally it would be desirable to perform studies on CBMCs from our cohort to establish the effect of viruses on IL-2 production; however, due to the methods of cord blood processing and sample storage, such studies are not possible.

We acknowledge that our results are based on relatively small number of children with severe exacerbations/hospitalizations. However, rather than basing our conclusions on the number of individuals with hospital admissions/exacerbations, we carried out a longitudinal study assessing the number of occurrences of acute exacerbations and admissions over time (49 exacerbations amongst 33 children and 22 admissions amongst 11 children). Also, to take into account the relatively low numbers, we used a modified robust variance estimator for generalized estimating equations with improved small-sample performance, which is robust to model misspecification due to small numbers [26].

We attempted to minimize false-positive results due to multiple testing (hypothesis-driven study limited to several carefully defined phenotypes); however, we acknowledge that the potential impact of multiple testing on the reliability of conclusions cannot be fully eliminated.

We acknowledge the importance of other cytokines in the response to virus infection and asthma development. We focussed only on IL-2, as to the best of our knowledge this is the

only relevant cytokine for which there is a well-described single CpG site essential for epigenetic regulation of transcription [14].

Strengths include availability of accurate data from children's medical records with precise information on the timing of hospital admissions, emergency department visit and prescriptions of oral steroids (i.e. we did not rely only on parentally reported wheezing, which may be unreliable [27]), and the prospective nature of data collection, which reduces the likelihood of recall bias.

Interpretation

Increasing methylation in cord blood of the functional *IL-2* CpG site was associated with the increased likelihood of severe exacerbations and hospital admissions with asthma.

While the impact of chronic asthma is important, the major cause of morbidity and mortality are exacerbations. We found that the risk of hospital admission increased with increasing methylation of this site after the first year of life (we used exacerbations and hospitalizations after age 1 year as primary outcome measures to account for potential confounding by RSV bronchiolitis). It is possible that altered methylation of the functional *IL-2* CpG site and the subsequent altered IL-2 expression may affect specific response to viruses, in particular rhinovirus. *IL-2* is implicated in the response to rhinovirus infection [13, 28], and *in vitro* stimulation of T cells with rhinovirus increases the production of cytokines, including IL-2 [28]. An inverse relationship has been observed between IL-2 production in PHA-stimulated mononuclear cells and duration of rhinovirus shedding and nasal mucus production [13]; individuals with low immunoproliferative responses have more virus shedding [29]. However, there is a substantial interindividual variability in the response to rhinovirus [29], and we speculate that epigenetic mechanisms such as the one we describe here may contribute to this heterogeneity. We found that decreased methylation of the functional *IL-2* CpG site was associated with higher CBMC immunoproliferation in response to PHA. This is in agreement with observations that low endogenous IL-2 results in reduced PHA immunoproliferation *in vitro* [30], which can be reversed by the addition of exogenous IL-2 [31].

Increased methylation of the functional *IL-2* CpG site was not associated with the increased risk of atopy or the presence or persistence of wheeze. However, we observed that increasing *LINE-1* methylation was associated with an increased risk for the development of atopic sensitization. As methylation of *LINE-1* is a measure of global methylation levels [20], this indicates that other potentially novel CpG sites may underlie this observation. Our data suggest that different factors (including epigenetic mechanisms) are important in the development of sensitization and chronic asthma compared with those which increase the risk of severe exacerbation.

In conclusion, increasing methylation in cord blood of a single functional CpG site in the *IL-2* promoter is associated with increased likelihood of acute exacerbations and hospital admission for asthma/wheeze during childhood. This effect was specific for asthma/wheeze, and was not seen for hospital admissions for other reasons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Funding: MAAS was supported by Asthma UK Grant No 04/014 and JP Moulton Charitable Foundation, and is currently supported by MRC Grant G0601361. FDM conceived the idea for this study; AC is a principal investigator of MAAS; JC designed the assays and measured methylation of the *IL-2* promoter-enhancer region at birth; ASJ extracted the primary care data; DB and JC analysed the data; all authors interpreted the data; AC, AS and JC wrote the report with input from the other authors. The authors would like to thank the children and their parents for their continued support and enthusiasm. We greatly appreciate the commitment they have given to the project. We would also like to acknowledge the hard work and dedication of the study team (postdoctoral scientists, research fellows, nurses, physiologists, technicians and clerical staff)

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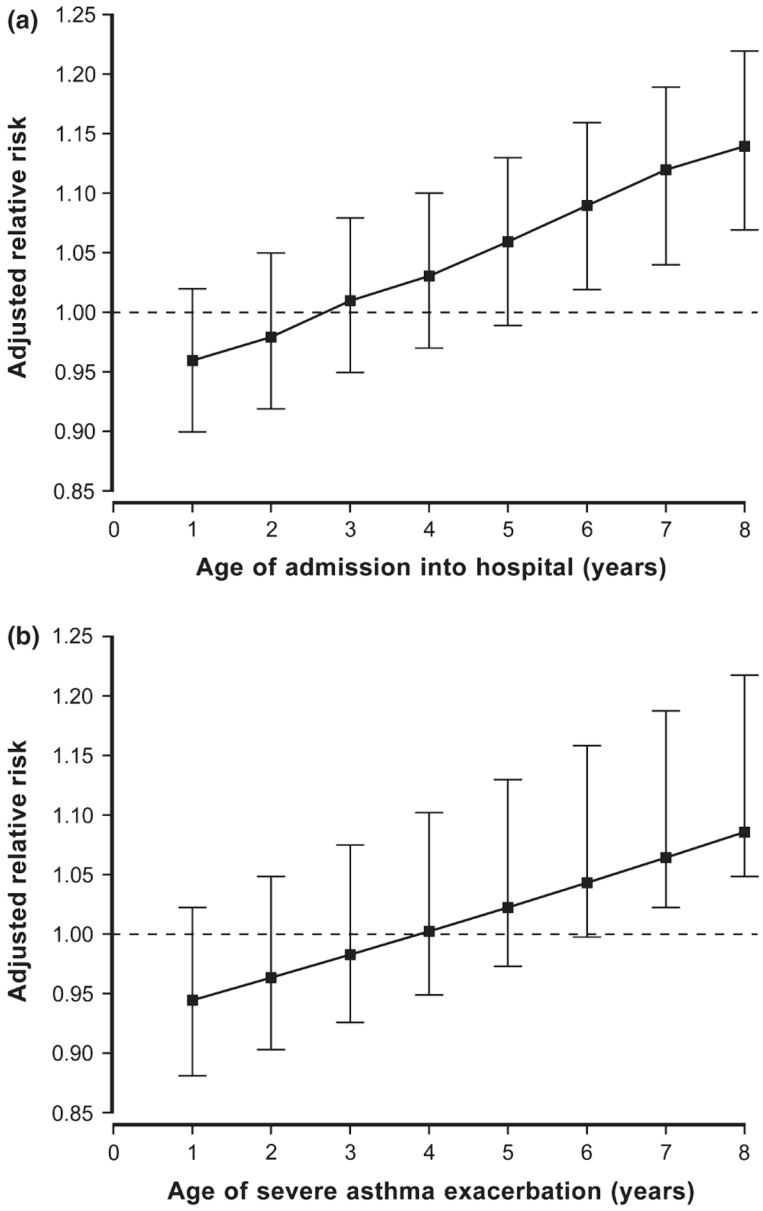

Fig. 1.

Conditional odds ratios for the change in the risk of hospital admission with asthma (a) and severe asthma exacerbation (b) with a unit increase in methylation level of *IL-2 Site1*.

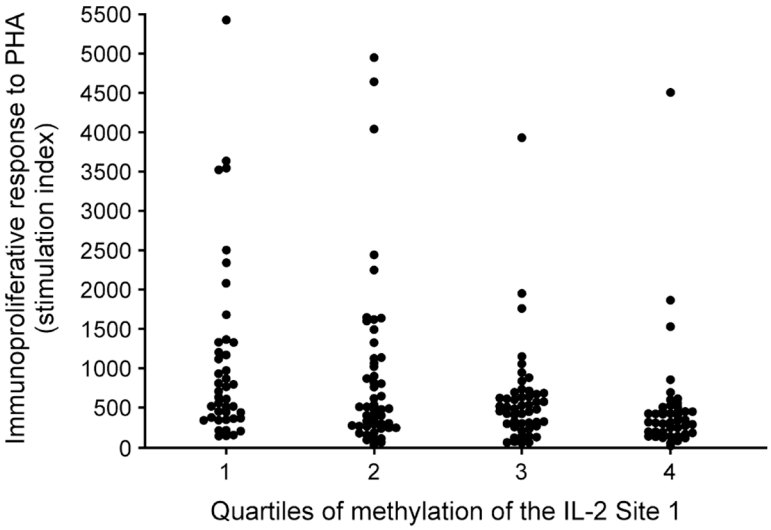

Fig. 2.

IL-2 Site1 methylation and mononuclear cell lymphoproliferative response at birth.

Table 1.

Description of the characteristics of subjects included in the study

	n	*percent*
Male	159/303	52%
Maternal Asthma	72/303	24%
Maternal Atopy	225/303	74%
Cat owner at recruitment	48/303	16%
Dog owner at recruitment	40/303	13%
Atopic sensitization		
age1	27/267	10%
age3	52/242	21%
age5	69/219	32%
age8	70/220	32%
Current Wheeze		
age1	102/273	37%
age3	39/259	15%
age5	56/251	22%
age8	30/238	13%
Wheeze Phenotypes		
Never wheezed	122/238	51%
Transient wheezers	53/238	22%
Intermittent wheezers	34/238	14%
Late-onset wheezers	8/238	3%
Persistent wheezers	21/238	9%

Table 2.

Perinatal factors and methylation of CpG sites (categorical and ordinal variables)

Mother atopic	**Mean [95% CI] Non-atopic**	**mean [95% CI] atopic**			***P*-value**
IL-2 Site 1	55.6 [54.1–57.2]	52.7 [51.6–53.8]			0.010
IL-2 Site 7	91.6 [91.1–92.0]	91.6 [91.3–92.0]			0.880
LINE-1	78.8 [78.5–79.2]	79.7 [79.5–80.0]			0.000
Maternal Asthma	no	yes			
IL-2 Site 1	53.1 [51.8–54.4]	52.0 [50.0–54.0]			0.39
IL-2 Site 7	91.7 [91.3–92.2]	91.5 [90.9–92.1]			0.56
LINE-1	79.8 [79.5–80.2]	79.5 [79.1–79.9]			0.20
Cat owner	no	yes			
IL-2 Site 1	53.9 [53.0–54.9]	51.0 [48.3–53.7]			0.02
IL-2 Site 7	91.6 [91.3–91.9]	91.7 [91.0–92.3]			0.94
LINE-1	79.5 [79.3–79.8]	79.3 [78.7–79.9]			0.44
Dog owner	no	yes			
IL-2 Site 1	53.4 [52.4–54.4]	53.9 [51.9–56.0]			0.71
IL-2 Site 7	91.9 [91.2–92.6]	91.9 [91.2–92.6]			0.50
LINE-1	79.4 [79.2–79.7]	79.9 [79.3–80.5]			0.20
Paternal income (£/annum)	≤ 10000	10 K–20 K	20 K–30 K	≥ 30 K	
IL-2 Site 1	53.4 [51.4–55.4]	52.6 [51.5–54.1]	55.0 [53.3–56.7]	54.8 [52.9–56.7]	0.14
IL-2 Site 7	91.7 [9.9–91.6]	91.5 [91.1–92.0]	91.8 [91.2–92.40]	92.4 [91.7–93.0]	0.01
LINE-1	79.9 [79.4–80.5]	79.5 [79.2–79.8]	79.6 [79.1–80.1]	79.2 [78.7–79.7]	0.29