

Pet keeping and tobacco exposure influence *CD14* methylation in childhood

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Abstract

Background: Several *CD14* gene–environment interactions in relation to the development of allergic diseases have been reported, but the underlying biological mechanisms are unclear. We recently showed that *CD14* methylation increased during childhood, paralleling a decreased impact of *CD14* polymorphisms on soluble *CD14* levels. Here, we aim to explore whether environmental stimuli during childhood affects *CD14* methylation, thereby providing a biological mechanism through which environment may modulate genetic effect.

Methods: *CD14* methylation levels were quantified in 157 children from the prospective Environment and Childhood Asthma birth cohort at ages 2 and 10. Associations between *CD14* methylation levels and house dust levels of endotoxin, $\beta(1,3)$ -glucans (at 2 yr only), allergens (dog, cat, and house dust mite), pet keeping and tobacco smoke exposure (TSE; questionnaire data) at 2 and 10 yr were explored.

Results: Children in homes without pets had *larger* increases in *CD14* methylation through childhood (2–10 yr) compared with children with pets (2.1% increase ($p = 0.003$) vs. 0.4% decrease (n.s.), global $p = 0.04$). At 10 yr of age, *lower* *CD14* methylation values were found in children with pets compared with children without pets at both 2 and 10 yr (5.4% vs. 7.5% [$p = 0.02$]). A similar trend was detected for TSE; children not exposed show *larger* increases in *CD14* methylation, most pronounced in school-age girls exposed vs. not exposed to tobacco (5.5% vs. 7.5% methylation, $p = 0.037$).

Conclusion: Pet keeping and TSE appears to limit increase in *CD14* methylation from 2 to 10 yr of age. This may partly explain the diverging *CD14* allele associations with allergic diseases detected in different environments.

CD14 is a pattern recognition receptor for environmental endotoxin (lipopolysaccharide [LPS]), and exists as a membrane-anchored protein (m*CD14*) on the surface of monocytes, macrophages, and neutrophils, and a monocyte or liver-derived soluble serum protein (soluble *CD14* [s*CD14*]) that lacks the anchor. Both molecules are critical for endotoxin-dependent signal transduction, and s*CD14* confers LPS sensitivity to cells lacking m*CD14* (1). Engagement of the *CD14*–LPS complex activates innate host defense mechanisms (2), presumably through inducing a Th1 cytokine profile and down-regulation a Th2 response in lymphocytes (3). Accordingly, levels of s*CD14* have been implicated in modulation of allergic disease (4).

CD14 polymorphisms (in particular rs2569190 [C159T]) have been associated with s*CD14* levels, but the associations have varied according to geographic location, age, and environmental exposures (5–8). This has resulted in increased focus on gene–environment interactions (9) and thereby epigenetic regulation, a mechanisms by which environmental stimuli may influence gene expression through modifications such as DNA methylation (10). Murine studies have demonstrated that *in utero* supplementation of methyl donors increased methylation of specific allergy-protecting genes, reducing transcriptional activity, thereby enhancing allergic airway disease (11). Recent studies in humans have further

shown that prenatal tobacco smoke exposure (TSE) (12), prenatal stress (13), famine (14), and traffic particle exposure (15), all influence DNA methylation. In previous work, we found that the impact of *CD14* polymorphisms on sCD14 levels decreased from birth to 10 yr of age in a prospective birth cohort and that this decrease in genetic impact paralleled a significant increase in *CD14* methylation in peripheral blood cells from age 2 to 10 yr (16). Furthermore, *CD14* methylation levels were inversely associated with sCD14 levels in the 10-yr-old children, suggesting that increased *CD14* methylation ultimately influences levels of sCD14.

The main objective of the present study was to explore whether methylation of the *CD14* gene body in peripheral blood in childhood is influenced by TSE, pet keeping, or house dust levels of microbial components and allergens, and whether such epigenetic pathways may help explain *CD14* gene–environment interactions in development of allergic disease.

Methods

Study design and subjects

The present study is based on a follow-up investigation of 1019 children at age 10 from the prospective birth cohort 'Environment and Childhood Asthma' (ECA) study in Oslo, described in details elsewhere (17). The present study includes the 157 children with available blood samples for *CD14* methylation measurements at both 2 and 10 yr of age (for paired analyses), with available exposure data as depicted in Fig. 1. Written informed consent was obtained from parents of all subjects, the study was approved by the Regional Medical Ethics Committee and the Norwegian Data Inspectorate, and notified to the Norwegian Biobank Registry.

DNA extraction

From the 2-yr-old subjects, DNA was extracted from blood clots with the Autopure LS extractor (Gentra) (by HUNT research centre, Verdal, Norway) obtained from the 2 yr investigation (1993–95) and stored at -70°C , as described elsewhere (16). From the 10-yr-old subjects DNA was extracted from peripheral whole blood consecutively as blood samples were drawn, using mainly a magnetic bead-based method on MagnaPure LC (Roche, Oslo, Norway), while some of the earliest samples were extracted on a 340A Nucleic Acid Extractor.

Bisulfite sequencing

DNA methylation was quantified by bisulfite sequencing, described elsewhere (16). Genomic DNA was converted using MethylEasy Xceed (Human Genetic Signatures, Randwick, NSW, Australia) according to manufacturer's instructions. Primers were designed in Methyl Primer Express v1.0 (Applied Biosystems, Carlsbad, CA, USA) to amplify the intragenic CpG island (consisting of 87 CpG sites) covering most of the *CD14* coding region (Fig. 2). PCR conditions, primer sequences, and cycling conditions are available upon request. Primers and dNTPs were enzymatically eliminated by ExoSAP-IT (USB Corporation, High Wycombe, UK). Amplicons were sequenced directly using a 1:32 dilution of BigDye Terminator v3.1 chemistry (Applied Biosystems) in reverse direction using M13 primer and separated on an ABI 3730 capillary sequencer. Methylation levels were quantified based on four-dye trace data from direct sequencing of PCR amplicates using Epigenetic Sequencing Methylation analysis software ESME (Epigenomics) (18).

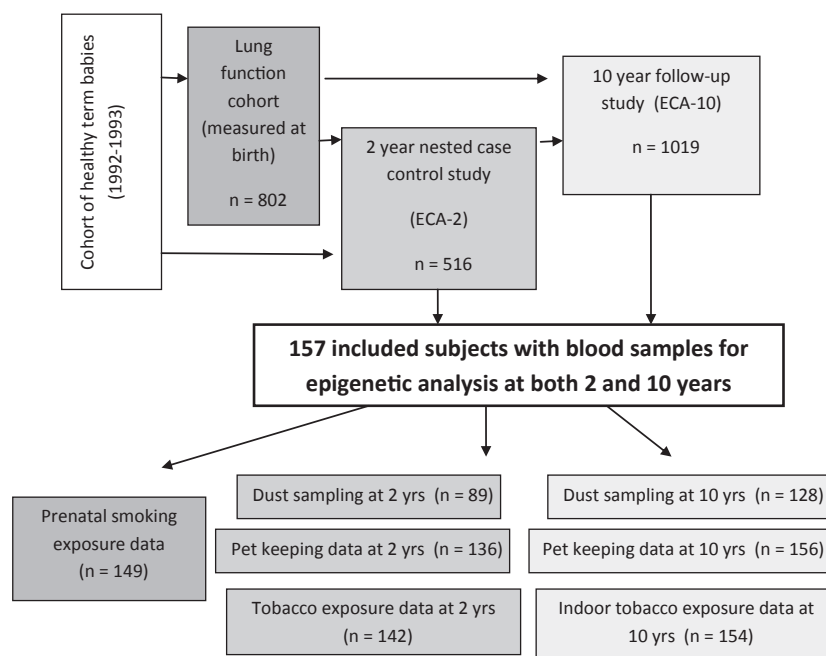


Figure 1 Study design.

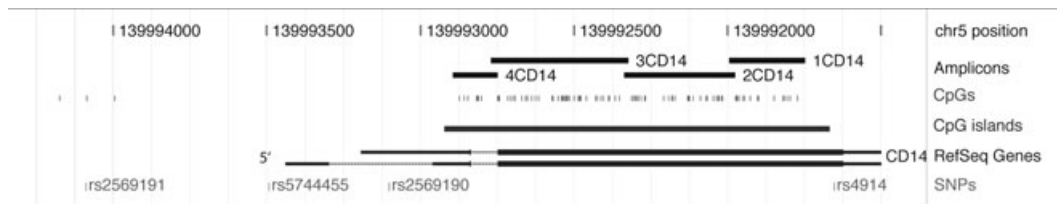


Figure 2 A map of the *CD14* gene showing the location of the individual CpG sites, the CpG island, *CD14* polymorphisms, and the amplicons used in the bisulfite sequencing.

Main outcomes

Mean *CD14* methylation (the average of % methylation at each of the 87 CpG sites in the CpG island per individual, portrayed in Fig. 2) at 2 and 10 yr, and delta (Δ)-methylation (the difference between mean methylation at 2 and 10 yr).

Explanatory variables

Prenatal TSE (yes/no) was obtained from questionnaires completed at time of birth.

Tobacco exposure at 2 yr (defined as parental smoking; TSE yes/no) and pet keeping by age 2 yes/no (including cats, dogs, birds, and other furry pets) was obtained from the 2 yr investigation questionnaires. House dust sampling was performed by parents vacuuming the living area of the homes at 2 yr of age (according to instructions (19)). The collected dust was stored at -20°C and extracted at the Norwegian Institute of Public Health (NIPH). Dust samples were analyzed by measuring levels of endotoxin by the kinetic chromogenic Limulus Amebocyte Lysate test, $\beta(1,3)$ -glucans by an inhibition enzyme immuno assay, dog allergen (Can f1), cat allergen (Fel d1), and house dust mite allergen (Der p1) by ELISA as described elsewhere (19). Detection limits for the assays were 12.5 ng/ml for dog allergen, 5 ng/ml for cat allergen, and 6.25 ng/ml for mite allergen.

Tobacco exposure at 10 yr (defined as indoor smoking by any household member) (TSE yes/no) and present keeping of cat, dog, or pet (including cats, dogs, other furry pets, and birds) yes/no, at 10 yr, was based on doctor-guided interviews with parents at the 10 yr investigation. Mattress dust was collected by the parents according to standardized instructions by vacuuming sleeping mattresses of the children (20). The fine fraction of the dust was extracted and analyzed at NIPH for concentration of cat, dog, and house dust mite allergens by ELISA following manufacturer's instructions (Indoor Biotechnologies, Clewyd, UK) (20).

Clinical possible confounders

Parental atopy was defined as parental asthma or rhinoconjunctivitis, as reported in the parental structured interview at the 10 yr investigations, described elsewhere (17).

Asthma ever by age 10 was defined as at least two of three criteria being fulfilled from the interview at the 10 yr investigation: (1) dyspnea, chest tightness, and/or wheezing ever; (2) a

physician's diagnosis of asthma; and (3) use of asthma medication.

Allergic sensitization at 2 yr was defined as a positive Phadiatop Infant[®] panel test (Phadia, Uppsala, Sweden; cut-off level for specific IgE [sIgE] 0.35 kU/l), including the allergens Der p1, cat, dog, egg white, cows milk, peanut, shrimp, timothy, birch, common ragweed, wall pellitory, lower detection limit of 0.1 PAU/l.

Allergic sensitization at 10 yr was defined as sIgE > 0.35 kU/l to at least one allergen. In addition to Phadiatop, the allergens Dermatophagoides farinae, German cockroach, rabbit dander, mugwort pollens, molds (Cladosporium herbarium and Alternaria alternata), and codfish were included (ImmunoCAP according to manufacturer's instruction; Phadia), lower detection limit of 0.1 kUA/l.

Statistical analysis

Based on a paired situation (change from age 2 to 10) and previously detected change (observed at 2 yr-mean (s.d.) of 5.5 (2.5) and at 10 yr-mean of 6.8 [3.8]) (16), the study power is $>84\%$, simulated under the assumption of a correlation of at least 0.20 between measurements.

Possible influence of different DNA extraction methods were tested for in a separate study (to be published), showing that extraction method did not influence methylation results.

Methylation levels were analyzed as continuous variables. Methylation levels at 10 yr were not normally distributed and log-transformed values at 2 and 10 yr were applied for analyses, but crude methylation values are presented in results and tables for sake of clarity. In addition, 'a sliding door' analyses across the entire CpG island was performed to evaluate where in the CpG island change from 2 to 10 was most pronounced, using four CpG sites per door, Pearson's correlation test was applied where explanatory variables were continuous, while ANOVA was applied when they were dichotomous.

To analyze change in methylation levels from 2 to 10 yr, data were stratified according to explanatory dichotomous variables, and change in methylation was analyzed using paired *t*-test within each group. To test for between-group differences, Δ -methylation was analyzed against environmental variables. Possible environmental interactions were explored using linear regression (including interaction steps) with mean methylation levels at ages 2 and 10 as dependent variables, respectively. In addition, the analyses were repeated using Z-scores (reference

group of unexposed subjects at 2 and 10 yr) to correct for the possibility of signal inflation.

The study population was by selection biased toward asthma and allergy. Thus, significant findings were controlled for confounding by parental atopy, asthma at 10 yr, and allergic sensitization at 2 and 10 yr. For this, an analysis of covariance (ANCOVA) was applied, defining a confounding effect as changing the *B* value of the fixed factors (potential confounders) by >20% when excluded from the analysis.

Analyses were performed in SPSS16 with a 5% statistical significance level. As the study objective is to explore influences on results from an earlier study (16), we have not corrected for multiple testing and present uncorrected *p*-values.

Results

Demographic information on included subjects is presented in Table 1. Of the 157 children included, 25 (21%) were allergically sensitized at 2 yr of age, while at 10 yr, 63 (42%) had become allergically sensitized, and 55 (35%) of the children had asthma ever. At 2 yr of age, 39 (29%) kept pets, increasing to 62 (40%) by age 10. At age 2 yr, 56 (39%) were exposed to tobacco while this decreased to 23 (15%) at age 10.

Methylation values at 2 yr

CD14 methylation levels at 2 yr were not associated with prenatal tobacco exposure, allergen, or microbial levels, pet keeping at 2 yr only, or tobacco exposure at 2 yr, data not shown. Regression analyses including all variables did not yield additional information.

Methylation values at 10 yr

Pet exposure was significantly associated with lower methylation values at 10 yr; by pet ownership at 2 and 10 yr ($p = 0.023$), by dog allergen levels at 10 yr in girls ($p = 0.018$) and by cat allergen levels at 10 yr in boys ($p = 0.041$; Table 2).

Tobacco exposure did not significantly influence *CD14* methylation in boys, whereas girls exposed to indoor smoking at the time of investigation had lower methylation levels ($p = 0.037$; Table 2).

The detected associations with *CD14* methylation at 10 yr were not influenced by the possible confounders (parental atopy, asthma, or allergies), and regression analyses including all variables did not yield additional information.

Change in *CD14* methylation from 2 to 10 yr of age

The average increase in *CD14* methylation from 2 to 10 yr ($n = 153$) was 1.3% (from 5.5% to 6.8%, $p = 0.001$) (16). Children in homes with any pets at 2 or 10 yr had consistently smaller increases in *CD14* methylation (all < 1% increases, all statistically non-significant) than those without the pets (1.4–2.1% increase, all $p < 0.005$) (Table 3). Δ -methylation in groups with and without pets reached statistical significance for those with and without pets at 2 yr of age ($p = 0.02$), and for those with or without pets at both 2 and 10 yr of age

Table 1 Demographic characteristics of study population

Demographic variable/ number ascertained in n (%) (of total 157 children) or mean (s.d.)	Boys (n = 88)	Girls (n = 69)	All children
Parental atopy (at child's age 10)/153: n (%)*	50 (58)	35 (53)	85 (56)
Allergic sensitisation at 2 yr/121: n (%)*	20 (30)	5 (9)	25 (21)
History of asthma at 10 yr/157: n (%)*	38 (43)	17 (25)	55 (35)
Allergic sensitisation at 10 yr/150: n (%)*	44 (51)	19 (30)	63 (42)
Pet keeping at 2 yr /136: n (%)	24 (32)	15 (25)	39 (29)
Dog keeping: n (%)	10 (13)	6 (10)	16 (10)
Cat keeping: n (%)	3 (4)	7 (12)	10 (6)
Endotoxin level at 2 yr EU/mg: mean (s.d.)	65 (117)	87 (136)	74 (124)
β (1,3)-glucans level at 2 yr: mean (s.d.)	3764 (2290)	3525 (2496)	3672 (2359)
Cat allergen at 2 yr ng/ml: mean (s.d.)	2 (13)	19 (70)	9 (45)
Dog allergen at 2 yr ng/ml: mean (s.d.)	5 (20)	5 (16)	5 (19)
Mite allergens at 2 yr ng/ml: mean (s.d.)	0.2 (0.13)	0.3 (0.41)	0.2 (0.3)
Pet keeping at 10 yr /156: n (%)	35 (40)	27 (40)	62 (40)
Dog keeping: n (%)	20 (29)	26 (30)	46 (29)
Cat keeping: n (%)	12 (18)	15 (17)	27 (17)
Cat allergen at 10 yr μ g/g: mean (s.d.)	7 (23)	31 (151)	18 (104)
Dog allergen at 10 yr μ g/g: mean (s.d.)	17 (52)	11 (27)	15 (42)
Mite allergens at 10 yr μ g/g: mean (s.d.)	0.5 (2.2)	0.5 (1.8)	0.5 (2)
TSE prenatally/149: n (%)	27 (32)	29 (45)	56 (38)
TSE at 2 yr/142: n (%)	29 (38)	27 (42)	56 (39)
TSE at 10 yr/154: n (%)	11 (13)	12 (18)	23 (15)

TSE, tobacco smoke exposure.

*Statistically significant difference between boys and girls ($p < 0.05$).

($p = 0.04$; Fig. 3 and Table 3), and was not influenced by the possible clinical confounders or changed by *Z*-score analyses.

A similar, but non-significant trend was seen for tobacco exposure after the prenatal period, where exposed children had a smaller increase in methylation than those not exposed (Table 3).

The average increase in *CD14* methylation from 2 to 10 yr was most pronounced at CpG site 13–16 (from the 5' end in amplicon one; Fig. 2) with a 5.1% increase ($p < 0.001$), but the increase was not significantly different between the groups stratified according to pet keeping or TSE.

No significant correlation was found between change in methylation from 2 to 10 yr (Δ -methylation) and allergen levels or the clinical confounders (parental atopy, asthma, or allergies).

Table 2 The relationship between degree of *CD14* methylation at 10 yr of age and environmental variables

	Methylation values at 10 yr		Methylation values girls only		Methylation values boys only	
	Degree (%) methylation yes vs. no	p-Value	Degree (%) methylation yes vs. no	p-Value	Degree (%) methylation yes vs. no	p-Value
Dog keeping 10 yr	5.8 vs. 7.0	0.197	5.4 vs. 7.4	0.117	6.0 vs. 6.6	0.716
Cat keeping 10 yr	6.1 vs. 6.9	0.251	6.9 vs. 7.2	0.769	5.5 vs. 6.7	0.195
Pet keeping 10 yr	6.3 vs. 7.1	0.131	7.0 vs. 7.3	0.411	5.8 vs. 7.0	0.197
Pets always vs. never	5.4 vs. 7.5	0.023	4.6 vs. 7.6	0.007	5.8 vs. 7.5	0.318
TSE* in pregnancy	7.0 vs. 6.7	0.510	7.1 vs. 7.2	0.870	6.9 vs. 6.3	0.634
TSE at 2 yr	6.6 vs. 7.1	0.900	6.2 vs. 7.8	0.150	6.9 vs. 6.7	0.200
TSE preschool	6.0 vs. 6.9	0.537	5.3 vs. 7.4	0.107	6.5 vs. 6.5	0.663
TSE school	6.1 vs. 6.9	0.373	5.5 vs. 7.5	0.037	6.9 vs. 6.5	0.404
	<i>B</i> value	p-Value	<i>B</i> value	p-Value	<i>B</i> value	p-Value
Dog allergen 10 yr	−0.164	0.064	−0.308	0.018	−0.105	0.385
Cat allergen 10 yr	0.092	0.302	0.170	0.198	−0.245	0.041
House dust 10 yr	0.039	0.665	0.106	0.427	−0.001	0.991

TSE, tobacco smoke exposure. Significant differences in bold.

Table 3 The change in degree of *CD14* methylation from 2 to 10 yr according to different explanatory environmental variables

Explanatory variables (n) for each group, respectively	Increase in methylation (%) in the respective groups	p-Values within the respective groups	Difference between groups (p-value)
Boys (86) vs. girls (67)	1% vs. 1.8%	0.09 vs. 0.002	0.22
Cat 2 yrs yes (9) vs. no (123)	0.1% vs. 1.5%	0.959 vs. 0.001	0.38
Dog 2 yrs yes (16) vs. no (116)	1.0% vs. 1.5%	0.339 vs. 0.002	0.63
Pets 2 yrs yes (38) vs. no (94)	−0.1% vs. 2.0%	0.855 vs. 0.000	0.02
Cat 10 yrs yes (26) vs. no (126)	0.7% vs. 1.4%	0.285 vs. 0.002	0.53
Dog 10 yrs yes (22) vs. no (130)	0.7% vs. 1.4%	0.417 vs. 0.001	0.52
Pet 10 yrs yes (61) vs. no (91)	0.1% vs. 1.5%	0.072 vs. 0.005	0.45
Pets always (22) vs. never (64)	−0.4% vs. 2.1%	0.502 vs. 0.003	0.04
Prenatal TSE* yes (54) vs. no (91)	1.7% vs. 1.1%	0.006 vs. 0.029	0.46
TSE 2 yrs yes (52) vs. no (86)	0.8% vs. 1.7%	0.077 vs. 0.005	0.29
TSE preschool yes (19) vs. no (132)	0.5% vs. 1.4%	0.454 vs. 0.001	0.46
TSE school yes (22) vs. no (128)	0.8% vs. 1.4%	0.161 vs. 0.002	0.62

TSE, tobacco smoke product exposure. The increase in methylation and respective p-values are measured by applying a paired *t*-test for methylation from 2 to 10 yr old in groups stratified according to explanatory variables in first column.

Discussion

The present study demonstrates that the increase in *CD14* methylation from 2 to 10 yr of age is significantly influenced by pet ownership and tobacco smoke. While environmental factors were not associated with *CD14* methylation at 2 yr of age, 10-yr-old children *exposed* to pets or tobacco had *lower* *CD14* methylation levels compared with children not exposed.

Pet keeping and *CD14* methylation

We have previously shown that increased *CD14* methylation parallels a decreasing impact of *CD14* polymorphisms on s*CD14* levels (16). The present finding that pet keeping and tobacco exposure are associated with lower *CD14* methylation

and thus (by inference to earlier work) an increased impact of gene polymorphisms, is consistent with several reported gene–environment interactions. Eder et al. (21) described opposite effects of *CD14* polymorphisms on sIgE depending on whether children had regular contact with pets or lived on farms, but no association in children with no contact with pets. Smit et al. (22) showed stronger effects of *CD14* genotypes on asthma in young subjects when they were born and raised on a farm compared with those who were not. Gern et al. (23) report a *CD14* genotype effect on atopic dermatitis among infants exposed to dogs, but no association in infants without dogs, and Leynaert et al. (24) found that *CD14* genotypes were associated with allergic sensitisation in young adults exposed to farm living in early life, but no genotype effect in non-farming individuals. Based on these studies, we suggest that certain

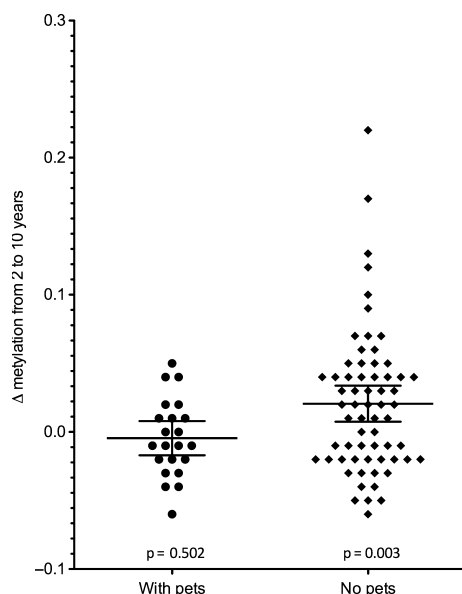


Figure 3 The figure shows Δ -methylation (change in methylation from 2 to 10 yr, with 95% CI) in children with pets at 2 and 10 compared with children without pets at either ages analyzed by paired *t*-test for the two time points in each group separately. The two groups are significantly different from each other, $p = 0.04$.

gene–environment interactions may be governed through *CD14* methylation. The fact that the association between *CD14* methylation and pet keeping became evident first at 10 yr of age could suggest that these epigenetic changes are manifested over longer time periods, or that older children possibly are in closer contact with pets than children below 2 yr of age; however, our study could not answer this. Furthermore, the increased *CD14* methylation may be a direct result of not owning pets or an indirect result of gene transcription activity (where lack of pet-driven gene transcription leads to a more ‘silent’ gene, which subsequently becomes methylated).

Tobacco exposure and *CD14* methylation

CD14 methylation from 2 to 10 yr of age increased significantly only in children not exposed to tobacco, and unexposed girls had significantly higher *CD14* methylation levels at 10 yr than exposed girls. These findings are consistent with those of Chaudhry et al. (25), where *CD14* genotypes were associated with IgE levels only when exposed to tobacco. It has been suggested that TSE affects methylation through endotoxin (an active component of cigarette smoke (26)); however, we found no association between endotoxin levels and TSE or *CD14* methylation.

Endotoxin and *CD14* methylation

In many previous reports, there is evidence suggesting that the C159T-C allele is a risk factor for allergic phenotypes at low levels of endotoxin exposure, while a protective factor at high

levels of exposure (27). Our *CD14* methylation analyses cannot explain these observations, as we did not detect any association between endotoxin and *CD14* methylation levels at 2 yr. However, endotoxin levels in our study were low compared with others (i.e. the lowest quartile of endotoxin levels in Boston metropolitan homes were above the highest levels measured in the ECA study (28)). Furthermore, endotoxin levels did not correspond to pet keeping (previously also observed in other urban populations compared with rural populations (28)). One explanation may be that endotoxin interacts with *CD14* polymorphism through other regulatory mechanisms. It has, for instance, been shown that endotoxin-induced tolerance may be mediated through microRNAs, illustrating the complex network of epigenetic regulation (29).

Gender effects

We found that environmental effects on methylation appeared more pronounced in girls than boys. In the same cohort, we have previously found that 10-yr-old girls had significantly higher levels of cat and dog allergens in their mattress dust compared with 10-yr-old boys, even when exclusively examining children without pets at home (20). This suggests that the 10-yr-old girls appear to be in closer contact with pets (also when not keeping pets at home). Thus, higher exposure to pet allergens among girls may explain the stronger associations with *CD14* methylation detected. However, we were not able to confirm this through our allergen analyses.

Implication for development of allergic disease

The focus of the present study was to explore environmental impact on *CD14* methylation in peripheral blood cells. Due to the limited number of children included ($n = 157$ with blood samples from both 2 and 10 yr of age), further stratification into groups with different clinical phenotypes or *sCD14* levels resulted in inadequate statistical power for detecting impact of our findings on allergic phenotype. However, previous studies report associations between allergic disease and both *sCD14* levels and *CD14* polymorphisms (7), although the relationship between the three has not been consistent and causality difficult to define. In our previous study, we showed that genetic impact on *sCD14* expression was modulated by the degree of *CD14* gene methylation, where increased methylation decreased the impact of *CD14* polymorphisms (16). If pet keeping, and possibly tobacco exposure, result in decreased *CD14* methylation (as proposed in the present study), this should increase genetic impact on *sCD14* levels. Indeed, this appears to be the case in many of the studies reviewed previously. Hence, we hypothesize that *sCD14* levels and subsequent effect on allergic disease will depend on both genetic predisposition, and environmental exposures that modify these genetic predispositions, through epigenetic mechanisms.

Strengths and limitations

The ECA study is characterized by a high follow-up rate, well-described phenotypes, and ample environmental data from

different periods in childhood. It is also one of few studies that have analyzed methylation patterns in the same individuals through childhood. Furthermore, level of *CD14* gene methylation is based on sequencing, that is, analyzing all the 87 CpG sites in the *CD14* CpG island, hence providing a comprehensive methylation degree of the gene. As such, the cohort is particularly well suited for exploring *CD14* epigenetic and environmental interactions during childhood.

Main challenges of the present study include the fact that methylation patterns are cell-line specific. Separating cells were not planned from the beginning of the study, and we were thus unable to overcome the issue of epigenetic heterogeneity. Being limited to ascertain methylation patterns in whole blood might conceal possible cell-specific methylation changes both with age and according to the environmental exposures. Another challenge was that endotoxin and $\beta(1,3)$ -glucan analyses were not performed in house dust from age 10. Although endotoxin levels were not associated with pet keeping, TSE exposure or *CD14* methylation levels at 2 yr, this still needs to be evaluated later in childhood. Finally, we do not have RNA from the ECA subjects and are therefore not able to extrapolate the effect of *CD14* methylation changes directly on gene expression.

In conclusion, we demonstrate that pet keeping and TSE were associated with both a smaller increase in *CD14* methylation during childhood and lower *CD14* methylation at 10 yr. Together with previous data indicating that *CD14* genetic impact on s*CD14* levels is inversely proportional to *CD14* methylation, this provides one possible biological mechanisms for reported *CD14* gene–environment interactions in an allergic disease.

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