

CD14 polymorphisms and serum CD14 levels through childhood: A role for gene methylation?

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Background: CD14 is a pattern-recognition receptor for environmental LPS, and engagement of the CD14-LPS complex activates innate host defense mechanisms. Single nucleotide polymorphisms (SNPs) in the *CD14* gene have been associated with soluble CD14 (sCD14) levels, but inconsistencies between studies suggest the presence of regulatory mechanisms hitherto not well understood.

Objective: We sought to investigate possible associations between *CD14* SNPs and sCD14 levels at different time points in childhood (at birth [cord blood] and 2 and 10 years) and to explore whether these associations were related to *CD14* gene methylation.

Methods: Four SNPs, rs2569191 (–1145GA), rs5744455 (–550CT or –651CT), rs2569190 (–159CT or –260CT), and rs4914 in *CD14* were genotyped in 762 children from the Environmental and Childhood Asthma study. Genotype frequencies were analyzed for association with sCD14 levels in 660 babies, 346 children at age 2 years, and 360 children at age 10 years. In a subgroup of 157 children with DNA available at both 2 and 10 years of age, *CD14* methylation patterns were determined and analyzed against detected *CD14* gene–sCD14 associations. **Results:** rs2569191, rs5744455, and rs2569190 were associated with sCD14 levels at birth and 2 years, but only rs5744455 was

associated with sCD14 levels at 10 years. *CD14* methylation increased significantly from age 2 to 10 years, and the level of methylation was inversely correlated with sCD14 levels at 10 years. **Conclusion:** The reduced effect of *CD14* polymorphisms on sCD14 levels from early to late childhood paralleled a small but significant increase in *CD14* methylation during the same period. (J Allergy Clin Immunol 2010;■■■■:■■■■–■■■■.)

Key words: Allergy, association study, asthma, *CD14*, child, cohort study, epigenetics, genetic analysis, methylation patterns

CD14 on chromosome 5q31 encodes 2 protein isoforms: a membrane-anchored protein (membrane-bound CD14) on the surface of monocytes, macrophages, and neutrophils and a monocyte- or liver-derived soluble serum protein (sCD14) that lacks the anchor. Both molecules are critical for LPS-dependent signal transduction, and sCD14 confers LPS sensitivity to cells lacking membrane-bound CD14.¹ Engagement of the CD14-LPS complex results in activation of innate host defense mechanisms,² presumably through inducing a T_H1 cytokine profile and downregulating a T_H2 response in lymphocytes.³ Accordingly, increased sCD14 levels have been implicated in conditions such as inflammatory infectious diseases,⁴ as well as in the modulation of allergic disease.⁵

In 1999, Baldini et al⁶ reported an association between the rs2569190 (159CT) polymorphism in the *CD14* promoter region and sCD14 levels in children, where the C allele was found to be associated with low sCD14 levels. A functional study supported this finding by showing increased *CD14* transcriptional activity for the rs2569190 T allele.⁴ Later studies, however, challenged the idea of a simple relationship between genotype and sCD14 levels. A longitudinal study from birth to 1 year of age suggests that the effect of *CD14* polymorphisms on sCD14 levels varies according to age.⁷ In adults from Singapore, *CD14* genotypes were not associated with sCD14 levels.⁸ Another study demonstrated that the significant association between *CD14* genotype and sCD14 levels disappeared after endotoxin inhalation.⁹ Hence the relationship between *CD14* genetics/sCD14 levels and later clinical manifestations appears equally complex. The same rs2569190-C allele has been positively, neutrally, and negatively associated with atopy or lung function,^{10–12} and both high and low levels of sCD14 have been related to clinical conditions such as septicemia,¹³ coronary heart disease,¹⁴ and allergic phenotypes.¹⁵

To reconcile these findings, it has been suggested that associations between *CD14* polymorphisms, sCD14 levels, and later clinical phenotypes might be governed by complex interactions between gene transcription, environment,^{16–18} and age.^{7,19} The field of epigenetics (heritable changes in gene expression that

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The study was performed within the Oslo Research Group of Asthma and Allergy in Childhood (ORAACLE), which is part of the Global Allergy and Asthma European Network (GA²LEN).

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Abbreviations used

LD: Linkage disequilibrium
 sCD14: Soluble CD14
 SNP: Single nucleotide polymorphisms

occur in the absence of alterations in DNA sequences) might provide a biological framework wherein these interactions can be understood.²⁰ Environmental exposures during specific phases of life appear to regulate gene transcription through altering DNA methylation patterns (covalent addition of a methyl group to the nucleotide cytosine a CpG dinucleotide) or histone acetylation.²¹ It has been demonstrated that *in utero* supplementation of methyl donors increased methylation of specific allergy-protecting genes, reduced transcriptional activity, and thereby enhanced allergic airway disease in offspring mice.²² Furthermore, CD14 expression in keratinocytes was influenced by epigenetic modifications through histone acetyltransferase activity, leading to altered antimicrobial functions important for the innate immune responses in the skin.²³

The main aim of the present study was therefore first to determine the relationship between *CD14* polymorphisms and sCD14 levels at 3 time points (ie, birth [cord blood] and 2 and 10 years of age) in children from the prospective birth cohort study Environment and Childhood Asthma. Second, we aimed to explore whether the relationships between *CD14* polymorphisms and sCD14 levels at different time periods were associated with methylation patterns in the *CD14* gene. Finally, the effect of our findings on specific atopic traits in the children was briefly explored.

METHODS**Study design and subjects**

The present study is based on a 10-year follow-up of children in the prospective birth cohort Environment and Childhood Asthma study in Oslo, Norway.²⁴ Briefly, a generalized birth cohort of healthy term babies was established in Oslo (1992-1993) with routine sampling of cord blood. A subpopulation of 802 children with lung function measured at birth, as well as 526 children examined in a nested case-control study for recurrent bronchial obstruction at 2 years of age, was asked to attend a 10-year follow-up study (Fig 1). Written informed consent was obtained from the parents of all subjects, and the regional medical ethics committee and the Norwegian Data Inspectorate approved the study.

The present study includes the 762 children with available DNA at 10 years of age and either available DNA from 2 years of age or sCD14 measurements at any age (Fig 1). Because of genotyping failure in 24 children and unavailable sCD14 levels for 15 children, genotype-sCD14 association analyses were performed in 723 children. Of these, sCD14 levels were measured in cord blood from 660 children, in 346 children at 2 years, and in 360 children at 10 years, with the 3 groups overlapping. The genotype-sCD14 level associations were analyzed in different atopic phenotype and sex groups for possible effects of confounding, as described in detail in the statistical section.

The methylation analyses were performed in 157 children with DNA available for epigenetic analyses at both 2 and 10 years of age. Of these, methylation analysis failed in 1 subject at 2 years of age and 3 subjects at 10 years of age.

Finally, the relationship between genotype, gene methylation, and sCD14 levels was briefly investigated for its effect on defined atopic phenotypes (specific IgE panel test at 2 years and asthma and specific IgE level measurements at 10 years).

sCD14

sCD14 levels were determined in a quantitative sandwich ELISA according to the manufacturer's recommendations (catalog no. DC140; R&D Systems Europe, Abingdon, Oxon, United Kingdom). Samples (diluted 1:200) were added in duplicate to a microplate precoated with monoclonal anti-sCD14 antibodies, followed by incubation with, in succession, enzyme-linked polyclonal antibody specific for sCD14, substrate solution, and stop solution. Optical density was measured at 450 nm in a MRX Microplate Reader connected to a PC with Revelation Software (Dynateck Laboratories, Chantilly, Va). A soluble CD14 standard (250-8000 pg/mL) was included on each plate for standard curve production. A control sample (normal adult serum) was also included on each plate.

DNA extraction

From the 2-year subjects, DNA extraction from blood clots stored at -70°C from the 2-year investigation (1993-1995) was performed at HUNT research center, Verdal, Norway. Before cell lysis, the samples were thawed, and serum and gel were removed. Homogenization was performed, and Red Blood Cell Lysis Solution and Proteinase K were added consecutively. Protein Precipitation Solution was used to form a protein suspension. Finally, isopropanol was added, and DNA was separated from the solution. After precipitation of DNA, the samples were rinsed with EtOH, and Tris-EDTA buffer was added.

From the 10-year subjects, DNA was extracted from peripheral whole blood consecutively as blood samples were drawn with a magnetic bead-based method on MagnaPure LC (Roche, Mannheim, Germany).

Single nucleotide polymorphism information and genotyping methods

Single nucleotide polymorphism (SNP) selection was based on data from HapMap accessible at the time of selection²⁵ and performed according to the following criteria. All validated SNPs in *CD14* (including 2,000 bp flanking the transcribed gene) with a minor allele frequency of greater than 5% (Hap-Map CEU population²⁵) were identified. The linkage disequilibrium (LD) patterns from the HapMap CEU population were evaluated to avoid selecting SNPs in strong LD (cutoff, $r^2 = 0.8$). Four SNPs were identified from the 5' end: rs2569191 (−1145GA), rs5744455 (−550CT or −651CT), rs2569190 (−159CT or −260CT), and rs4914. Of these, rs4914 is a synonymous SNP in the exon, and the 3 remaining SNPs are in the 5' flanking region. These SNPs are assumed to capture the majority of the known common variations in the gene. Allele distribution, minor allele frequencies, and LD patterns from our population are portrayed in Fig 2.

Genotyping was performed with the SNPlex genotyping system, a capillary electrophoresis-based assay that enables simultaneous genotyping of up to 48 SNPs in a single sample (<https://products.appliedbiosystems.com/>). The genotyping failure rate was 4.1% for rs2569191, 3.4% for rs5744455, 3.5% for rs2569190, and 3.9% for rs4914.

Bisulfite sequencing

In the bisulfite sequencing method genomic DNA is treated with sodium bisulfite, which converts cytosines to uracils but leaves methylated cytosines unchanged. The converted DNA is subject to PCR with primers designed to amplify the region of interest, and the PCR product is sequenced to quantify the level of DNA methylation.²⁶ In the present study DNA methylation was quantified by means of bisulfite sequencing in the 157 children with DNA available both at 2 and 10 years of age. Genomic DNA (in most cases 500 ng) was converted with MethylEasy Xceed (Human Genetic Signatures), according to the manufacturer's instructions (DNA input range, 50 pg to 5 μg). Primers were designed to amplify the intragenic CpG island covering most of the coding region of *CD14* and the rs2569191 locus in the 5' region of the transcription start, itself a CpG site when existing as a G allele (Fig 2). Primer design was done with the Methyl Primer Express v1.0 software (Applied Biosystems, Foster City, Calif). PCRs were performed in 384-well plates containing 1 $\times$ Gold buffer, 1.4 mmol/L deoxyribonucleotide triphosphate mix, 1.5 mmol/L MgCl_2 , 0.5 U of Gold polymerase (Applied Biosystems),

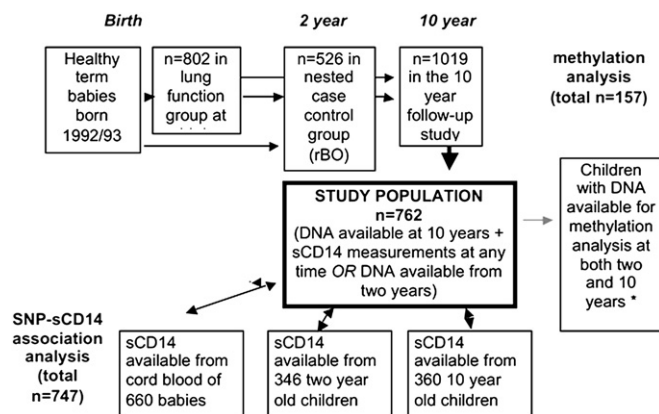


FIG 1. Study design and subjects included. The different groups overlap; that is, there are children included in more than 1 analysis.

1 μ mol/L each of forward and reverse primers, and 4 to 8 ng of bisulfite-treated genomic DNA. Primer sequences are shown in Table I, and cycling conditions are available on request. For PCRs covering the intragenic region, unincorporated primers and deoxyribonucleotide triphosphates were enzymatically eliminated by adding 2 μ L of ExoSAP-IT (USB Corporation) to each 5- μ L reaction. The PCRs covering rs2569191 were cleaned up with CleanSEQ magnetic beads (Agencourt). Amplicons were sequenced directly by using a 1:32 dilution of BigDye Terminator v3.1 chemistry (Applied Biosystems) in reverse direction with M13 primer and separated on an ABI 3730 capillary sequencer. Levels of methylation were quantified based on 4-dye trace data from direct sequencing of PCR amplicates by using Epigenetic Sequencing Methylation analysis software (Epigenomics, Berlin, Germany).²⁷

Clinical atopic parameters

Information regarding symptoms of airway obstruction was obtained from parental structured interviews at the 2- and 10-year investigations, as described in detail elsewhere.²⁴

Specific IgE analysis

Blood was drawn into SST vacutainer tubes between 10 AM and 2:15 PM and allowed to clot for 1 hour at stable room temperature before centrifugation. Serum was placed in pipettes and stored at -70°C for further analyses. One nurse handled all blood samples, according to standardized procedures.

Serum was analyzed for total IgE by using the UniCAP fluoroenzyme immunoassay (FEIA), according to the manufacturer's instructions (Phadia, Uppsala, Sweden).¹⁵ In the 2-year-old children specific IgE was analyzed by using the panel test Phadiatop Infant (Phadia), including 11 allergens (*Dermatophagoides pteronyssinus*, cat, dog, egg white, cow's milk, peanut, shrimp, timothy, birch, common ragweed, and wall pellitory [*Parietaria judaica*]). The lower limit of detection was 0.1 PAU/L. In the 10-year-old children specific IgE levels were measured by using the panel test Phadiatop in addition to the following allergens: *Dermatophagoides farinae*, German cockroach, rabbit dander, mugwort pollens, molds (*Cladosporium herbarum* and *Alternaria alternata*), and codfish by means of ImmunoCAP, according to the manufacturer's instructions (Phadia). The lower detection limit was 0.1 kUA/L.

Main outcomes

sCD14 levels. The mean value for 2 parallel wells was used.

Confounders/atopic phenotypes

A history of asthma at 10 years of age was defined as at least 2 of the following 3 criteria being fulfilled²⁴: (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 years was defined as a positive Phadiatop result (cutoff, 0.35 kU/L), whereas allergic sensitization at 10 years was defined by a specific IgE concentration of greater than 0.35 kU/L to at least 1 allergen.

Statistical analyses

CD14 genotypes were assessed for Hardy-Weinberg equilibrium with Haploview 3.32.²⁸ Demographic data are presented as counts (percentages) or mean values with SDs. sCD14 levels at all 3 time points were normally distributed, and raw values are used. Differences in mean sCD14 levels for the various *CD14* SNP genotypes were analyzed by using 1-way ANOVA. Each outcome was controlled for confounding by sex, history of asthma (at 10 years), and allergic sensitization by using specific IgE measurements (at both 2 and 10 years of age). For this, an analysis of covariance was applied, defining a confounding effect as changing the B value of the fixed factors (potential confounders) by greater than 20% when excluded from the analysis. *CD14* haplotype analyses were performed with Unphased.²⁹ Genotype-phenotype analysis at both 2 and 10 years was performed with the Pearson χ^2 test. The *P* values reported in the association studies have been corrected according to the Bonferroni method and multiplied by the number of phenotype tests (3 in the present study).

Mean levels of methylation (percentages) were determined at each CpG site and in the CpG island as a whole. The difference in methylation level between age 2 and age 10 years at the defined sites was calculated (methylation level at age 10 years – methylation level age 2 years), yielding a Δ methylation value for every site (Δ -methylation). The value for methylation levels at 10 years was not normally distributed, and the log-transformed methylation values at 2 and 10 years were used in the analyses, whereas the Δ -methylation value (both positive and negative values) was normally distributed and was therefore not transformed.

Changes in methylation from 2 to 10 years (at individual CpG sites and for the whole CpG island) were determined by using the paired-samples *t* test. Single CpG site analyses were corrected for multiple testing *ad modum* Bonferroni (multiplied by number of tests, $n = 87$). Associations between CpG island methylation (2 years, 10 years, and Δ -methylation) and *CD14* genotypes and atopic phenotypes were explored by using ANOVA. Correlation between CpG island methylation level and sCD14 level was tested by using bivariate correlation analysis (the Pearson correlation test).

All statistical tests were performed with SPSS version 14 software (SPSS, Inc, Chicago, Ill). The level of significance was set to .05 (5%).

RESULTS

In the present prospective cohort study the clinical characteristics for all children included are presented in Table II. In the 2 years of age study, 55% were boys, 61% had recurrent bronchial obstruction, and 16% had allergic sensitization. The corresponding percentages at 10 years were 56% boys, 51% with a history of asthma, and 37% with allergic sensitization. *CD14* alleles for tested SNPs were in Hardy-Weinberg equilibrium in both the 2- and 10-year-old populations.

Genetic association study (sCD14 vs genotypes)

Three of the 4 tested SNP genotypes (rs2569191, rs5744455, and rs2569190) were associated with sCD14 levels at birth and 2 years of age. Only rs5744455 was still associated with sCD14 levels at 10 years of age (Table III). Haplotype analyses supported the detected SNP associations; the haplotype including the 3 associated SNP alleles (haplotype G-C-T-C) was most strongly associated with high sCD14 levels early in childhood, whereas the haplotype including the opposite alleles (haplotype A-T-C-C) was likewise most strongly associated with low sCD14 levels early in childhood (Table IV).

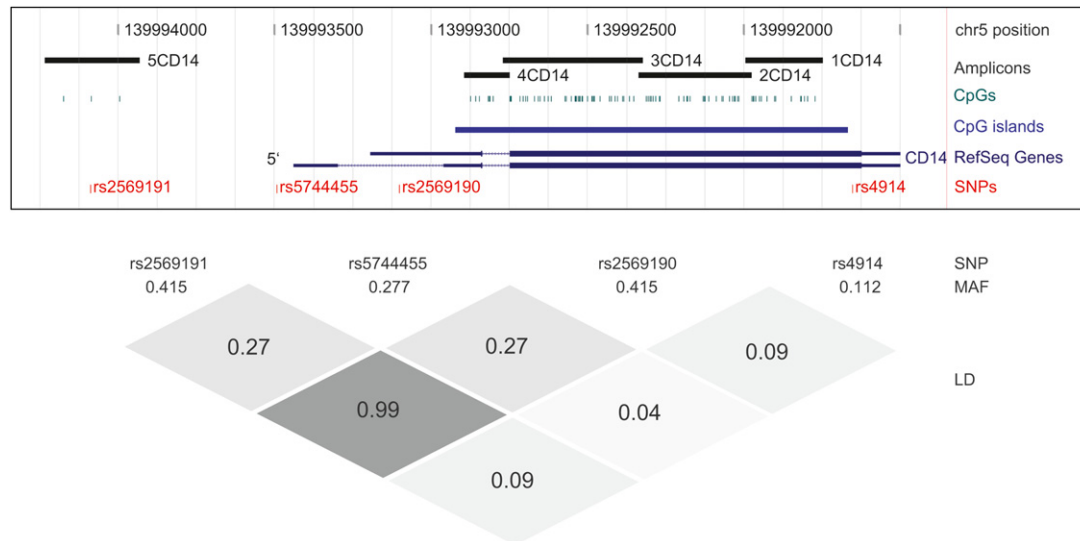


FIG 2. A map of *CD14* showing the location of the SNPs, the amplicons and CpGs in the bisulfite sequencing, and SNP minor allele frequencies with LD plotting.

TABLE I. PCR primer sequences for the methylation assay: Amplicons 1 through 5

Amplicon 1*	Forward	tgtaaacgacggccagctTTTATAGGGATTAGGAAGGGATT
	Reverse	caggaaacagctatgaccAACCTAAACCTCAACCACAACCT
Amplicon 2	Forward	tgtaaacgacggccagctAGTTGTGGTTGAGGTTTAGGTT
	Reverse	caggaaacagctatgaccACCACAAAACCTTACACTTTCCA
Amplicon 3	Forward	tgtaaacgacggccagctGTGTAAAGTTTGTGGTTTGTAGAGG
	Reverse	caggaaacagctatgaccTAAACCTCTACTCTCTCCCA
Amplicon 4	Forward	tgtaaacgacggccagctTTTGGGGAGAGAGTAGAGGT
	Reverse	caggaaacagctatgaccTAAACACTTCCAAAACCTATCC
Amplicon 5	Forward	tgtaaacgacggccagctTTTTTTGTTTGTAAATTGTGAA
	Reverse	caggaaacagctatgaccCTTTCCTCCCAAAATCTAAAAAT

*For amplicon location, see Fig 2.

All SNP associations (genotypes vs sCD14) were tested for confounding by sex in addition to allergic sensitization at 2 years and “history of asthma” and allergic sensitization at 10 years. Neither sex, asthma at 10 years, nor allergic sensitization at 2 or 10 years significantly altered the identified associations (data not shown).

Epigenetic methylation analysis

A total of 87 CpG sites were analyzed, of which 86 CpG sites were located within the intragenic CpG island; the remaining CpG site was the rs2569191 SNP site. There were no significant changes in levels of methylation from age 2 to 10 years at individual CpG sites when correcting for multiple testing (data not shown).

The intragenic CpG island was found to be mainly unmethylated. A small but significant increase from 5.5% to 6.8% in methylation levels was detected from age 2 to 10 years ($P = .001$, Table V). There was no association between Δ -methylation of the intragenic CpG island and any of the tested genotypes or changes in sCD14 levels from 2 to 10 years of age (data not shown). However, a significant correlation between level of methylation and sCD14 levels at 10 years was detected, showing that higher levels of methylation were correlated with lower sCD14 levels at 10 years of age ($P = .013$, Fig 3). No association

between methylation levels and sCD14 levels at 2 years of age was detected.

The rs2569191 CpG site in the *CD14* promoter was, as expected, associated with the rs2569191 genotypes, where having the G allele was associated with methylation ($P < .0001$). Apart from this, there were no significant changes in methylation levels from 2 to 10 years of age (Table VI) and no association between level of methylation or Δ -methylation and the other genotypes or sCD14 levels at 2 or 10 years of age.

Effect on atopic phenotypes

The tested *CD14* genotypes alone were not significantly associated with asthma at 10 years. However, the rs25691910 and rs2569191 genotypes and the G-C-T-C haplotype associated with low sCD14 levels in early life tended toward overrepresentation in children with allergic sensitization at 2 years ($P = .099$, $P = .092$, and $P = .068$, respectively) but not at 10 years of age. There were no significant associations between level of methylation at 2 or 10 years or Δ -methylation and the atopic phenotypes.

DISCUSSION

The present longitudinal study demonstrates that *CD14* genotypes are more closely associated with sCD14 levels at birth

TABLE II. Clinical characteristics of all children included and characteristics of children divided into subpopulations according to genetic association and epigenetic studies

	Babies in genetic association analysis at birth (n = 660)	Children in genetic association analysis at 2 y (n = 346)	Children in genetic association analysis at 10 y (n = 360)	Children in epigenetic analyses (n = 157)	All children included (n = 762)
Sex (male), no. (%)	350 (53)	191 (55)	202 (56)	88 (56)	404 (53)
Parental atopy* (at child's age 10), no. (%)	334 (51), n = 660†	196 (57), n = 346	202 (44), n = 360	85 (56), n = 153	392 (52), n = 758
Allergic sensitization at 2 y, no. (%)	42 (16), n = 257	56 (16), n = 343	56 (17), n = 335	25 (21), n = 121	57 (17), n = 346
History of asthma at 10 y, no. (%)	202 (31), n = 660	181 (52), n = 346	185 (51), n = 360	55 (35), n = 157	249 (33), n = 762
Allergic sensitization at 10 y, n (%)	234 (36), n = 655	125 (36), n = 343	133 (37), n = 358	63 (42), n = 150	268 (36), n = 751
sCD14 levels in cord blood (ng/mL), mean (SD)	703 (203), n = 660	711 (205), n = 259	701 (200), n = 276	706 (199), n = 116	703 (203), n = 660
sCD14 levels at 2 y (ng/mL), mean (SD)	2007 (446), n = 259	1996 (435), n = 346	2002 (438), n = 338	2028 (426), n = 121	1996 (435), n = 346
sCD14 levels at 10 y (ng/mL), mean (SD)	2050 (453), n = 276	2081 (449), n = 338	2066 (445), n = 360	2071 (456), n = 125	2066 (445), n = 360

Values in italics refer to retrospective or prospective data at the given time period and are thus not applicable for the given analyses. The different groups presented (birth, 2 years, 10 years, and epigenetic analysis) overlap; that is, there are children included in more than 1 analysis.

*Parental atopy is defined as parental asthma or rhinoconjunctivitis recorded when the index child was 10 years old.

†The numbers (n) in each data box indicate the number of children with available data for the analysis.

TABLE III. Relationship between serum levels of CD14 and *CD14* genotype

Rs no.	Alternative nomenclature	Genotypes	CD14 cord blood levels (n* = 660)		CD14, 2 y (n = 346)		CD14, 10 y (n = 360)	
			No. (%)	Mean (SD)	No. (%)	Mean (SD)	No. (%)	Mean (SD)
rs4914		CC	500 (79)	700 (207)	267 (80)	1975 (433)	277 (80)	2049 (441)
		CG	123 (19)	707 (193)	62 (19)	2120 (430)	66 (19)	2139 (475)
		GG	11 (2)	735 (177)	3 (<1)	1967 (133)	3 (<1)	2106 (416)
		P value/corrected	.822/NS		.059/NS		.337/NS	
rs2569190	-159CT -260 CT	TT	109 (17)	729 (229)	62 (19)	2100 (445)	63 (18)	2180 (454)
		CT	311 (49)	717 (206)	153 (46)	2033 (468)	159 (46)	2035 (436)
		CC	217 (34)	662 (181)	116 (35)	1908 (361)	122 (35)	2040 (454)
		P value/corrected	.003/.009		.009/.027		.073/NS	
rs5744455	-550CT -651	CC	341 (53)	728 (210)	169 (51)	2073 (447)	173 (50)	2153 (469)
		CT	250 (39)	678 (197)	132 (40)	1945 (419)	142 (41)	1976 (413)
		TT	47 (7)	623 (161)	30 (9)	1860 (356)	29 (8)	1991 (389)
		P value/corrected	.0003/.0009		.007/.021		.001/.003	
rs2569191	-1145GA	GG	108 (17)	730 (230)	62 (19)	2100 (445)	63 (18)	2180 (454)
		GA	309 (49)	720 (209)	153 (46)	2041 (464)	158 (46)	2033 (436)
		AA	216 (34)	660 (177)	115 (35)	1904 (361)	121 (35)	2038 (455)
		P value/corrected	.001/.003		.006/.018		.068/NS	

NS, Not significant.

*n, Number of total subjects included in analysis (varies slightly within the different genotypes because of genotyping errors).

and in early childhood compared with those at 10 years of age and is the first to demonstrate an increase in *CD14* methylation during the same period. Furthermore, an inverse relationship between *CD14* methylation level and sCD14 levels at 10 years of age was demonstrated.

Association analyses

The associations between *CD14* genotypes (in particular the T allele in rs2569190) and sCD14 levels detected in the present study are supported by many,^{6,30} but not all,⁸ *CD14* gene association studies. These inconsistencies might partly be due to inadequate statistical power and differences in ethnicity and study design but might also be related to the complex gene-environment

interactions in the *CD14* gene expression relationship.^{17,31} Hence although several studies suggest that gene-expression associations depend on the stage of disease activity,^{9,18,32} we observed a significant interaction between *CD14* gene expression and age (in childhood). The observed reduced effect of *CD14* polymorphisms on sCD14 levels through childhood is in line with the longitudinal analyses from birth by LeVan et al,⁷ suggesting that *CD14* polymorphisms modulate sCD14 levels up to 1 year of age. Although no associations between genotypes and sCD14 levels were observed until 3 months of age in LeVan et al's study, the present study includes a larger number of subjects at birth than LeVan et al's study (660 vs 387) and follows children up to age 10 years, suggesting that the effect of *CD14* polymorphisms might in fact be present earlier and also last longer than the first year of life.

TABLE IV. Relationship between sCD14 haplotypes and sCD14 levels

Age	Haplotypes				sCD14 levels Mean (SD)	Haplotype test* P value/corrected
	rs2569191	rs5744455	rs2569190	rs4914		
Cord blood	G - C - T - C				520 (41)	.0015/.0045
	A - C - C - C				248 (20)	.89/NS
	A - T - C - C				340 (27)	.00003/.00009
	A - C - C - G				145 (11)	.55/NS
	Rare haplotypes				1 (<1)	—
2 y	G - C - T - C				275 (42)	.002/.006
	A - C - C - C				123 (19)	0.09/NS
	A - T - C - C				189 (29)	.0009/.0027
	A - C - C - G				68 (10)	.04/NS
	Rare haplotypes				1 (<1)	—
10 y	G - C - T - C				282 (41)	.08/NS
	A - C - C - C				129 (19)	.72/NS
	A - T - C - C				198 (29)	.001/.003
	A - C - C - G				72 (10)	.14/NS
	Rare haplotypes				1 (<1)	—

NS, Not significant.

*The individual haplotype test is chosen for the present analysis. This test scores the difference in risk for high or low sCD14 levels between the given haplotype and all the levels of other haplotypes pooled together.

†The number of haplotype alleles tested is lower than the number of individual SNP alleles tested because haplotypes were only made when all SNP alleles were called.

TABLE V. Methylation analyses of the *CD14* gene at 2 and 10 years of age

	Methylation level in intragenic CpG island (n = 153)	Methylation level at CpG site (rs2569191) (n = 121)*
2 y, mean (SD)	5.5% (2.5)	32.3% (31.4)
10 y, mean (SD)	6.8% (3.8)	35.2% (31.1)
Change in methylation from 2-10 y	+1.3% P = .001	+2.9% P = .138

*The amplicon covering the promoter CpG site was difficult to amplify, thus explaining the lower number of subjects included in this analysis.

Epigenetic analyses

The present study demonstrates that the decreasing effect of *CD14* polymorphisms on sCD14 levels is paralleled by a small but significant increase in *CD14* methylation from 2 to 10 years of age. Furthermore, higher *CD14* methylation is correlated with lower sCD14 levels at 10 years. This is the first study, to our knowledge, exploring the relationship between methylation status in the *CD14* gene, gene variation, and gene expression. The present results are in line with studies proposing that methylation patterns develop and change throughout life³³ and also provide support for the belief that epigenetic modifications might affect the relationship between genes and susceptibility to disease in an age-related context. It has been proposed that *CD14* expression is partly regulated by the transcription factor Sp protein, where Sp-3 preferentially binds to the rs2569190 C allele, thereby decreasing *CD14* transcription.⁴ We propose that methylation of the *CD14* gene itself might reduce the differential influence of promoter polymorphisms and their interactions with transcription factors. Thus varying results in previous studies regarding *CD14*

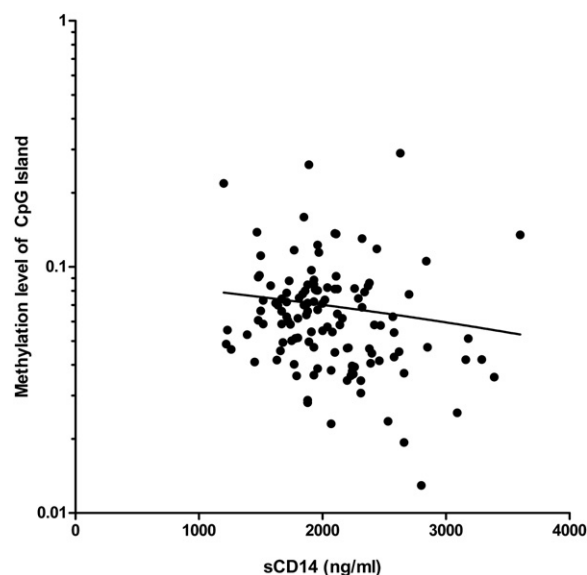


FIG 3. Scatter plot showing correlation between methylation level of the CpG island and sCD14 levels at 10 years. The values shown on the y-axis are the antilogs to the log methylation values. Pearson correlation = -0.224 , $P = .013$.

polymorphism–sCD14 level associations might in part reflect differential methylation status of the *CD14* gene.

Implications for development of disease

The focus of the present study was to explore the relationship between *CD14* genotypes, *CD14* methylation, and sCD14

expression. However, we also found that children with the genotypes/haplotypes associated with low sCD14 values tended to more often have allergic sensitization at 2 years of age but not at 10 years of age. This is indirectly supported by an Australian study detecting association between *CD14* polymorphisms and atopy during midchildhood but not by early adulthood.¹⁹ Together this provides support for the existence of a window of opportunity in early childhood during which the effect of *CD14* polymorphisms on disease is gradually reduced by methylation over time. Thus interactions between gene polymorphisms and gene methylation can thereby modulate development of conditions associated with high or low sCD14 levels.

We found no association between *CD14* methylation and atopic status, suggesting that other external factors influence gene methylation, with examples possibly being tobacco smoke products, endotoxin, or diet. Exploring the effect of environmental factors on methylation was outside the scope of the present study; however, recent studies in mice by Hollingsworth et al²² show that a folate-rich diet during pregnancy results in increased methylation of several specific gene promoters in their offspring, decreasing gene expression and thereby enhancing allergic immune responses in these offspring. Hence further studies exploring the relationship between *CD14* methylation and environmental factors would have consequences reaching into the cause, environmental influences, prevention, and treatment of CD14-related inflammatory conditions.

Strengths and limitations of the present study

The present study includes children through their first 10 years of life, a cohort characterized by a high follow-up rate and well-described phenotypes. The study is therefore particularly well suited for exploring genetic associations during different stages of childhood and is one of very few studies that have analyzed methylation patterns in the same subjects over time. However, because epigenetic studies were not planned from the beginning of the study (1992), we had to use available serum and blood stored at the time of examination for the present research questions. This limited the number of complete and optimal data sets substantially but should not have a major effect on the main results.

The main challenges of the present study were lack of consensus regarding how to report and analyze methylation patterns, and the well-established fact that methylation patterns in genes are organ specific. We examined methylation patterns in whole blood cells without differentiating cell types but could not ascertain whether the methylation patterns would be similar in the different blood cell types or reflect patterns present in hepatocytes (where sCD14 also is produced). Therefore in the planning of future epigenetic studies, selection of pure cell types of relevant tissue for the trait under study should be of high priority.

In conclusion, we demonstrate for the first time that reduced effects of *CD14* gene polymorphisms on sCD14 levels from early to late childhood paralleled an increase in methylation of the *CD14* gene, thus presenting a possible biological explanation for the differential influence of gene polymorphisms on gene transcription and subsequent disease.

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Clinical implications: The epigenetic changes indicated by gene methylation imply gene-environment interactions in early life that can explain reported inconsistencies in the role of *CD14* polymorphisms and the risk of atopic disease in childhood.

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