

DNA Methylation in Inflammatory Genes among Children with Obstructive Sleep Apnea

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Background: Pediatric obstructive sleep apnea (OSA) leads to multiple end-organ morbidities that are mediated by the cumulative burden of oxidative stress and inflammation. Because not all children with OSA exhibit increased systemic inflammation, genetic and environmental factors may be affecting patterns of DNA methylation in genes subserving inflammatory functions.

Methods: DNA from matched children with OSA with and without high levels of high-sensitivity C-reactive protein (hsCRP) were assessed for DNA methylation levels of 24 inflammatory-related genes. Primer-based polymerase chain reaction assays in a case-control setting involving 47 OSA cases and 31 control subjects were conducted to confirm the findings; hsCRP and myeloid-related protein (MRP) 8/14 levels were also assayed.

Measurements and Main Results: Forkhead box P3 (FOXP3) and interferon regulatory factor 1 (IRF1) showed higher methylation in six children with OSA and high hsCRP levels compared with matched children with OSA and low hsCRP levels ($P < 0.05$). In the case-control cohort, children with OSA and high CRP levels had higher log FOXP3 DNA methylation levels compared with children with OSA and low CRP levels and control subjects. IRF1 did not exhibit significant differences. FOXP3 DNA methylation levels correlated with hsCRP and MRP 8/14 levels and with apnea-hypopnea index (AHI), BMI z score, and apolipoprotein B levels. A stepwise multiple regression model showed that AHI was independently associated with FOXP3 DNA methylation levels ($P < 0.03$).

Conclusions: The FOXP3 gene, which regulates expression of T regulatory lymphocytes, is more likely to display increased methylation among children with OSA who exhibit increased systemic inflammatory responses. Thus, epigenetic modifications may constitute an important determinant of inflammatory phenotype in OSA, and FOXP3 DNA methylation levels may provide a potential biomarker for end-organ vulnerability.

Keywords: obstructive sleep apnea; epigenetics; DNA methylation; T regulatory lymphocytes; inflammation

Obstructive sleep apnea (OSA) is characterized by repeated events of partial or complete upper airway obstruction during sleep that lead to disruption of normal ventilation, hypoxemia, and sleep fragmentation. In the last decade, the emergence of increasing obesity rates has led to remarkable increases in the prevalence of OSA and increased awareness of the prominent

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The determinants to phenotypic variance in pediatric obstructive sleep apnea (OSA) are unknown. We explored the hypothesis that epigenetic changes in inflammatory genes may be associated with divergent inflammatory phenotypes in children with OSA.

What This Study Adds to the Field

Children with OSA with elevated systemic inflammatory markers were more likely to display increased DNA methylation in the Forkhead box P3 gene. DNA methylation of this gene was also independently associated with the severity of OSA and with the variance in systemic inflammatory markers, suggesting that epigenetic modifications in selected genes underlie individual susceptibility components.

neurocognitive, behavioral, cardiovascular, and metabolic morbidities associated with this condition (1–5). Although the underlying mechanisms leading to OSA-induced morbidities are likely multifactorial and remain to be fully elucidated, the cumulative burden of oxidative stress and inflammation has emerged as the most likely contributor to the occurrence and magnitude of OSA-associated morbidity (6–8). However, not all children with OSA exhibit increased systemic inflammation (9, 10), suggesting that genetic and environmental factors could play a role in determining the inflammatory phenotype among children with OSA (11–13). Myeloid-related protein (MRP) 8 and MRP 14 are calcium-binding proteins of the S100 family. These proteins form a heterodimeric complex in a calcium-dependent manner and regulate myeloid cell function and control inflammation through activation of the receptor for advanced glycation end products (14, 15). MRP 8/14 has been identified as an important predictor of cardiovascular disease (16, 17), and MRP 8/14 levels are increased in obese children and in children with OSA in a dose-dependent fashion (18).

The field of epigenetics has gained substantial interest in the past few years as a potential mechanism underlying the etiology and phenotypic variation of multiple diseases, such as cancer and metabolic and inflammatory diseases (19–24). Although epigenetic adaptations may alter gene activity and are heritable through many cell divisions, they do not alter the primary DNA sequence. Molecular mechanisms underlying epigenetic alterations of DNA, such as DNA methylation, histone acetylation, and chromatin remodeling, have been extensively studied (22). One of the key epigenetic phenomena, DNA methylation, mainly occurs at CpG dinucleotides and involves the enzymatic addition of a methyl group to the cytosine residue without changing the primary DNA sequence. Such modifications, particularly within regulatory genomic regions such as the promoter region, may alter the

transcriptional state of the gene, thereby modifying its expression and the expression of gene-related products (25, 26). To the best of our knowledge, no study has explored the potential changes in epigenetic DNA methylation of specific genes in children with OSA and whether such changes, if present, are associated with the observed variance in the inflammatory phenotype. DNA methylation levels in specific genes of interest, such as known inflammatory genes, may assist in the assessment of risk for OSA-related complications among children afflicted with this condition. Therefore, we hypothesized that discrepant inflammatory responses in the context of pediatric OSA reflect different patterns of DNA methylation in genes subserving inflammatory functions.

MATERIALS AND METHODS

Subjects

The study was approved by the University of Louisville Human Research Committee (protocol #474.99), and informed consent was obtained from the legal caregiver of each participant. Consecutive children (5–10 yr of age) with a diagnosis of OSA according to polysomnographic criteria were invited to participate in the study. For the screening study aiming to identify specific highly methylated inflammatory genes, we prospectively identified and carefully matched children with OSA for apnea-hypopnea index (AHI), BMI *z* score, age, sex, and ethnicity and allocated them into two groups based on the presence of high or low levels of high-sensitivity C-reactive protein (hsCRP) serum (high hsCRP > 1.50 mg/dl). Of the 87 children with OSA (AHI \geq 1 per h of total sleep time [hrTST]), six children with high hsCRP levels and six children matched for age, sex, BMI *z* score, and AHI with low hsCRP levels were identified and served as the basis of the initial screen for highly methylated inflammatory genes.

In the second phase of the study, a case-control study involving 47 children with OSA and 31 control subjects was conducted to verify in a larger cohort whether the highly methylated genes identified from the screening study would confirm the differences identified during the screening process. Age-, sex-, and ethnicity-matched healthy, nonsnoring children without OSA who underwent overnight polysomnography as part of a community-based study were invited to participate. Children were excluded if they had known diabetes or prediabetes (<http://www.diabetes.org/pre-diabetes/pre-diabetes-symptoms.jsp>) or any defined genetic abnormality or underlying systemic disease or if they were within a month from any acute infectious process.

Overnight Polysomnography

Polysomnography was conducted and scored as previously reported (27–30). Central, obstructive, and mixed apneic events were counted. Obstructive apnea was defined as the absence of airflow with continued chest wall and abdominal movement with duration of at least 2-second breaths. Hypopneas were defined as a decrease in oronasal flow of 50% or greater with a corresponding decrease in SpO₂ of 4% or more and/or an arousal. The obstructive apnea hypopnea index was defined as the number of obstructive apneas and hypopneas per hour of total sleep time. Arousals were defined according to the American Academy of Sleep Medicine Scoring Manual (31, 32). Mild OSA was defined by the presence of an obstructive AHI \geq 1 per hrTST and AHI < 5 per hrTST, and moderate to severe OSA was defined as AHI \geq 5 per hrTST. Control subjects were nonsnoring children who had AHI < 1 per hrTST.

Anthropometry

Children were weighed in a calibrated scale to the nearest 0.1 kg, and height (to 0.1 cm) was measured with a stadiometer (Holtain, Crymch, UK). Body mass index (BMI) was calculated, and BMI *z* score was computed using CDC 2000 growth standards (www.cdc.gov/growthcharts) and online software (www.cdc.gov/epiinfo). Children with a BMI *z* score above 1.04 (85th percentile) were considered as overweight children, and children with a BMI *z* score above 1.65 were considered as obese.

Sphygmomanometry

Arterial blood pressure was measured noninvasively in all children using an automated mercury sphygmomanometer (Welch Allyn, Skaneateles Falls, NY) at the brachial artery using a guidelines-defined appropriate cuff size on the nondominant arm (33). Blood pressure measurements were made in triplicate in the morning immediately after awakening. Systolic and diastolic blood pressures were calculated as the mean values, and mean blood pressure was calculated.

DNA Methylation Profile and FOXP3 DNA Methylation Levels

Genomic DNA was extracted from blood samples using the QIAamp Spin Column protocol (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. DNA samples were frozen at -80°C until assay. DNA methylation profiling of a panel of 24 inflammatory-associated genes was measured using the EpiTech methyl quantitative PCR (qPCR) array according to the manufacturer's instructions (SABiosciences, Qiagen, Chatsworth, CA). Briefly, the method is based on the detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (34, 35). After digestion, the remaining DNA is quantified by qPCR in each individual enzyme reaction using primers that flank a promoter region of interest. The relative fractions of hypermethylated, intermediately methylated, and unmethylated DNA are determined by comparing the amount in each digest with that of mock (no enzyme added) digest as a control. Based on our findings in this first phase of the study, we conducted quantitative PCR primer-based DNA methylation assays to confirm the methylation patterns of the gene of interest using specific primers (NM_0140089; SABiosciences, Qiagen) in a case-control setting involving 47 OSA cases and 31 control subjects. The cutoffs for FOXP3 DNA hypermethylation were defined as 24 and 33%, which corresponded to the mean methylation values +1 and +2 SD among non-OSA control subjects. All measures were performed in duplicate, with a correlation coefficient for the duplicates of $r = 0.987$ and an average coefficient of variation for pair sets of 1.1%.

FOXP3 DNA Methylation Pyrosequencing

To quantify the percentage of FOXP3 DNA methylation within individual CpG sites, bisulfite-converted DNA was sequenced using a pyrosequencing system (PSQ 96HS; Biotage, Uppsala, Sweden) as described previously (36). This assay was designed to target the first intron of the FOXP3 gene (Assay ID: ADS 783, Human FOXP3, intron 1; Ensembl Transcript ID: ENST00000376207, EpigenDX, MA), which includes 11 CpG sites in a Treg-specific DNA demethylation region (TSDR) (37, 38). This region was selected because FOXP3 TSDR methylation status plays a critical role not only in the regulation of inflammatory processes but also in the determination of the characteristics and development of Treg lymphocytes (37, 38). In brief, this technique is a sequencing-by-synthesis method that quantitatively monitors the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal. After bisulfite treatment and PCR, the degree of methylation of each CpG position in a sequence was determined from the ratio of T (thymine) and C (cytosine). We included a non-CpG cytosine in the region for pyrosequencing because it provides the internal control of the completeness of bisulfite treatment, the major criterion for reliability of DNA methylation analysis. PCR reactions were performed with HotStarTaq Mater Master Mix kit (Qiagen). The target CpGs were evaluated by converting the resulting pyrograms into numerical values for peak heights and calculating the average of all CpG sites analyzed (*see* Figure E1 in the online supplement).

Serum Levels of hsCRP, MRP 8/14, and Lipids

Fasting blood samples were drawn by venipuncture in the morning after the sleep study. Blood samples were immediately centrifuged and frozen at -80°C until assay. Plasma MRP 8/14 levels was measured using a commercial ELISA kit (ALPCO Diagnostics, Salem, NH for MRP 8/14) following the manufacturer's instructions. The MRP 8/14 assay has a sensitivity of 0.4 $\mu\text{g/ml}$. The interassay and intraassay coefficients of

TABLE 1. CHARACTERISTICS OF CHILDREN WITH OBSTRUCTIVE SLEEP APNEA AND HIGH OR LOW HIGH-SENSITIVITY C-REACTIVE PROTEIN

	OSA with High hsCRP (n = 6)	OSA with Low hsCRP (n = 6)
Age, yr	7.3 ± 2.7	8.5 ± 1.4
Male, %	50	66.6
BMI z score	2.55 ± 0.61	2.25 ± 0.23
AHI, events/h	15.7 ± 8.7	11.8 ± 10.5
hsCRP, mg/dl	3.13 ± 1.40	1.05 ± 0.42*

Definition of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; hsCRP = high-sensitivity C-reactive protein; OSA = obstructive sleep apnea;

* $P < 0.01$, OSA with low hsCRP vs. OSA with high hsCRP.

variability for MRP 8/14 were 6.4 and 4.8%, respectively. Serum levels of lipids, including total cholesterol, high-density lipoprotein, calculated low-density lipoprotein, and triglycerides were assessed with a Flex reagent cartridge (Date Behring, Newark, DE). Apolipoprotein B levels were measured by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). High sensitive CRP levels were measured within 2 to 3 hours after collection using the Flex reagent Cartridge (Date Behring), which is based on a particle-enhanced turbidimetric immunoassay technique. This method has a detection level of 0.05 mg/dl and exhibits linear behavior up to 255 mg/dl, with intraassay and interassay coefficients of variability of 9 and 18%, respectively. High hsCRP levels were defined as CRP level above 1.5 mg/dl, corresponding to the 95th percentile among non-OSA control subjects.

Statistical Analysis

Data are expressed as mean ± SD. Significant differences within groups were analyzed using ANOVA for continuous variables and χ^2 tests for categorical variables. Bonferroni corrections were applied for multiple comparisons. Data that were not normally distributed were logarithmically transformed. Because obesity could be expected to contribute to increases in FOXP3 DNA methylation levels as a confounding variable, we performed ANCOVA to examine whether there were significant differences between groups while adjusting for BMI z score as a covariate. Spearman's correlation analyses were conducted to examine

potential associations between FOXP3 DNA methylation levels and other variables. Univariate and stepwise multivariate linear regression analyses were then conducted while treating FOXP3 DNA methylation level as a dependent variable in relation to AHI and other covariates. Statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL). All P values reported are two-tailed with statistical significance set at < 0.05 .

RESULTS

Study Population

In the first phase of study, which identified highly methylated inflammatory genes in OSA children with high CRP levels, six children with OSA and high CRP levels and six children with OSA and low CRP levels who were matched for age, sex, BMI z score, and AHI were tested (Table 1). Seventy-eight children were then prospectively recruited to confirm the previously identified highly methylated gene(s) using a case control design. Based on the presence of high and low hsCRP levels, the presence or absence of habitual snoring, and AHI cut-off values in the polysomnography, 31 children in the second group had OSA and high hsCRP levels, 16 children had OSA and low hsCRP levels, and 31 were control subjects. The demographic, polysomnographic, and biochemical characteristics of the three groups are shown in Table 2. Mean age, sex, and ethnic distribution were similar across the three groups ($P > 0.05$). However, log FOXP3 DNA methylation patterns, log MRP 8/14 levels, and log hsCRP levels showed significant group differences (Table 2).

DNA Methylation Profiling and FOXP3 DNA Methylation Levels

The heatmap for DNA methylation profile, which included 24 inflammatory-related genes in the six high hsCRP- and low hsCRP-matched children with OSA, is shown in Figure 1. These initial screening experiments revealed that FOXP3 and IRF1 genes were more likely to be highly methylated in children with OSA and high hsCRP levels (children with OSA and high

TABLE 2. DEMOGRAPHIC, RESPIRATORY, AND METABOLIC CHARACTERISTICS OF CHILDREN WITH OBSTRUCTIVE SLEEP APNEA AND MATCHED CONTROL SUBJECTS*

	OSA with High hsCRP (n = 31)	OSA with Low hsCRP (n = 16)	Control (n = 31)
Age, yr	8.20 ± 1.85	8.07 ± 1.65	8.56 ± 1.42
Male, %	54.8	75.0	58.1
Caucasian, %	64.5	62.5	74.2
BMI z score	2.03 ± 0.77 ^{†,‡}	0.36 ± 1.52 [§]	1.11 ± 1.01
Systolic blood pressure, mm Hg	107.6 ± 8.9	99.4 ± 9.57 [¶]	104.2 ± 9.07
Diastolic blood pressure, mm Hg	64.2 ± 6.9	61.1 ± 5.4	60.3 ± 7.3
Apnea hypopnea index, events/h	6.79 ± 9.80 ^{**}	3.62 ± 2.35	0.45 ± 0.26
Sa _{O₂} nadir, %	86.4 ± 9.40	85.8 ± 10.8	91.0 ± 6.75
Total cholesterol, mg/dl	174.1 ± 37.0	173.9 ± 28.2	157.3 ± 25.4
HDL cholesterol, mg/dl	50.4 ± 10.8	56.6 ± 9.2	50.6 ± 11.8
LDL cholesterol, mg/dl	105.8 ± 30.7	104.2 ± 25.6	90.9 ± 22.2
Tryglycerides, mg/dl	89.1 ± 50.9	65.1 ± 21.9	78.2 ± 47.4
Log MRP 8/14	0.06 ± 0.29 [†] (1.42 ± 0.69 μg/ml) ^{††}	-0.22 ± 0.25 [‡] (0.69 ± 0.40 μg/ml)	-0.19 ± 0.31 (0.81 ± 0.67 μg/ml)
Log hsCRP	0.37 ± 0.74 [†] (1.90 ± 1.48 mg/dl) ^{††}	-0.16 ± 0.09 [‡] (0.31 ± 0.03 mg/dl)	-0.77 ± 0.57 (0.58 ± 0.66 mg/dl)
Log FOXP3 DNA methylation	3.03 ± 0.60 [†] (24.2 ± 12.1%) ^{††}	2.58 ± 0.55 [¶] (15.5 ± 9.5%)	2.61 ± 0.58 (15.9 ± 8.6%)

Definition of abbreviations: BMI = body mass index; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; MRP = myeloid-related protein.

* All data are expressed as mean ± SD.

[†] $P < 0.01$, control subjects vs. children with OSA and high hsCRP.

[‡] $P < 0.01$, children with OSA and low hsCRP vs. children with OSA and high hsCRP.

[§] $P < 0.05$, control subjects vs. children with OSA and low hsCRP.

^{||} $P < 0.01$, differences between three groups (ANOVA).

[¶] $P < 0.05$, children with OSA and low hsCRP vs. children with OSA and high hsCRP.

^{**} $P < 0.05$, control subjects vs. children with OSA and high hsCRP.

^{††} Actual values and units.

TABLE 3. UNIVARIATE AND MULTIVARIATE ANALYSES BETWEEN APNEA-HYPOPNEA INDEX AND FORKHEAD BOX P3 DNA METHYLATION AND COVARIATES

Independent Variables	FOXP3 DNA Methylation*					
	Univariate			Stepwise Multivariate		
	β	SE	P Value	β	SE	P Value
Age	0.049	0.036	0.170	–	–	–
Sex	0.763	0.118	<0.001	0.789	0.114	<0.001
Race	0.132	0.069	0.076	–	–	–
BMI z score	0.037	0.046	0.429	–	–	–
Apolipoprotein B	0.006	0.003	0.071	–	–	–
AHI*	0.227	0.100	0.027	0.233	0.098	0.021

Definition of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; FOXP3 = Forkhead box P3.

*Data were log transformed.

hsCRP levels vs. children with OSA and low hsCRP levels: FOXP3: $41.4 \pm 30.4\%$ vs. $12.9 \pm 1.3\%$, $P < 0.05$; IRF1: $26.6 \pm 16.0\%$ vs. $8.1 \pm 5.5\%$, $P < 0.05$).

Therefore, in a subsequent verification stage, we conducted qPCR assays with specific primers for the two candidate genes. Although no significant differences emerged for IRF1, children with OSA and high hsCRP levels had higher log FOXP3 DNA methylation levels compared with children with OSA and low hsCRP levels and control subjects (children with OSA and high hsCRP levels vs. children with OSA and low hsCRP levels: 3.03 ± 0.60 vs. 2.58 ± 0.55 with log-transformed FOXP3 DNA methylation levels, $24.2 \pm 12.1\%$ vs. $15.5 \pm 9.5\%$ with actual FOXP3 DNA methylation levels, $P < 0.05$) (Table 2). Log FOXP3 DNA methylation levels were not significantly different between children with OSA and low hsCRP levels and control subjects. Moreover, the percentage of cases with increased FOXP3 DNA methylation, which corresponded to the mean methylation values plus 1 or 2 SD among children without OSA, was highest in children with OSA and high hsCRP levels (children with OSA and high hsCRP vs. children with OSA and low hsCRP vs. control subjects [+1 SD definition], 54.8 vs. 25.0 vs. 19.4%, $P < 0.01$; children with OSA and high hsCRP vs. children with OSA and low hsCRP vs. control subjects [+2 SD definition], 32.3 vs. 1.25 vs. 3.2%, $P < 0.01$) (Figure 2). Similar findings emerged for OSA severity (moderate to severe OSA vs. mild OSA vs. control [+1 SD definition]: 62.5 vs. 41.0 vs.

19.4%, $P < 0.05$; moderate to severe OSA vs. mild OSA vs. control [+2 SD definition]: 50.0 vs. 20.5 vs. 3.2%, $P < 0.01$) (Figure 3). Because obesity could be expected to contribute to increases in FOXP3 DNA methylation levels as a confounding variable, we compared FOXP3 DNA methylation levels in overweight and normal weight children. No significant differences emerged in FOXP3 DNA methylation levels in these two BMI z score assigned groups (overweight children vs. normal weight children: 2.8 ± 0.61 vs. 2.6 ± 0.61 with log-transformed FOXP3 DNA methylation levels, $20.4 \pm 11.4\%$ vs. $16.8 \pm 9.9\%$ with actual FOXP3 DNA methylation levels, $P > 0.05$). The proportion of cases in which FOXP3 DNA hypermethylation was present did not differ in the two BMI z score categories (overweight children vs. nonoverweight children: 37.3 vs. 29.6%, $P > 0.05$). Furthermore, FOXP3 DNA methylation levels were significantly different in children with OSA with high and low CRP and control subjects (ANCOVA; $P < 0.05$) after adjusting for BMI z score as a covariate. Similar findings emerged for OSA severity (ANCOVA; $P < 0.05$; Table 3).

FOXP3 DNA Pyrosequencing Analysis

To quantify the percentage of FOXP3 DNA methylation in the first intron of the FOXP3 gene, we examined 11 CpG sites within the TSDR (see Table E2 in the online supplement) and performed pyrosequencing analysis in 10 children with OSA and 10 matched control subjects. The second (–2322 bp from ATG) and tenth positions (–2281 bp from ATG) of the 11 CpG sites within the intronic 1 region were significantly different between OSA children and control subjects (children with OSA vs. control subjects [second position]: 92.02 ± 2.10 vs. 86.9 ± 3.48 , $P < 0.01$; children with OSA vs. control subjects [ninth position]: 89.22 ± 2.62 vs. 86.48 ± 2.48 , $P < 0.05$), thereby confirming the hypermethylation findings described above.

Correlations between FOXP3 DNA Methylation Levels, Inflammatory Markers, Serum Lipids, and Polysomnographic Measures

A significant linear correlation between log FOXP3 DNA methylation levels and inflammatory markers, including log CRP and log MRP 8/14 levels (CRP: $r = 0.362$, $P < 0.01$; MRP 8/14: $r = 0.304$, $P < 0.001$), emerged (Figure 4). Moreover, log FOXP3 DNA methylation levels were not only correlated with log AHI ($r = 0.230$, $P < 0.05$) (Figure 5A) but were also significantly

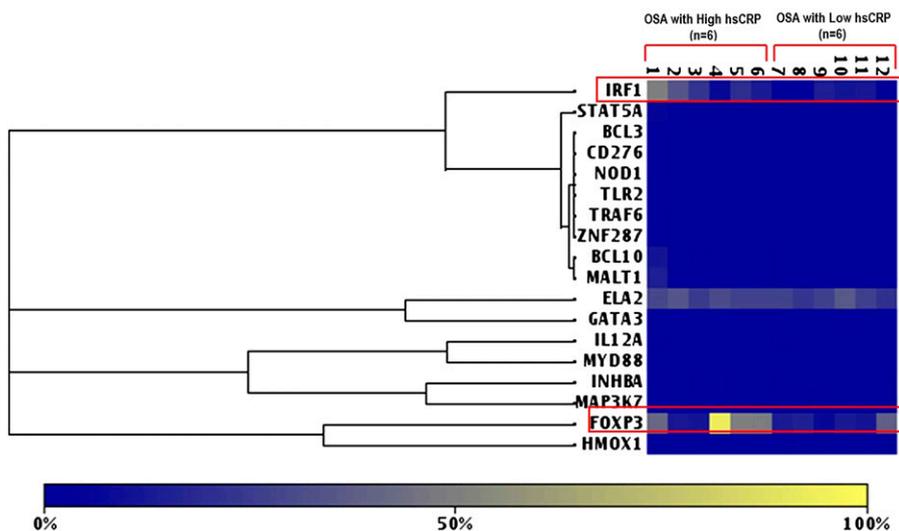


Figure 1. Semi-DNA methylation array data. Heatmap of DNA methylation profile that includes 24 inflammatory related genes in children with obstructive sleep apnea (OSA) and high levels of high-sensitivity C-reactive protein (hsCRP) ($n = 6$) and matched children with OSA and low hsCRP levels ($n = 6$) (see Table E1).

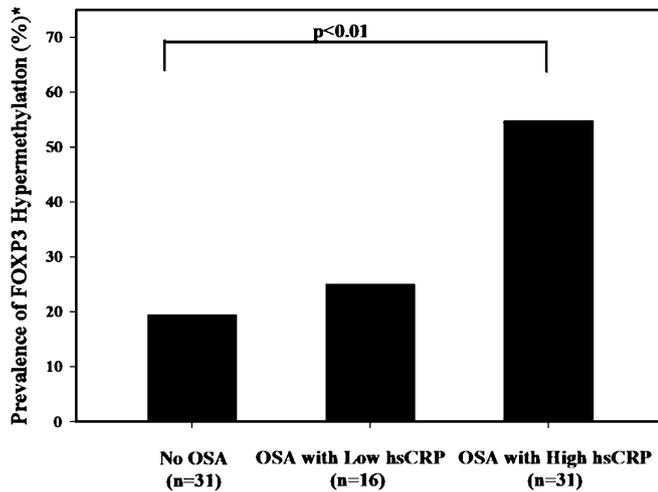


Figure 2. Prevalence of Forkhead box P3 (FOXP3) DNA hypermethylation in children with obstructive sleep apnea (OSA) and control subjects. *FOXP3 DNA hypermethylation is defined as > 24%. hsCRP = high-sensitivity C-reactive protein.

associated with BMI z score ($r = 0.2684$, $P < 0.05$) (Figure 5B) and apolipoprotein B levels ($r = 0.422$, $P < 0.01$) (Figure 4C). However, Sa_{O_2} nadir was not statistically significant ($r = -0.213$, $P = 0.087$). To further explore the independent predictors of log FOXP3 DNA methylation levels in children, we performed stepwise regression analyses (Table 3). In the initial univariate analysis, log FOXP3 DNA methylation levels exhibited significant correlations with sex ($\beta \pm SE$, 0.763 ± 0.118 ; $P < 0.001$) and log AHI ($\beta \pm SE$, 0.227 ± 0.100 ; $P < 0.05$). In the stepwise multiple regression model, AHI was independently associated with FOXP3 DNA methylation levels ($\beta \pm SE$, 0.233 ± 0.098 ; $P < 0.05$) (Table 3), which accounted for 54.7% of the variance after controlling for age, sex, race, BMI z score, and apolipoprotein B.

DISCUSSION

In the present study, we report that the FOXP3 gene exhibits a higher probability for high methylation levels in children with OSA who manifest an inflammatory phenotype. The prevalence of FOXP3 DNA hypermethylation was dose-dependently increased in relation to the severity of OSA, as defined by the AHI. Moreover, FOXP3 DNA methylation levels were not only correlated with inflammatory biomarkers and serum lipids, such as hsCRP, MRP 8/14, and apolipoprotein B, but were also associated with AHI and BMI z scores. However, even after adjusting for all potential confounding factors, AHI was positively and independently associated with FOXP3 DNA methylation levels in the stepwise multiple regression models (Table 3).

Considering the ever-increasing body of evidence, it has become clear that OSA should be viewed as a chronic and low-grade systemic inflammatory disease in children and adults (12, 13). hsCRP, which is produced in the liver through IL-6 activity, is a robust biomarker for underlying systemic inflammation, serves as a relatively good predictor for future cardiovascular disease (39, 40), and has been shown to participate in atheromatous lesion formation through induction and enhanced expression of adhesion molecules (41). Considering the compelling evidence indicating that OSA confers incremental cardiovascular morbidity risk independent from other confounders, it is not surprising that increased levels of hsCRP have been reported in adults (42–44) and children with OSA (45, 46) and that such levels were reduced after OSA treatment (47, 48). However, not

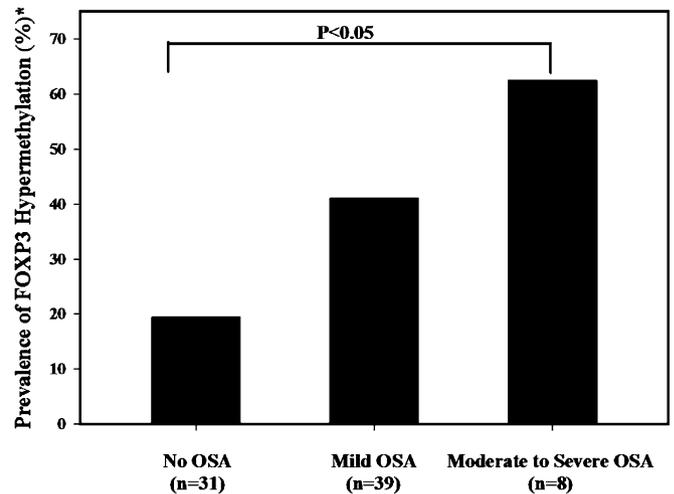


Figure 3. Prevalence of Forkhead box P3 (FOXP3) DNA hypermethylation in children according to the categorical severity of obstructive sleep apnea (OSA). *FOXP3 DNA hypermethylation was defined as > 24%.

all studies in adults (49, 50) or in children (9, 10) have confirmed the putative association between hsCRP levels and OSA, suggesting that interactions between the severity of OSA and lifestyle and environmental conditions and genetically determined variance may influence the individual magnitude of resultant inflammatory responses as induced by OSA (13). Furthermore, we have previously shown that the magnitude of hsCRP increases in the context of pediatric OSA appears to be a major determinant of neurocognitive morbidity (5), thereby emphasizing the importance of the inflammatory phenotype in the context of end-organ vulnerability.

Dyugovskaya and colleagues (51) reported that CD4 and CD8 T cells of patients with OSA undergo phenotypic and functional changes and acquire cytotoxic activity, with a shift in CD4 and CD8 T lymphocytes toward a Th1 cytokine dominance. Conversely, the Th2 cytokine IL-10 was negatively correlated with the severity of OSA in T lymphocytes, whereas TNF- α was positively correlated with AHI (52). Similarly, studies from our laboratory have shown that plasma IL-10 levels in children with OSA are reduced with reciprocal increases in IL-6 (53). Thus, OSA seems to shift the Th1:Th2 balance to a Th1 predominance. FOXP3 is one of four members of the FOXP superfamily of forkhead transcription factors. Although the precise control mechanism has not been established, FOX proteins belong to the forkhead/winged-helix family of transcriptional regulators and are presumed to exert control via similar DNA binding interaction during transcription (54). The gene encoding for Foxp3 is a key transcription factor in T-cell lymphocyte lineage differentiation, where it regulates a subset of regulatory T cells (Tregs) and plays a major role in their development (55). Tregs are a small subset of T cells, usually constituting only 5 to 15% of the peripheral CD4+ T-cell compartment in humans (56). As such, Foxp3 is a critically important regulator of the Th1 and Th2 cytokine balance (56, 57). Although various signals that affect the expression of FOXP3 have been identified, the precise mechanisms by which the expression of this protein is controlled in Tregs are not well understood. Generally, Tregs control effector T-cell responses and influence the activities of cells in the innate immune system (56). A precise understanding of the immunosuppressive mechanism of Treg cells remains incomplete, even though there is increasing evidence that Treg cells modulate the secretion of immunosuppressive soluble factors, such as IL-9, IL-10, and TGF- β .

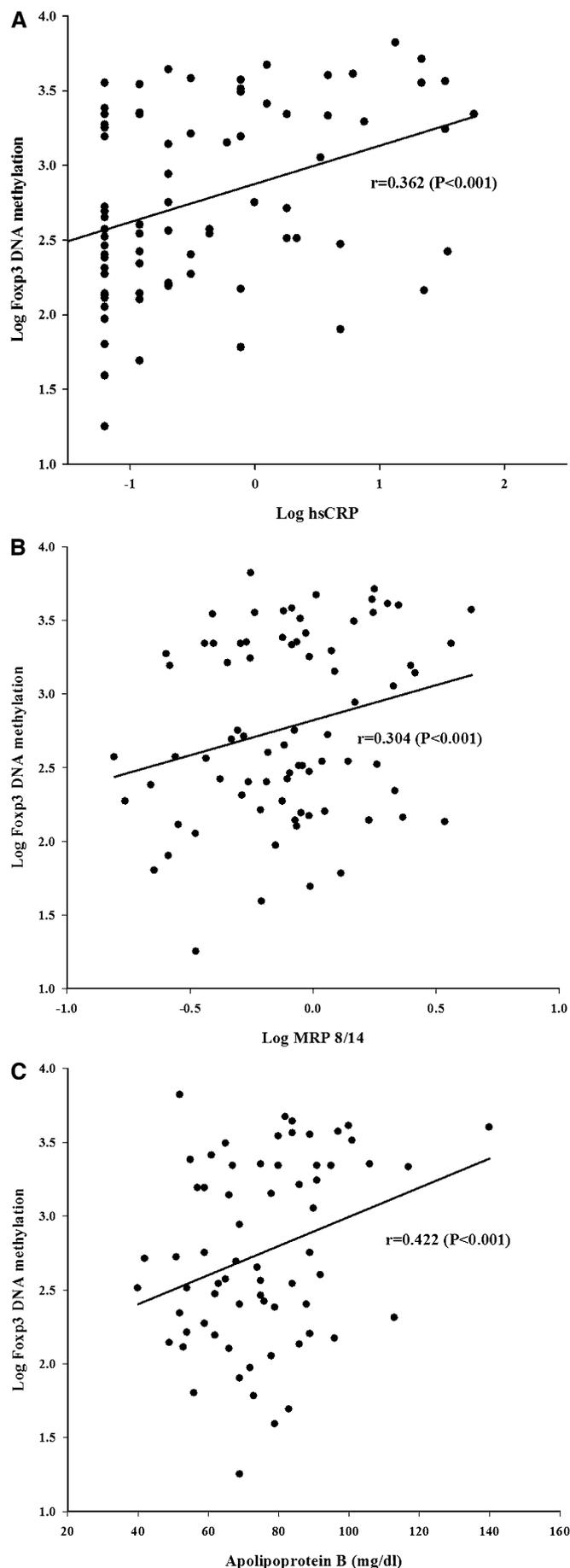


Figure 4. Correlation between log high-sensitivity C-reactive protein (hsCRP), myeloid-related protein (MRP) 8/14, and apolipoprotein B levels and log Forkhead box P3 (FOXP3) DNA methylation levels in children with obstructive sleep apnea and control subjects. (A) Correlation between serum log hsCRP levels and log FOXP3 DNA methylation levels. (B) Correlation between plasma log MRP 8/14 levels and log FOXP3 DNA methylation levels. (C) Correlation between serum apolipoprotein B and log FOXP3 DNA methylation levels.

In the present study, we found that FOXP3 and IRF1 genes were significantly more likely to be highly methylated in a small group of children with OSA who exhibited marked increases in hsCRP. Both of these genes are involved in the development and regulation of T-cell subpopulations. Moreover, FOXP3 DNA methylation levels were not only confirmed in a larger cohort but also dose dependently increased as a function of the severity of OSA and showed independent associations with AHI. Accordingly, we postulate that high FOXP3 DNA methylation may favor the down-regulation of Foxp3 protein expression and thus reduce the number of Tregs, thereby favoring an imbalance of Th1/Th2 cytokines in pediatric OSA. Further studies are required to substantiate this hypothesis and to assess the functional and outcome significance of FOXP3 gene methylation patterns in the context of OSA in children.

Epigenetic modifications are being increasingly identified as imposing lifelong locus-specific changes acting at the single-gene level and are capable of reprogramming vast areas of the genome if needed. The epigenome is therefore the result of interactions between a given developmental genetic asset and the lifetime action of the environment (58). The main environmental factors able to cause this extended effect on the genome are not well characterized, but metabolic determinants of epigenetic alterations are emerging as important effectors (58). Indeed, the concurrence of metabolic events and epigenetic changes in gene expression has been recorded. Recent studies showed that several epigenetic markers, such as histone acetylation and DNA methylation in CpG dinucleotides, have been reported at the FOXP3 locus. In particular, CpG dinucleotides at the FOXP3 locus are methylated in naive CD4+CD25- T cells and TGF- β -induced adaptive Tregs, whereas they are completely demethylated in natural Tregs (57, 59). Methylation of CpG residues represses FOXP3 expression, whereas complete demethylation is required for stable FOXP3 expression (60). Furthermore, recent evidence indicates that induction and stabilization of FOXP3 expression are under epigenetic control, suggesting that selective interference with the underlying chromatin remodeling mechanisms might enable development of future therapeutic strategies in various diseases (25, 61). Another important epigenetic regulator is hypoxia. Indeed, epigenetics seem to play a crucial role in the cellular response to hypoxia, may closely interact with the hypoxia-induced transcription factor family, or may contribute to the maintenance of a hypoxia-adapted cellular phenotype even after hypoxia-induced transcription factor has initiated and activated many of the immediate cellular hypoxic response pathways. Indeed, hypoxia can induce hypermethylation or demethylation of specific genes, the latter via activation of specific demethylases (62–65). However, the impact of intermittent hypoxia methylation remains unknown. Finally, published evidence supports the finding that global DNA hypermethylation is associated with adverse cardiovascular outcomes (66), and the current study supports this assumption, particularly when considering the previously reported association between hsCRP and neurocognitive dysfunction and that of MRP 8/14 and hsCRP in endothelial dysfunction (5, 18). Thus, considering the morbid consequences

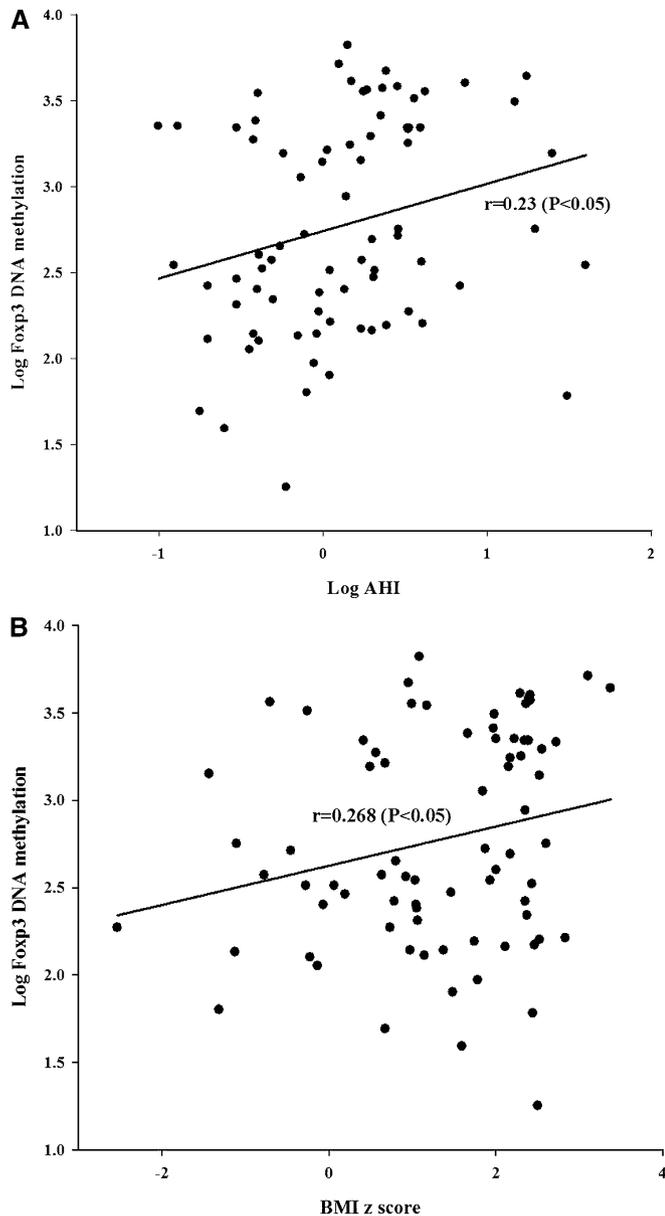


Figure 5. Correlation between apnea-hypopnea index (AHI), body mass index (BMI) z score, and Forkhead box P3 (FOXP3) DNA methylation levels in children. (A) Correlation between AHI and FOXP3 DNA methylation levels. (B) Correlation between BMI z score and FOXP3 DNA methylation levels.

of OSA and the potential impact of this condition on the epigenetic regulation of gene networks, there is a need to further explore the potential targets aiming to identify and reduce susceptibility to this disease.

Several limitations in the present study must be acknowledged. First, we did not measure specific T-cell lymphocyte subpopulations or assess whether OSA is associated with specific cytokine signatures to elucidate the potential links between increased FOXP3 DNA methylation levels and phenotypic manifestations in pediatric OSA. However, hsCRP and MRP 8/14, which have been previously identified as important inflammatory markers associated with OSA in children (18), exhibited significant relationships with FOXP3 DNA methylation levels, indicating that the latter could play an important role in the regulation of the inflammatory response in the context of OSA. Second, we did not

examine other markers of epigenetic modifications (67), such as histone acetylation, chromatin remodeling, and activation of modulating enzymes, all of which may provide valuable insights into the mechanisms of epigenetic alterations associated with OSA in children and add to the relationships between OSA and its phenotypes. Third, we did not explore FOXP3 DNA methylation levels in a longitudinal fashion to examine the possibility of accelerated increases or reductions in FOXP3 DNA methylation levels over time among patients with OSA and the effect of therapy. Finally, our study does not elucidate whether the increases in FOXP3 DNA methylation are a cause or a consequence of OSA or whether these observations represent an epiphenomenon. However, we show that specific FOXP3 intron 1 CpG islands were differentially methylated in OSA, and therefore, it will be important to examine in future studies whether such putative sites play a role in the regulation of the inflammatory phenotype pertaining to OSA and to explore the methylation state of regulatory regions in Th1 and Th2 genes. Similarly, we do not know whether FOXP3 DNA methylation levels are a marker of the cumulative oxidative and inflammatory burden through life or if FOXP3 DNA methylation plays an active pathogenetic role in the risk for adverse outcomes. Notwithstanding such limitations, the current findings provide initial exciting observations on the presence of selective epigenetic DNA modifications in the context of OSA and support the need for expanded exploration of this promising area.

In summary, IRF1 and FOXP3 genes were more likely to exhibit increased methylation in children with OSA who manifest increased inflammatory responses, suggesting that epigenetically mediated down-regulation of specific T-cell lymphocyte subpopulations, such as Tregs, may be an important determinant of the inflammatory and morbidity phenotype in OSA. Furthermore, this study shows that FOXP3 DNA methylation levels do not only correlate with inflammatory markers but are also independently associated with AHI and thus may serve as a potential biomarker of vulnerability in children with OSA. The clinical implications of FOXP3 DNA methylation in pediatric OSA merit future confirmatory and mechanistic studies in larger cohorts.

Author disclosures are available with the text of this article at www.atsjournals.org.

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