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Original Article

The methylation of the *LEPR/LEPROT* genotype at the promoter and body regions influence concentrations of leptin in girls and BMI at age 18 years if their mother smoked during pregnancy

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Abstract: To determine whether DNA methylation (DNA-M) of the leptin receptor genotype (*LEPR/LEPROT*) links gestational smoking and leptin serum levels and BMI later in life, we focused on female offspring, 18 years of age, from the Isle of Wight Birth Cohort (IOWBC). Leptin binds to the leptin receptor encoded by the *LEPR/LEPROT* genotype. Using general linear models, we tested a two-stage model. First, we investigated whether single nucleotide polymorphisms (SNPs) acting as methylation quantitative trait loci (methQTLs) depending on gestational smoking were related to differentially methylated cytosine-phosphate-guanine (CpG) sites. In stage 2, we tested whether the selected CpG sites, in interaction with other SNPs (modifiable genetic variants, modGV), are associated with serum leptin and BMI (stage 2). Children from the IOWBC were followed from birth to age 18. Information on gestational smoking was gathered upon delivery. SNPs tagging *LEPR* and *LEPROT* genes were genotyped. Data on *LEPR/LEPROT* DNA-M and leptin were obtained from blood samples drawn at age 18; to determine BMI, height and weight were ascertained. Blood samples were provided by 238 girls. Of the 21 CpG sites, interactions between gestational smoking and SNPs were detected for 16 CpGs. Methylation of seven of the 16 CpGs were, in interaction with modGVs, associated with leptin levels at age 18 years. Two CpGs survived a multiple testing penalty and were also associated with BMI. This two-stage model may explain why maternal smoking has a long-term effect on leptin levels and BMI in girls at age 18 years.

Keywords: LEPR, LEPROT, leptin, CpG sites, *in utero* smoking exposure, rs12059300, BMI

Introduction

Leptin, a 16 kDa pleiotropic cytokine, is associated with obesity via appetite and energy expenditure regulation [1] and with wheezing, asthma, and allergic responses [2-5]. Associations with the latter outcomes motivated us to better understand which genetic, epigenetic, and environmental factors regulate leptin levels in the body. Previous studies have shown that a single nucleotide polymorphism (SNP), 2548 G>A (rs7799039), on the leptin (LEP) gene was associated with increased plasma and serum leptin levels in the body [6-10]. Given that leptin binds to the leptin receptor (which is encoded by the leptin receptor gene, *LEPR*) [11, 12], it is important to also consider the role of the *LEPR* gene on circulating leptin levels. Studies in animals (cattle and pig) have detected associations between *LEPR* SNPs and serum leptin concentrations [13-17]. Previously the Q223R *LEPR* SNP (rs1137101) has been shown to be associated with increased leptin levels in Pacific Islanders [18], however two other studies observed no association between genetic variations of the *LEPR* gene and serum leptin levels [19, 20]. Given these conflicting reports, there is a need for further investigation

into the role that genetic variants may play in serum leptin levels. We focused on the *LEP*R and the leptin receptor overlapping transcript (*LEPROT*) genes.

Furthermore, there is a lack of understanding of the role of environmental factors that affect the association between genetic polymorphisms and leptin levels. In animal studies where rats were exposed to cigarette smoke in an enclosed space, a decrease in serum leptin levels were observed as compared to rats who were not exposed to cigarette smoke [21, 22]. In humans, obese Japanese men who smoked were observed to have lower serum leptin levels than obese subjects who did not smoke [23].

Leptin is known to play a role in the control of the body's fat stores [1] and serum leptin levels in humans have been associated with body mass index (BMI) [24, 25]. Studies also support a relationship between maternal smoking during pregnancy and obesity in the offspring (both during infancy and later in life) [26-31]. These findings motivated us to look at whether serum leptin levels are associated with maternal smoking during pregnancy.

DNA methylation (DNA-M) can serve as a footprint for past environmental exposures [32, 33], which, in conjunction with genetic variation, may influence leptin levels. Specifically, maternal smoking during pregnancy has been associated with differential DNA-M in epigenome-wide studies in Norwegian offspring [34]. Furthermore, it has been suggested that genetic variants, referred to as methylation quantitative trait loci (methQTLs), are considered to alter methylation levels [6, 35-40].

Using data from a subsample of female offspring from the Isle of Wight Birth Cohort study, we tested a two-stage model explaining the combined influence of genetic variants and DNA-M of the *LEPR/LEPROT* gene and maternal smoking during pregnancy, on leptin levels at age 18 in girls [41]. In Stage 1, we tested, for the first time, whether maternal smoking during pregnancy interacts with *LEPR/LEPROT* SNPs to change the DNA-M status of specific CpG sites in the *LEPR/LEPROT* gene. Following this screening/filtering stage to identify *LEPR/LEPROT* CpG sites modified by maternal smoking, these were then tested for association with leptin and BMI. The possibility that altered DNA methylation could then lead to differential activity (effects on promoter or splicing [42, 43]) of the gene led us to consider modifiable genetic variants (modGVs; e.g. SNPs whose effects on phenotype is modified by DNA methylation). In Stage 2, we therefore tested whether those same CpG sites on the *LEPR/LEPROT*, in interactions with modGVs in this locus, were associated with offspring serum leptin concentrations at age 18 years. To corroborate the leptin findings, we also tested the stage 2 model using offspring BMI at age 18 as the outcome.

Material and methods

Study population and characteristics

A whole population birth cohort was established on the Isle of Wight, UK, in 1989 to prospectively study the natural history of allergies from birth to 18 years of age. Ethics approvals were obtained from the Isle of Wight Local Research Ethics Committee (now the National Research Ethics Service Committee South Central – Southampton B). Of the 1,536 children born between January 1, 1989, and February 28, 1990, written informed consent was obtained from parents to enroll 1,456 newborns. Children were followed up at the ages of 1 (n=1,167), 2 (n=1,174), 4 (n=1,218), 10 (n=1,373), and 18 years (n=1,313). The IOW birth cohort has been described in detail elsewhere [44]. Detailed interviews and examinationswere completed for each child at each follow-up. When a visit was not possible, a telephone questionnaire was completed or a postal questionnaire was sent. Information on maternal smoking during pregnancy was obtained from mothers at the time recruitment (birth).

Leptin concentration and body mass index measurements

Leptin concentrations were obtained from blood samples collected at age 18 years. Aliquots of blood serum that were isolated from the blood samples were used in enzyme-linked immunosorbent assays from Biokit, S.A. (Barcelona, Spain). The assays were conducted according to the manufacturer's kit instructions. Each sample, including standards and the blank, was assayed in duplicate. As part of the repeated follow ups, the original questionnaire-based information were updated and

weight and height of the child were measured at age 18 years. Body mass index (BMI) was measured using the following formula: weight (kg)/height (m)^2.

LEPR/LEPROT genotyping and DNA methylation analysis

SNPs (n=21) that tagged the *LEPR* and neighboring *LEPROT* genes were identified using Tagger implemented in Haploview. We included the leptin receptor overlapping transcript (*LEPROT*) because it shares the same promoter and the first two exons as the *LEPR* gene. DNA was extracted from blood or saliva samples from cohort subjects (n=1,211). DNA samples were interrogated using Golden Gate Genotyping Assays (Illumina Inc, SanDiego, CA) on the Bead Xpress Veracode platform (Illumina, Inc, SanDiego, CA) per Illumina's protocol [45, 46]. In brief, samples were fragmented and hybridized to the pool of allele-specific primer sets. Following an extension/ligation reaction the samples were then hybridized to the Veracode bead pool and processed on the Bead Xpress reader. Data were analyzed using the genotyping module of the GenomeStudio Software package (Illumina, Inc, SanDiego, CA). DNA from each subject plus 37 replicate samples were analyzed. The quality threshold for allele determination was set at a GenCall score 0.25 (scores #0.25 were ''no calls'') with 98.3% retained for further analysis. Analysis of each locus included reclustering of genotyping data using our project data to define genotype cluster positions with additional manual reclustering to maximize both cluster separation and the 50th percentile of the distribution of the GenCall scores across all genotypes (50% GC score).

For measuring methylation levels, DNA was extracted from whole blood collected at age 18 years from 245 randomly selected female offspring [47]. One microgram of DNA was bisulfite-treated for cytosine to thymine conversion using the EZ 96-DNA methylation kit (Zymo Research, CA, USA), following the manufacturer's standard protocol. Genome-wide DNA-M was assessed using the Illumina Infinium Human Methylation 450 Bead Chip (Illumina, Inc., CA, USA), which interrogates>484,000 CpG sites associated with approximately 24,000 genes. Arrays were processed using a standard protocol as described elsewhere [48], with multiple identical control samples assigned to each bisulphite conversion batch to assess assay variability and samples randomly distributed on microarrays to control against batch effects. The Bead Chips were scanned using a Bead Station, and the methylation level (beta value) calculated for each queried CpG locus using the Methylation Module of Bead Studio software.

Statistical analysis

To identify haplotype blocks, linkage disequilibrium analysis was performed on fourteen *LEPR* SNPs and seven *LEPROT* SNPs with Haploview 4.2 [49], using the Gabriel et al. method [50]. We then selected one SNP from each block that best presented the inherited haplotype block for further statistical analyses. After cleaning the DNA-M data, beta (β) values were presented as the proportion of methylated (M) over methylated (M) and unmethylated (U) sites (β=M/[c+M+U], with c being constant to prevent dividing by zero) were used to estimate the effect of DNA-M [51]. The methylation levels of thirty-one CpG sites spanning the *LEPR* and *LEPROT* gene regions were analyzed in this study.

To identify *methQTL*s, modeling was performed by using *LEPR* and *LEPROT* SNPs and their interaction with *in utero* smoking exposure to predict specific DNA-M (stage 1). Each CpG site was modeled against 10 SNPs (one from each haplotype block and the five SNPs that were not in any blocks, **Figure 1**), with each SNP interacting with *in utero* smoking exposure. The most parsimonious model was determined via backward elimination using the 10% rule, first by removing interaction terms followed by individual SNPs. This process filters those CpG sites that were statistically significantly affected by the combined effect of intrauterine smoke exposure and SNPs identified as methQTLs (1st filtering process). Bonferonni adjustment for multiple testing was applied to the interaction terms of genetic variants with smoking, the main focus of stage 1. In the second step of the two-stage model, the selected CpG sites were then tested on whether they modified the association that other *LEPR/LEPROT* SNPs had with leptin serum concentrations at age 18 (2nd filtering process). In this step, our focus was again on the interaction effects and Bonferroni adjustment was

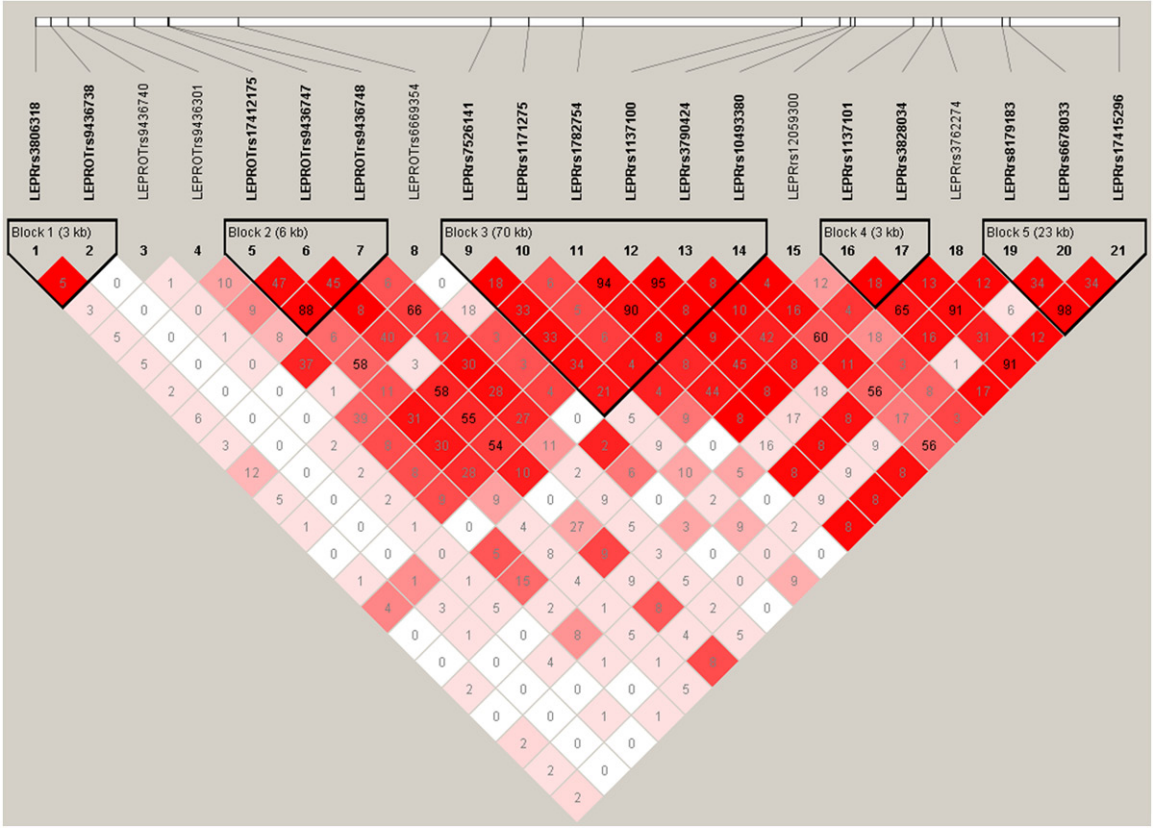

Figure 1. Linkage disequilibrium of LEPR and LEPR single nucleotide polymorphisms.

employed to correct for multiple testing among the tests for interaction effects between CpG sites and genetic variants. For both statistical analyses, the GLM procedure was used in SAS 9.2 (SAS, Gary, NC, USA). The knowledge that BMI plays a role in serum leptin levels [7, 52] allowed us to corroborate our leptin findings by repeating the stage 2 analysis with two CpG sites (cg11807188 and cg03050981) that survived the multiple testing penalty by testing offspring BMI at age 18 as an outcome.

Results

Population characteristics

There was no statistically significant difference between female offspring among the whole cohort and the 245 who were randomly selected for DNA methylation analysis (in terms of gestational smoke exposure, leptin concentrations, and allele proportions in *LEPR* and *LEPROT* genes, **Table 1**). Of the 21 SNPs that were analyzed, one *LEPR* SNP in the 5'UTR region preceded the overlapping *LEPROT* gene segment, which contained three *LEPROT* SNPs in the intron region, followed by four *LEPROT* SNPs in the 3'UTR region (**Table 2**). The remaining *LEPR* SNPs spanned the 5'UTR (located downstream of the *LEPROT* SNPs), intron, coding, and 3'UTR regions. In six SNPs (rs17412175, rs9436743, rs7526141, rs1137101, rs3762274, and rs6678033) the heterozygous genotype had the greatest frequency. Allele and genotype frequencies were similar to those previously reported for the Caucasian population and no deviation from Hardy-Weinberg equilibrium was observed. The haplotype analysis revealed 5 blocks and 5 SNPs that did not have strong linkage disequilibrium with other SNPs (were not in a block with other SNPs, **Figure 1**).

The measurement of methylation of CpG sites (cg) found on the *LEPR* and *LEPROT* genes revealed that the methylation sites were either highly methylated (mean $\beta>0.65$) or lowly methylated (mean $\beta\leq0.2$, **Table 3**). Given that the

Table 1. Population characteristics of offspring

		Sub population with methylation data	Whole cohort population	*P*-Value
SNP		N (%)	N (%)	
rs3806318	AA	116 (50.7)	294 (51.1)	
	AG	92 (40.2)	234 (40.7)	0.77
	GG	21 (9.2)	47 (8.2)	
rs9436738	AA	1 (0.4)	5 (0.9)	
	AG	54 (23.1)	130 (22.4)	0.95
	GG	179 (76.5)	445 (76.7)	
rs9436740	AA	15 (6.5)	50 (8.7)	
	AT	89 (38.7)	224 (38.8)	0.38
	TT	126 (54.8)	303 (52.5)	
rs9436301	TT	140 (59.6)	342 (57.2)	
	CT	86 (36.6)	216 (37.5)	0.85
	CC	9 (3.8)	23 (5.4)	
rs17412175	AA	53 (22.9)	132 (21.1)	
	AT	114 (49.4)	276 (48.7)	0.77
	TT	64 (27.7)	169 (30.2)	
rs9436747	AA	32 (13.7)	83 (15.1)	
	AG	97 (41.6)	247 (44.1)	0.67
	GG	104 (44.6)	248 (40.8)	
rs9436748	AA	120 (20.9)	120 (18.7)	
	AC	269 (47.0)	269 (48.6)	0.79
	CC	184 (32.1)	184 (32.7)	
rs6669354	AA	412 (78.3)	412 (78.7)	
	AC	104 (19.8)	104 (19.5)	0.72
	CC	10 (1.9)	10 (1.9)	
rs7526141	AA	135 (23.4)	135 (22.2)	
	AG	276 (47.8)	276 (48.2)	0.73
	GG	166 (28.8)	166 (29.6)	
rs1171275	AA	11 (1.9)	11 (2.8)	
	AG	169 (29.3)	169 (29.0)	0.96
	GG	397 (68.8)	397 (68.2)	
rs1782754	AA	300 (51.6)	300 (50.8)	
	AG	235 (40.5)	235 (40.7)	0.50
	GG	46 (7.9)	46 (8.4)	
rs1137100	AA	296 (51.4)	296 (51.3)	
	AG	238 (41.3)	238 (40.3)	0.34
	GG	42 (7.3)	42 (8.4)	
rs3790424	AA	292 (51.3)	292 (50.7)	
	AG	230 (40.4)	230 (40.0)	0.38
	GG	47 (8.3)	47 (9.4)	
rs10493380	AA	390 (68.1)	390 (69.5)	
	AC	167 (29.1)	167 (27.8)	0.60
	CC	16 (2.8)	16 (2.7)	
rs12059300	AA	21 (3.6)	21 (3.2)	0.38
	AG	190 (32.6)	190 (32.7)	0.60
	GG	372 (63.8)	372 (64.1)	
rs1137101	AA	169 (29.4)	3169 (30.2)	
	AG	292 (50.8)	292 (48.5)	0.46
	GG	114 (19.8)	114 (21.4)	
rs3828034	AA	390 (68.1)	390 (67.8)	
	AG	163 (28.5)	163 (28.8)	0.76
	GG	20 (3.5)	20 (3.4)	
rs3762274	AA	218 (38.9)	218 (37.8)	
	AG	265 (47.3)	265 (47.2)	0.31
	GG	77 (13.8)	77 (15.0)	

rs8179183	CC	18 (3.1)	18 (3.2)	
	CG	162 (28.2)	162 (28.0)	0.85
	GG	395 (68.7)	395 (68.8)	
rs6678033	AA	76 (13.2)	76 (14.0)	
	AG	261 (45.5)	261 (46.8)	0.65
	GG	237 (41.3)	237 (39.2)	
rs17415296	AA	19 (3.3)	19 (3.3)	
	AC	166 (28.5)	166 (28.6)	0.55
	CC	398 (68.3)	398 (68.2)	
In utero smoking exposure (Yes)		47 (19.3)	188 (25.3)	0.055
Variable		N (Median; 5,95%)	N (Median; 5,95%)	
Leptin at age 18 years (ng/ml)		239 (13.1; 2.8, 54.6)	265 (13.1; 2.4, 54.6)	0.85
BMI at age 18 years (kg/m^2)		240 (22.9; 19.1, 32.9)	499 (22.9; 18.3, 33.7)	0.89

Table 2. Location of *LEPR* and *LEPROT* SNPs

SNP	Alleles	Gene	Location	Coordinate
rs3806318	A, G	*LEPR*	5'UTR	65885357
rs9436738	A, G	*LEPROT*	Intron	65888560
rs9436740	A, T	*LEPROT*	Intron	65891901
rs9436301	T, C	*LEPROT*	Intron	65895927
rs17412175	A, T	*LEPROT*	3'UTR	65904886
rs9436747	A, G	*LEPROT*	3'UTR	65911607
rs9436748	A, C	*LEPROT*	3'UTR	65911672
rs6669354	A, C	*LEPROT*	3'UTR	65925349
rs7526141	A, G	*LEPR*	5'UTR	65975275
rs1171275	A, G	*LEPR*	5'UTR	65982633
rs1782754	A, G	*LEPR*	5'UTR	65993348
rs1137100	A, G	*LEPR*	Coding	66036441
rs3790424	A, G	*LEPR*	Intron	66044013
rs10493380	A, C	*LEPR*	Intron	66046117
rs12059300	A, G	*LEPR*	Intron	66047072
rs1137101	A, G	*LEPR*	Coding	66058513
rs3828034	A, G	*LEPR*	Intron	66062325
rs3762274	A, G	*LEPR*	Intron	66064113
rs8179183	C, G	*LEPR*	Coding	66075952
rs6678033	A, G	*LEPR*	Intron	66077624
rs17415296	A, C	*LEPR*	3'UTR	66099013

LEPROT gene shares the same promoter and first two exons of the *LEPR* gene, the body of the *LEPROT* gene is between the 5'UTR methylated segments (**Table 3**). Low methylation levels were observed from the transcription start site (TSS) 1500 to the beginning of the 5'UTR region (cg26876444). Higher methylation was observed from cg15466952 to cg14199090 (all in the 5'UTR region). Low methylation again was observed in the next cluster of five CpG sites (cg08234308 to cg03514351), followed by high methylation going into sites on the body and 3'UTR region of the gene (**Table 3**).

MethQTL analysis and DNA methylation predicting serum leptin concentrations

Of the 31 methylation sites that were modeled, 16 sites were found to be influenced by adjacent SNPs (methQTLs) in interaction with maternal smoking during pregnancy (**Table 4**). Nine of these methQTLs were found in the intron region of the *LEPR* and *LEPROT* genes, three were in the flanking 5'UTR region, one was in the 3'UTR region, and 4 were found in the coding region. Fourteen of the 16 CpG sites with methQTLs were located in the promoter region of the *LEPR* and *LEPORT* genes, one at the end of the promoter region and into the body and one on the body of the gene (**Table 4**).

Among the CpG sites in the promoter region, five of them had rs12059300 (located in the intron region) as a methQTL (**Table 4**). In these sites, with the exception of site cg11807188, girls, who were exposed to smoking and had the heterozygous genotype of rs12059300, presented a lower proportion of methylation than those children who had the GG or AA genotype.

Table 3. Distribution of methylation on CpG sites on the *LEPR* gene

Gene	CPG site	Location	Coordinate	Mean Methylation	5%	95%
LEPROT; LEPR	cg03853587	TSS1500	65885364	0.12	0.078	0.17
LEPROT; LEPR	cg25307371	TSS1500	65885547	0.079	0.05	0.096
LEPROT; LEPR	cg10062258	TSS1500	65885702	0.033	0.02	0.049
LEPROT; LEPR	cg23055818	TSS1500	65885868	0.077	0.049	0.11
LEPROT; LEPR	cg07342512	TSS1500	65886002	0.06	0.04	0.087
LEPROT; LEPR	cg14976592	TSS200	65886160	0.11	0.067	0.17
LEPROT; LEPR	cg13202122	TSS200	65886182	0.041	0.021	0.068
LEPROT; LEPR	cg07921092	Exon 1, 5'UTR	65886266	0.026	0.01	0.044
LEPROT; LEPR	cg27502791	Exon 1, 5'UTR	65886279	0.051	0.035	0.066
LEPROT; LEPR	cg08610741	Exon 1, 5'UTR	65886304	0.023	0.01	0.041
LEPROT; LEPR	cg13446852	5'UTR	65886635	0.052	0.04	0.065
LEPROT; LEPR	cg08922075	5'UTR, Body	65886896	0.092	0.058	0.13
LEPROT; LEPR	cg26876444	5'UTR	65887413	0.23	0.16	0.31
LEPROT; LEPR	cg15466952	5'UTR	65889855	0.67	0.56	0.74
LEPROT; LEPR	cg01933519	5'UTR	65891704	0.89	0.86	0.91
LEPR	cg03050981	5'UTR	65906670	0.69	0.6	0.79
LEPR	cg11807188	5'UTR	65924844	0.75	0.69	0.8
LEPR	cg23688719	5'UTR	65935654	0.81	0.76	0.86
LEPR	cg11228758	5'UTR	65976172	0.81	0.76	0.85
LEPR	cg26342890	5'UTR	65988782	0.82	0.76	0.86
LEPR	cg06558372	5'UTR	65990448	0.9	0.88	0.93
LEPR	cg14199090	5'UTR	65990564	0.87	0.84	0.9
LEPR	cg08234308	5'UTR	65991176	0.067	0.044	0.095
LEPR	cg00630958	5'UTR	65991461	0.12	0.089	0.16
LEPR	cg16987305	5'UTR	65991596	0.034	0.016	0.057
LEPR	cg03401738	5'UTR	65991664	0.11	0.088	0.13
LEPR	cg03514351	5'UTR	65991765	0.15	0.11	0.18
LEPR	cg16265717	5'UTR	65993149	0.88	0.85	0.91
LEPR	cg07959978	5'UTR	65994091	0.82	0.77	0.86
LEPR	cg21683619	Body	66070348	0.7	0.59	0.77
LEPR	cg03607891	Body, 3'UTR	66099615	0.88	0.83	0.92

The CpG sites are presented in the order they appear on the *LEPR* and *LEPROT* genes from 5' to 3'. TSS1500=1500 base pairs from the transcription start site. TSS200=200 base pairs from the transcription start site.

Table 4. Interaction effect of prenatal exposure to smoking with methylation quantitative trait loci for *LEPR* and *LEPROT* genes

Variable		Estimate	STD	*P* value
cg10062258[a] Bonferroni adjusted cutoff: 0.01				
In utero smoking exposure*rs17412175	AA	0.02	0.007	0.01
	AT	0.01	0.006	0.06
	TT	0.0	-	-
In utero smoking exposure*rs1137101	AA	-0.04	0.01	0.0009
	AG	-0.03	0.01	0.004
	GG	0.0	-	-
cg01933519[b] cutoff: 0.05				
In utero smoking exposure*rs12059300	AA	0.0	-	-
	AG	-0.02	0.009	0.0098

	GG	0.0	-	-
cg21683619[c] cutoff: 0.05				
In utero smoking exposure*rs9436301	TT	-0.14	0.05	0.004
	CT	-0.09	0.05	0.07
	CC	0.0	-	-
cg16987305[d] cutoff: 0.05				
In utero smoking exposure*rs8179183	CC	0.03	-	-
	CG	0.01	0.007	0.05
	GG	0.0	-	-
cg14199090[e] Bonferroni adjusted cutoff: 0.017				
In utero smoking exposure*rs12059300	AA	0.0	-	-
	AG	-0.04	0.01	0.003
	GG	0.0	-	-
cg11807188[f] Bonferroni adjusted cutoff: 0.0125				
In utero smoking exposure*rs12059300	AA	0.0	-	-
	AG	0.07	0.02	0.001
	GG	0.0	-	-
cg03050981[g] cutoff: 0.05				
In utero smoking exposure*rs12059300	AA	0.0	-	-
	AG	-0.59	0.02	0.01
	GG	0.0	-	-
cg26876444[h] Bonferroni adjusted cutoff: 0.025				
In utero smoking exposure*rs8179183	CC	0.0	-	-
	CG	0.06	0.03	0.02
	GG	0.0	-	-
cg14976592[i] Bonferroni adjusted cutoff: 0.017				
In utero smoking exposure*rs7526141	AA	-0.06	0.02	0.01
	AG	0.0006	0.02	0.97
	GG	0.0	-	-
cg13446852[j] Bonferroni adjusted cutoff: 0.013				
In utero smoking exposure*rs7526141	AA	0.01	0.006	0.048
	AG	0.02	0.005	0.001
	GG	0.0	-	-
cg08610741[k] Bonferroni adjusted cutoff: 0.008				
In utero smoking exposure*rs7526141	AA	0.0	-	-
	AG	0.02	0.008	0.005
	GG	0.0	-	-
In utero smoking exposure*rs12059300	AA	0.0	-	-
	AG	-0.02	0.006	0.004
	GG	0.0	-	-
cg00630958[l] Bonferroni adjusted cutoff: 0.017				
In utero smoking exposure*rs8179183	CC	0.0	-	-
	CG	0.05	0.01	0.001
	GG	0.0	-	-
cg08922075[m] Bonferroni adjusted cutoff: 0.05				
In utero smoking exposure*rs9436740	AA	0.04	0.02	0.002
	AT	0.02	0.01	0.03
	TT	0.0	-	-
cg23055818[n] Bonferroni adjusted cutoff: 0.05				
In utero smoking exposure*rs9436740	AA	0.004	0.01	0.74
	AT	0.02	0.009	0.01
	TT	0.0	-	-

cg06558372[o] Bonferroni adjusted cutoff: 0.05				
In utero smoking exposure*rs8179183	CC	0.0	-	-
	CG	0.02	0.008	0.009
	GG	0.0	-	-
cg03514351[p] Bonferroni adjusted cutoff: 0.05				
In utero smoking exposure*rs9436301	TT	0.08	0.02	0.0002
	CT	0.07	0.02	0.002
	CC	0.0	-	-

Controlled for: in utero smoking exposure: a-p. rs3806318: a, c, d, g, i, k, p. rs3806318**in utero* smoking exposure: a. rs9436740: a, b, c, d, j, k, m, n. rs9436740**in utero* smoking exposure: e, j, k, m, n. rs9436301: a, c, f, i, j, k, l, p. rs9436301**in utero* smoking exposure: a, f, j, p. rs17412175: a, h, k. rs17412175* *in utero* smoking exposure: h, k. rs6669354: a, b, d, e, g, h, j, k, l. rs6669354**in utero* smoking exposure: a, j. rs12059300: a, b, e, f, g, i, k. rs12059300**in utero* smoking exposure: a, e, f, l. rs1137101: a, c, g, i, k, l. rs1137101**in utero* smoking exposure: i, l. rs3762274: a, c, d, f, h, k, l, o. rs3762274**in utero* smoking exposure: f. rs7526141: d, e, g, h, i, j, k, l. rs7526141**in utero* smoking exposure: f, i, k. rs8179183: d, e, h, k, l, o. rs8179183**in utero* smoking exposure: k, l, o. rs6436740: j, p. rs6436740**in utero* smoking exposure.

Two methQTLs were detected in the coding region of the genes, of which one (rs1137101) influenced methylation at the TSS1500 region of *LEPR* and *LEPROT*, which was 84,356 base pairs (bp) downstream from the CpG site (cg10062258, **Table 4**). This was the only methQTL in the coding region that had a negative association with DNA-M. In four other CpG sites in the promoter region, all downstream from cg10062258, children with the heterozygous genotype (AG) for the rs8179183 SNP and exposure to smoking during pregnancy had higher methylation than those with the AA genotype and mothers who did not smoke during pregnancy (**Table 4**).

The methQTL that was detected in the 3'UTR region (rs174121475), in interaction with *in utero* smoking exposure, was associated with increased DNA-M in the TSS1500 region (cg10062258, **Table 4**). This is the same CpG site observed to have decreased methylation with at methQTL located in the coding segment of the gene and in interaction with maternal smoking during pregnancy. Lastly, the methQTL in the flanking 5'UTR region (rs7526141) tended to be positively associated (in interaction with maternal smoking during pregnancy) with DNA-M at the 5'UTR region (**Table 4**). However, when considering DNA-M upstream in the TSS200 region, a negative association was observed (**Table 4**).

In most of the models we observed that, although the main effects of both gestational smoking and SNPs were not strongly associated with DNA-M at the promoter region, their interactions had a stronger and statistically significant influence even after adjustment for multiple testing. In the first filtering process, representing stage 1 of the 2-stage model, we identified sixteen CpG sites of 21 potential CpGs that were differentially methylated by the interaction of maternal smoking during pregnancy and the child's *LEPR/LEPROT* genotype.

Addressing stage 2, we then tested these sixteen CpG sites to determine whether the smoking fingerprint changed the role of other SNPs in the *LEPR* and *LEPROT* genes on their association with leptin concentrations at age 18 years. SNPs that identified as being modified are named modifiable genetic variants (*modGV*). Again, modifying effects (interactions) of all tag SNPs with these 16 CpG sites were tested. The most parsimonious model yielded thirty-two tests (i.e.-thirty two SNP-CpG interactions). Applying Bonferroni adjustment for these thirty-two tests resulted in a *p*-value cut off point of 0.00156. We found that two of the sixteen CpG sites, in interaction with modGVs, were statistically significantly associated with serum leptin concentrations at age 18 years (**Table 5**). Both of these CpG sites were in the 5'UTR region of the gene and both interacted with rs12059300 (located in the intron region) to influence serum leptin levels. The CpG sites cg11807188 and cg03050981 were upstream from rs12059300 by 122,228 and 140,402 base pairs respectively. In those with the AA genotype of rs12059300, serum leptin levels were observed to decrease as proportion of methylation at cg11807188 and cg03050981 increased as compared to those

Table 5. Methylation of cg11807188 and cg03050981 associated with leptin concentrations (ng/mL) and BMI (kg/m^2) at age 18 years

Leptin at age 18 years (ng/mL)				
CpG-genotype interaction		Estimate	STD	*P* Value
cg11807188*rs12059300[a]	AA	-1281.6	381.5	0.001
	AG	164.7	119.3	0.17
	GG	0.0	-	-
cg03050981*rs12059300[b]	AA	-549.2	158.3	0.0007
	AG	-132.5	53.7	0.01
	GG	0.0	-	-
Body mass index at age 18 years				
CpG-genotype interaction		Estimate	STD	*P* Value
cg11807188*rs12059300[c]	AA	-1174.4	500.4	0.0005
	AG	33.2	124.5	0.79
	GG	0.0	-	-
cg03050981*rs12059300[d]	AA	-33.2	8.6	0.0002
	AG	-6.49	2.9	0.027
	GG	0.0	-	-

Bonferroni adjusted cutoff: 0.00156. Controlled for: a, c: Cg11807188, rs3806318, rs9436301, rs17412175, cg11807188*rs17412175, rs6669354, cg11807188*rs6669354, rs7526141, rs12059300, cg11807188*rs12059300, rs1137101. b, d: cg03050981, rs3806318, cg03050981*rs3806318, rs12059300.

with the GG genotype. Additionally, for cg03050981, those with the AG genotype showed a similar pattern as those with the AA genotype (**Table 5**).

The serum leptin levels from those with the AA genotype of rs12059300 ranged from 2.90 ng/mL to 60.6 ng/mL; decreasing as the proportion of methylation at cg03050981 increased (**Figure 2**). Those with the AG genotype were observed to have a similar pattern although the difference was not as dramatic. Regarding methylation at cg11807188, those with the AA genotype had serum leptin concentrations ranging from 3.29 ng/mL to 78.1 ng/mL; the leptin levels decreased as proportion of methylation at that site increased. Leptin levels for those with the AG and GG genotype remained constant across different methylation levels (**Figure 3**).

Previous studies have suggested that body mass index (BMI) plays a role in serum leptin levels [7, 51]. We therefore repeated the stage 2 analysis with the same two statistically significant CpG sites in interaction with rs12059300 predicting standardized BMI levels at age 18 years. We found the same significant associations. BMI was observed to decrease as proportion of methylation increased in those with the AA genotype as compared to those with the GG genotype (**Table 5**).

Discussion

We detected seven methQTLs working in interaction with *in utero* smoking exposure to influence DNA methylation at 16 sites on the *LEPR* and *LEPROT* genes (**Table 4**). Two of these same CpG sites were found to modify the effect of *LEPR* and *LEPPROT* SNPs on serum leptin concentration levels at age 18 years (**Table 5**). This is the first study to identify SNPs (methQTLs) on the leptin receptor gene that in interaction with *in utero* smoking exposure change the DNA methylation of sixteen CpG sites, which in turn modifies the association that genetic variants (SNP) had on serum leptin concentrations. This association was then corroborated with BMI as an outcome of the stage 2 analysis.

The probability for selection bias influencing our analysis is minimal since the samples from the 245 female offspring were randomly selected. Furthermore, there were no differences observed between the subset population and the female population from the whole cohort. However, the small sample size in our analysis is a potential weakness of our study. Regarding the exclusion of male offspring in the analysis, given that boys and girls in our study have sig-

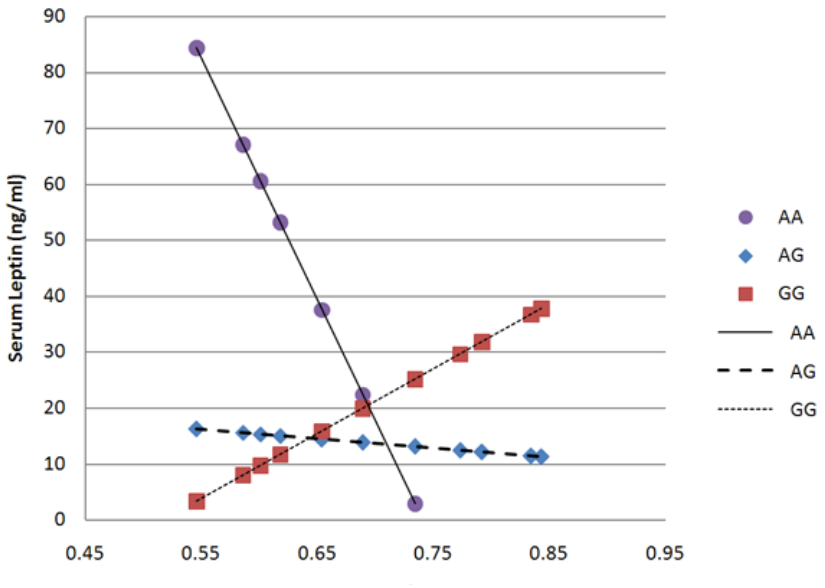

Figure 2. Association of methylation at cg03050981 with serum leptin concentrations across different rs12059300 genotypes.

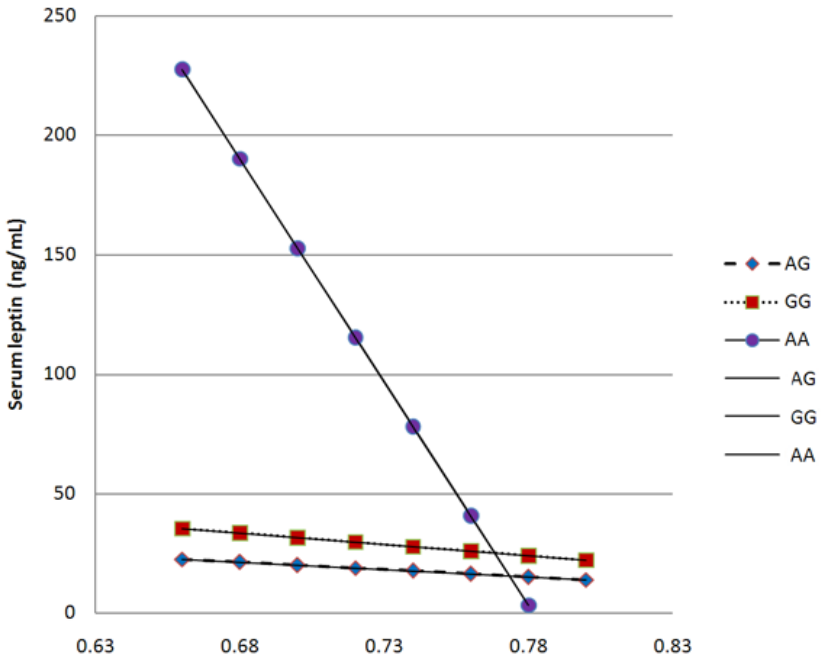

Figure 3. Association of methylation at cg11807188 with serum leptin concentrations across different rs12059300 genotypes.

nificantly different leptin levels, especially following puberty (1.2 ng/mL and 13.1 ng/mL in boys and girls, respectively; data not shown), this same analysis needs to be done in the future separately in boys. A strength of our study is the use of the Infinium Human Methylation 450 array to obtain DNA methylation profiles, which has been reported to have strong reproducibility and validity [45, 46]. Additionally, the SNPs that were detected to be associated with DNA-M levels in this study do not overlap with the probes on the methylation array, suggesting that our findings are actual methQTLs and not technical artifacts caused by differential probe binding.

The distance between the methylation site and the SNPs used in our analysis is sometimes greater than 10,000 bp (**Tables 1** and **2**). Methylation in the promoter region of the *LEPR* gene is influenced by SNPs either in the same region or downstream. This is plausible biologically as well as within the confines of cis-acting regulation [6, 36].

We focused on the *LEPR* gene because it codes for the leptin receptor, which is known to bind to leptin [53, 54]. Our analysis shows that two of the methylation sites that are influenced by a methQTL in interaction with a prenatal environmental exposure, are in turn associated with leptin concentrations in the body at age 18 years. This suggests a potential pathway from a prenatal smoke exposure altering DNA methylation, and the DNA methylation subsequently influencing a phenotypic outcome in adulthood. Given that the environmental factor in this case is a modifiable risk factor (maternal smoking during pregnancy), our findings sup-

port other studies that have tested intervention methods for obesity [55] and point to the development of further efforts.

Our findings fit into a two-stage model [41]: Stage 1 addresses SNPs that make the DNA at some CpG sites more or less prone to DNA methylation conditional of exposure (maternal smoking during pregnancy). Stage 2 emphasizes that these CpG sites then regulate the activity of other genetic variants of the gene, either masking it or facilitating more penetrance.

Given that leptin levels and BMI are correlated, we attempted to corroborate our findings in stage 2 using BMI at age 18 as the final outcome. The two SNP - DNA methylation interactions found for leptin were also statistically significantly associated with BMI. We know that *in utero* exposure to maternal smoking is associated with offspring BMI into late childhood [26-30] but the mechanism behind this relationship is not understood. Our findings linking maternal smoking during pregnancy with leptin suggest an intermediary role of leptin in providing a possible explanation. Future studies will address the leptin gene and whether we can find similar effects in boys and in the offspring of the birth cohort.

Conclusion

A two-stage model consisting of genetic variants in the *LEPR/LEPROT* loci, gestational smoke exposure, and DNA methylation provides an explanation of how maternal smoking during pregnancy can be linked to increased serum leptin levels and BMI at age 18 years. Results of the first stage demonstrate that DNA methylation which sets the offspring at a higher risk of increased leptin and BMI can be avoided by reducing maternal smoking during pregnancy. The second stage, explaining serum leptin concentrations and BMI, shows strong interactions between DNA methylation and genetic variants suggesting that the study of DNA methylation and genetic variants is much more powerful than the single analyses of genetic variants.

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