RESEARCH PAPER

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Genome-wide methylation analysis identifies differentially methylated CpG loci associated with severe obesity in childhood

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Childhood obesity is a major public health issue. Here we investigated whether differential DNA methylation was associated with childhood obesity. We studied DNA methylation profiles in whole blood from 78 obese children (mean BMI Z-score: 2.6) and 71 age- and sex-matched controls (mean BMI Z-score: 0.1). DNA samples from obese and control groups were pooled and analyzed using the Infinium HumanMethylation450 BeadChip array. Comparison of the methylation profiles between obese and control subjects revealed 129 differentially methylated CpG (DMCpG) loci associated with 80 unique genes that had a greater than 10% difference in methylation (P-value < 0.05). The top pathways enriched among the DMCpGs included developmental processes, immune system regulation, regulation of cell signaling, and small GTPase-mediated signal transduction. The associations between the methylation of selected DMCpGs with childhood obesity were validated using sodium bisulfite pyrosequencing across loci within the *FYN, PIWIL4*, and *TAOK3* genes in individual subjects. Three CpG loci within *FYN* were hypermethylated in obese individuals (all $P < 0.01$), while obesity was associated with lower methylation of CpG loci within *PIWIL4* ($P = 0.003$) and *TAOK3* ($P = 0.001$). After building logistic regression models, we determined that a 1% increase in methylation in *TAOK3*, multiplicatively decreased the odds of being obese by 0.91 (95% CI: 0.86 – 0.97), and an increase of 1% methylation in *FYN* CpG3, multiplicatively increased the odds of being obese by 1.03 (95% CI: 0.99 – 1.07). In conclusion, these findings provide evidence that childhood obesity is associated with specific DNA methylation changes in whole blood, which may have utility as biomarkers of obesity risk.

Introduction

The prevalence of childhood obesity worldwide has increased over the past decade. An estimated 43 million preschool children were overweight or obese in 2010, a 60% increase since 1990.[1] Childhood obesity is linked to both short- and long-term health issues.[2,3] Obese children have a higher risk of obesity in later life as well as of co-morbidities, such as atherosclerosis, type-2 diabetes, hypertension, and some forms of cancer.[4-6] The rapid rise in prevalence of obesity over such a short time frame cannot be explained solely by fixed genetic factors but suggests that environmental factors, such as diet and the level of physical activity, are likely to play a major role.[7] There is now a substantial body of evidence from both animal and human studies linking environmental factors particularly in early life to later adiposity and the risk of metabolic disease.[8] For instance, in animal studies, variations in maternal diet have been linked to alterations in metabolism and body composition in the offspring,[9] while in humans, famine exposure during pregnancy,[10] maternal obesity,[11] or gestational diabetes,[12] were associated with an increased risk of obesity in the adult offspring.

The mechanisms by which early environmental factors may influence phenotype and disease risk have been suggested to involve the altered epigenetic regulation of genes.[13] Epigenetic processes, such as DNA methylation, induce heritable changes in gene expression without a change in nucleotide sequence. Epigenetic regulation is central to the control of gene expression, genomic imprinting, X chromosome inactivation, and cell

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specification. There is growing evidence that environmental factors can alter the epigenome and that such alterations are involved in the development and progression of non-communicable diseases. Epigenetic traits associated with type-2 diabetes,[14] adiposity,[15,16] cardiovascular disease,[17] stress responses,[18] and specific solid tumors,[19] have also been identified in peripheral tissues, and such epigenetic marks may have utility as biomarkers to identify individuals at increased risk of disease.[15,16] However, to date, there has been no systematic genome-wide screen to investigate whether childhood obesity is associated with differences in DNA methylation in peripheral blood. Identification of methylation differences in peripheral blood associated with severe childhood obesity may provide insights into the systemic changes associated with obesity in children and identify potential epigenetic biomarkers of childhood obesity. Here, we used the Illumina Infinium HumanMethylation450 BeadChip arrays (450K) in a case-control study using pooled DNA from obese children and age- and sex-matched controls to identify sites of differential methylation. We then sought to validate these sites using pyrosequencing in an extended number of individual subjects to investigate if variations in methylation status at identified CpG sites were associated with child's adiposity.

Results

Study participants

For the Infinium HumanMethylation450K BeadChip analysis, we used pooled DNA samples from 54 children (25 male) with severe obesity (median age: 12.6 years, age- and sex-adjusted BMI Z-score: 2.58) and from 54 controls (25 male) (median age: 12.4 years, age- and sex-adjusted BMI Z-score: 0.13) (**Table S1**). Validation of individual CpG loci selected from the 450K array was carried out by pyrosequencing on individual samples from an extended group of 78 obesity cases (40 male) and 71 controls (36 male); median ages of the obesity cases and the controls were 12.6 and 12.9 years, respectively, while median age- and sex-adjusted BMI Z-scores were 2.61 and 0.08, respectively (**Table 1**).

Table 1. Cohort characteristics of severe obesity cases and controls

	Severe Obesity Cases	Controls
	Subjects used for Validation	
Number	78	71
Sex	M:40 F:38	M:36 F:35
Age at Visit (years)	12.55 (9.4 to 13.73)	12.9 (10.7 to 14.10)
BMI Z-score	2.61 (2.33 to 2.79)	0.08 (−0.4 to 0.59)
Fasting Insulin	20 (8 to 27)	6 (3 to 10)
Fasting Glucose	4.6 (4.4 to 5.0)	4.7 (4.3 to 5.0)
SBP	118 (108 to 130)	101 (97 to 109)
DBP	64 (61 to 72)	60 (57 to 63)
Cholesterol	4.5 (3.6 to 5.2)	4.1 (3.6 to 4.7)
LDL	2.60 (2.15 t 3.25)	2.20 (1.85 to 2.98)
HDL	1.05 (0.8 to 1.32)	1.45 (1.20 to 1.60)
Triglycerides	1.2 (0.8 to 2.0)	0.6 (0.4 to 0.8)

For age, BMI Z-score (CDC growth charts),[52] and fasting insulin levels, the median and interquartile ranges are given

Identification of differentially methylated CpG loci associated with childhood obesity

A total of 1879 CpGs (associated with 1119 unique genes) were differentially methylated ($P < 0.05$) between the obese and control subject pools with a difference in methylation of more than 5% (**Table S2**). Of these, 129 CpGs (associated with 81 unique genes) had a greater than 10% difference in methylation between the case and control groups and were denoted differentially methylated CpGs (DMCpGs) (**Table 2**). As cellular heterogeneity can influence methylation profiles and drive some of the methylation differences detectable across individual blood samples,[20] blood cellular content was estimated in all the pooled samples using a previously reported signature.[21] Cellular composition was similar in the pooled obese and control samples (**Table S3**). Of the 1879 CpGs, 776 significantly covaried with cell type (**Table S2**), while 22 of the 129 DMCpGs with a 10% difference in methylation between the case and control groups significantly co-varied with one cell type (B-cells) (**Table 2**).

Among the DMCpGs independent of cellular composition, 50 showed hypomethylation and 57 showed hypermethylation in obese subjects, compared to the control group. CpGs located within *O(6)-methylguanine-DNA methyltransferase* (*MGMT*), *TAO kinase 3* (*TAOK3*), *piwi-like RNA-mediated gene silencing 4* (*PIWIL4*), *mir-125b*, and *aurora Kinase A* (*AURKA*) genes were hypomethylated in the obese pool, while sites located within *spondin2* (*SPON2*), *ras related protein Rab1A* (*RAB1A*), and *FYN oncogene related to SRC* (*FYN*) were hypermethylated in the obese pool. The genomic distribution of DMCpGs in comparison to all the probes located on the 450K BeadChip array with respect to gene structure or CpG islands is shown in **Figure 1**. There was no significant enrichment of DMCpGs within specific gene regions (**Fig. 1A**); however, in relation to CpG content and distance to CpG islands, there was an enrichment of hypomethylated DMCpGs within open seas ($P = 0.005$), while there were fewer hypermethylated DMCpGs within CpG islands ($P = 0.009$) (**Fig. 1B**).

Validation of differentially methylated regions by pyrosequencing

Validation of individual CpG loci within *FYN, PIWIL4*, and *TAOK3*, selected from the 450K array, was carried out by pyrosequencing individual samples from an extended group of 78 obesity cases (40 male) and 71 controls (36 male); these samples included the 54 obese and 54 lean subjects analyzed by the 450K array. These DMCpGs were selected according to the following criteria: i) contiguous CpGs that were differentially methylated with respect to obesity; ii) independent of cellular composition; and iii) having a difference of methylation of above 10%, with both hypo- and hyper-methylated CpGs being assessed. Pyrosequencing analysis of the DMCpGs in individual subject samples confirmed both the statistical significance and direction of the associations between methylation levels and childhood obesity. *FYN* (Cg26846943- CpG1) was hypermethylated in obese individuals [median: 12.2% (interquartile range: 10.0–25.7%)] compared to controls [10.8% (9.2–18.2%)] (P=0.012) (**Table 3**, **Fig. 2A**). For *FYN*, the

Table 2. Differentially methylated CpGs (DMCpGs) with a greater than 10% difference in methylation and a *P*-value < 0.05 for the difference between severe obesity cases and controls, as identified by the Human Methylation450 BeadChip array

Gene Name	Probe	CHR	Genomic Co-ordinate	*P*-value	Control	Obese
Hypomethylated DMCpGs						
LRIG1*	cg26131019	3	66550740	4.96E-05	0.244	0.0723
OIT3*	cg24769348	10	74692580	0.0002	0.4437	0.3271
MGMT	cg09993319	10	131529400	0.0003	0.7305	0.5051
PIWIL4	cg 16436762	11	94307970	0.0004	0.5275	0.4228
	cg04456492	7	20010780	0.0007	0.802	0.6206
PCBD1	cg05601623	10	72647650	0.001	0.228	0.1008
	cg23892028	6	27256340	0.0015	0.4042	0.2748
FER1L5*	cg20312012	2	97331030	0.0016	0.5836	0.4771
AMBRA1*	cg20090290	11	46543700	0.0016	0.4844	0.3821
A1CF	cg16531903	10	52645850	0.002	0.871	0.7431
ARNT*	cg00944785	1	150825400	0.002	0.4981	0.3921
FAM107B	cg14152591	10	14587920	0.0021	0.493	0.3628
	cg14192979	17	12562530	0.0025	0.4944	0.2951
	cg26261358	15	24043140	0.0026	0.4387	0.2601
	cg11557901	9	128022400	0.003	0.6408	0.5322
	cg26576353	10	126135200	0.0033	0.8465	0.7393
TMCC1*	cg12196294	3	129575100	0.0033	0.6321	0.5305
	cg22626683	1	172903100	0.0037	0.7357	0.6279
PRG2	cg15971518	11	57159180	0.0043	0.5815	0.393
	cg19699682	3	119349800	0.0045	0.552	0.425
B3GNT7	cg00424152	2	232263100	0.0045	0.7712	0.6492
IGF2BP3*	cg17209188	7	23387400	0.0048	0.5024	0.4001
TAOK3	cg17627898	12	118782500	0.0049	0.4411	0.3409
	cg07879897	17	66201170	0.005	0.2838	0.149
SULF2*	cg21130926	20	46415320	0.0054	0.6266	0.4702
	cg09196346	13	23499330	0.0054	0.2887	0.135
	cg04450797	8	337367	0.0057	0.7987	0.6379
FSCN2	cg05248234	17	79495520	0.0077	0.7439	0.6027
	cg11725581	7	6140990	0.0084	0.745	0.6439
AP3S1	cg03637218	5	115209100	0.0087	0.7055	0.593
ZNF827*	cg07091220	4	146752100	0.009	0.5674	0.467
NAPSB	cg25094735	19	50848020	0.0091	0.6449	0.5334
RIPK2*	cg15723028	8	90776470	0.0098	0.5502	0.4427
PDLIM7	cg15225325	5	176921800	0.0101	0.5126	0.3925
SH3PXD2B	cg24921943	5	171847600	0.0102	0.5018	0.3943
MIR125B1	cg26916936	11	121970600	0.0116	0.6221	0.5164
MATN2*	cg19935471	8	99048260	0.0116	0.6963	0.5957
SLC35E2	cg12213037	1	1666808	0.0122	0.7003	0.5419
C18orf62	cg23936477	18	73139740	0.0126	0.7775	0.6599
PCDHB3	cg23918315	5	140479000	0.0138	0.4064	0.2979
RALGPS1*	cg14306650	9	129829100	0.0141	0.705	0.6032
	cg27114706	12	92527250	0.0148	0.877	0.7548
DNAJC8	cg03040423	1	28560940	0.0157	0.9116	0.807
MEGF11*	cg00682263	15	66188800	0.0163	0.5975	0.4801
LOC652276*	cg06035616	16	2653306	0.0209	0.1801	0.0665
RAB36	cg05338731	22	23489040	0.0217	0.4992	0.3863
	cg00968488	5	103876200	0.0217	0.6865	0.5749
	cg16885113	6	29648510	0.0235	0.8421	0.7381
AURKA	cg09712306	20	54949300	0.0248	0.481	0.3621
	cg23661721	14	95991370	0.0271	0.5536	0.4314
LOC652276	cg03314158	16	2653280	0.0278	0.1674	0.0465
AGPAT1	cg17213381	6	32139500	0.029	0.8273	0.6971
	cg21852792	2	71678460	0.03	0.5511	0.4473
DEAF1	cg03951394	11	660455	0.0316	0.8874	0.7094
SORL1	cg10746778	11	121460800	0.0341	0.5232	0.3801

(*Continued on next page*)

Table 2. Differentially methylated CpGs (DMCpGs) with a greater than 10% difference in methylation and a *P*-value < 0.05 for the difference between severe obesity cases and controls, as identified by the Human Methylation450 BeadChip array *(Continued)*

Gene Name	Probe	CHR	Genomic Co-ordinate	*P*-value	Control	Obese
			Hypomethylated DMCpGs			
TOP1MT	cg00033213	8	144399300	0.0383	0.6014	0.4747
	cg06864789	6	139013000	0.0386	0.5387	0.3803
SNORA1*	cg09719956	11	93465910	0.0386	0.6563	0.5343
	cg05320460	15	63179550	0.039	0.7531	0.6475
	cg27245056	10	122356500	0.0396	0.6572	0.5472
	cg12799049	1	197811500	0.0401	0.4571	0.3469
CTSS	cg26891210	1	150703100	0.0402	0.7015	0.5925
CBX7	cg13306870	22	39527580	0.0408	0.3769	0.2444
LTBP1*	cg11918450	2	33359200	0.0425	0.645	0.5413
B4GALT6	cg11986743	18	29205360	0.0447	0.6977	0.5903
OTUD6B*	cg19586698	8	92097850	0.0463	0.7898	0.6607
TP53INP2	cg20592836	20	33292130	0.0496	0.5504	0.3536
			Hypermethylated DMCpGs			
SPON2	cg10852718	4	1167230	7.59E-06	0.734	0.8759
	cg24591913	10	47062880	0.0002	0.6864	0.8203
IL32*	cg16730716	16	3114986	0.0015	0.8161	0.9198
FYN	cg26846943	6	112165100	0.002	0.2063	0.33
TOX2	cg26365090	20	42574360	0.0021	0.1902	0.4129
TNXB	cg14188106	6	32063900	0.0021	0.4143	0.5163
	cg12342501	2	8530521	0.0021	0.6035	0.7998
	cg27596172	8	26727370	0.0034	0.7664	0.8698
UBE4A	cg25574849	11	118269800	0.0035	0.2916	0.4022
C7orf50	cg26542892	7	1133730	0.0035	0.6345	0.7703
TMEM71	cg10054641	8	133773100	0.0041	0.3066	0.429
	cg08506672	5	3959743	0.0059	0.2318	0.4672
KRTAP27-1	cg05809586	21	31709690	0.0063	0.5095	0.7153
	cg03240981	6	26615070	0.0063	0.4289	0.5301
	cg00696044	10	63240300	0.0063	0.3894	0.5672
SLC12A8	cg09866143	3	124861500	0.0078	0.565	0.7826
WTIP*	cg10771931	19	34972140	0.0083	0.7332	0.8829
SLC25A21	cg00814218	14	37445440	0.0084	0.6818	0.8043
CCDC88C	cg23165913	14	91880060	0.0086	0.413	0.5392
CCS	cg24851651	11	66362960	0.0087	0.3717	0.701
TRPM4	cg01997599	19	49689670	0.0103	0.4019	0.5334
ABCD3*	cg26908356	1	94929490	0.0111	0.4584	0.6008
	cg02479782	2	71033150	0.0113	0.3091	0.5286
	cg08754654	5	154026400	0.0117	0.2817	0.3824
NAV1	cg04287574	1	201619600	0.0124	0.2851	0.3895
CSMD2	cg21440084	1	34615960	0.0125	0.4898	0.6454
	cg10384133	9	45733080	0.0126	0.7488	0.8503
USP36	cg01385356	17	76837380	0.0139	0.2224	0.3224
	cg07371521	5	154026400	0.0142	0.2003	0.3382
	cg04653913	16	53407750	0.0145	0.588	0.6985
	cg18618432	19	34621910	0.0147	0.5521	0.6704
TRAPPC9	cg16191297	8	140926700	0.0156	0.7429	0.8567
	cg14580085	2	239553400	0.0172	0.5782	0.6973
	cg24643105	11	113928500	0.0189	0.6603	0.7965
	cg19373347	1	219634800	0.019	0.4338	0.5557
IL12RB1	cg26642774	19	18170380	0.0191	0.5503	0.7219
	cg02389264	16	88757750	0.0191	0.6135	0.7415
	cg10510935	1	4059661	0.0209	0.3138	0.4174
	cg09916840	16	87248610	0.0214	0.0156	0.1245
	cg02100397	19	646890	0.0223	0.4163	0.5673
SH3GL1	cg11592634	19	4370001	0.0224	0.1313	0.3691

(*Continued on next page*)

Table 2. Differentially methylated CpGs (DMCpGs) with a greater than 10% difference in methylation and a *P*-value < 0.05 for the difference between severe obesity cases and controls, as identified by the Human Methylation450 BeadChip array *(Continued)*

Gene Name	Probe	CHR	Genomic Co-ordinate	*P*-value	Control	Obese
			Hypomethylated DMCpGs			
C10orf110	cg12421087	10	1083304	0.0225	0.6924	0.8347
ANKH	cg14843632	5	14870590	0.0236	0.2696	0.3867
ARPC3	cg10738648	12	110888900	0.0238	0.5427	0.7118
IRGM	cg22000984	5	150226300	0.0239	0.1801	0.3087
SLC6A5*	cg14524936	11	20627600	0.0253	0.3286	0.4456
	cg01584086	11	10373720	0.0254	0.1171	0.2833
	cg07700233	4	39171230	0.0264	0.3269	0.4351
ATP9B*	cg09636756	18	77134250	0.0267	0.7454	0.8943
	cg02088292	1	235099200	0.0268	0.2089	0.3511
RAB1A	cg00570635	2	65355270	0.0318	0.1747	0.3775
SPDEF	cg16527629	6	34524700	0.0324	0.331	0.4785
C14orf119	cg08253809	14	23568020	0.033	0.1935	0.3079
HADHA	cg01188578	2	26464060	0.034	0.2665	0.4863
	cg18182981	6	1449399	0.037	0.6137	0.7345
MOSC2	cg12466610	1	220950200	0.0394	0.1258	0.3988
FLJ42875	cg08121686	1	2981840	0.0397	0.5734	0.714
	cg10588622	2	45397780	0.0421	0.2567	0.3627
SPTBN1	cg01286930	2	54783770	0.0458	0.4365	0.5427
	cg07044115	10	123100200	0.0478	0.5947	0.6962
PMF1	cg25465065	1	156198400	0.0486	0.5544	0.7344
	cg01118640	14	52816920	0.0494	0.6159	0.7683

The genes tagged by "*"are associated with B lymphocytes, counted by the Houseman method, with *P*-value < 0.05 in line regression

pyrosequencing assay also covered 2 adjacent CpG loci, where methylation levels were also higher in the obese group: For *FYN* CpG2 (GRCh37/hg19 112165053) the mean was 9.5% (8.2–24.2%) in the obese group vs. 8.7% (7.5–17.4%) in the control group (*P*=0.004); for *FYN* CpG3 (GRCh37/hg19 112165057) the mean was 16.6% (13.6–28.9%) in the obese group vs. 14.6% (12.3–21.5%) in the control group ($P = 0.031$). In contrast, methylation levels of CpG sites related to *PIWIL4* and *TAOK3* were lower in the obese group: median methylation levels for *PIWIL4* Cg 6436762 were 26.8% (20.2 – 31.9%) in obese cases and 32.3% (25.1 – 37.9%) in controls ($P = 0.003$) (**Table 3**, **Fig. 2B**); for *TAOK3* Cg17627898, methylation levels were 23.7% (19.7 – 28.3%) in the obese group compared to 27.2% (23.0 – 32.0) in the control group ($P = 0.001$) (**Table 3**, **Fig. 2C**). Genotyping analysis in all subjects excluded the presence of SNPs at the cytosine of the DMCpGs within *PIWIL4, FYN*, and *TAOK3*.

Logistic models were built using case vs. control status as the outcome, and methylation of the CpG and gender used as predictors for each CpG of interest. All models were found to differ significantly from the null models, with CpG methylation being a significant predictor of case vs. control status for each model and *P*-values of 0.001 for *TAOK3*, 0.009 for *PIWIL4*, and 0.011 for *FYN3*. Nagelkerke R squared (a measure of the variation explained by each model) was between 7 and 12%. Gender was not a significant predictor of case vs. control status when CpG methylation was controled for. These models indicated that for each 1% increase in methylation of *TAOK3*, the odds of being a case increased by a factor of 1.11 for both genders.

A scatterplot matrix of methylation for all CpGs indicated high correlation between *FYN* CpGs1, 2, and 3 and also between the identified CpG loci associated with *TAOK3* and *PIWIL4*, with Spearman correlation coefficients for highly correlated CpGs ≥ 0.89 (**Fig. S1** and **Table S4**). It was not possible to include all CpGs in a regression model as a result of co-linearity in predictor variables. One CpG from each group of highly correlated CpGs was entered into a logistic regression model including gender. A logistic regression model with case vs. control status as outcome and *FYN* CpG3, *TAOK3*, and sex as predictor variables was significantly different from the null model ($P = 0.002$) and explained 13.8% of the variance (Nagelkerke R square = 0.138). An increase in methylation of 1% in *TAOK3* multiplicatively decreased the odds of being a case by 0.91 (95% CI: 0.86 – 0.97) ($P = 0.005$), all other variables in the equation being held constant; an increase of 1% methylation in *FYN* CpG3 multiplicatively increased the odds of being a case by 1.03 (95% CI: 0.99 – 1.07) ($P = 0.114$).

Given the high correlation between the methylation status of *TAOK3* and *PIWIL4*, we run Gapped Local Alignment of Motifs (GLAM2), which is part of the motif-based sequence analysis suite MEME (http://meme.nbcr.net/meme),[22] to determine whether there was any commonality between the sequences surrounding these CpG sites. No statistically signicant common motif was found within 75 bp at either side of the identified CpGs associated with *PIWIL4* or *TAOK*.

Pathway analysis

The extended list of CpGs with $P < 0.05$ for control vs. obese and a methylation difference of more than 5%, excluding those

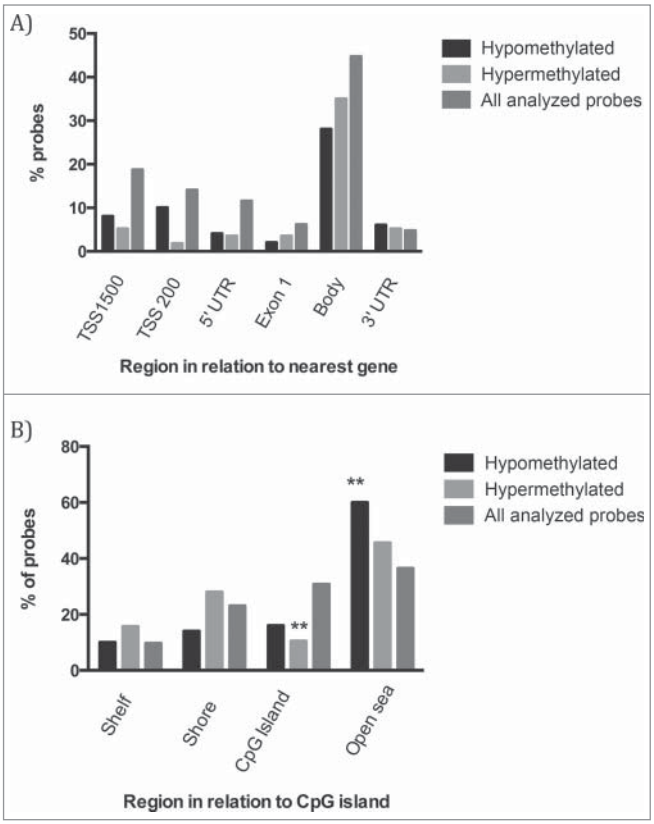

Figure 1. Distribution of hypo- and hypermethylated DMCpGs versus all analyzed CpGs sites on the Infinium HumanMethylation450 BeadChip in relation to (**A**) the nearest gene regions; (**B**) CpG island regions. Chi-square analysis was performed to test for over- or under-representation of sequence features among the DMCpGs. * = *P*-value < 0.05.

CpGs associated with cell type, was significantly enriched for multiple Gene Onotology (GO) processes involved in developmental processes, immune system regulation, regulation of cell signaling, and small GTPase-mediated signal transduction (**Table S5**). Among the significantly enriched GO molecular functions were ion binding, phosphotransferase activity, protein kinase activity, collagen binding, and Ras guanyl nucleotide exchange factor activity (**Table S6**).

Discussion

In this study, we identified 1879 CpG sites in peripheral blood cells with a greater than 5% difference in methylation between obese and control children, of which 129 CpGs had a greater than 10% difference in methylation. Both hypo- and hyper-methylation of CpGs loci in relation to obesity were observed. Interestingly, most of the differentially methylated CpGs were found within open seas or intergenic regions, with a paucity of DMCpGs within CpG islands. This is consistent with previous findings, which have suggested that DNA methylation may be more dynamically regulated outside CpG islands. For instance, tissue-specific DNA methylation often occurs in shores outside the CpG islands,[23] while disease associated DMCpGs and environmentally induced epigenetic changes, such as those induced by exercise intervention, have been shown to be enriched within intergenic regions.[24-26] The enrichment of DMCpGs within the intergenic regions may reflect the location of regulatory elements, such as enhancers or insulators, which are frequently located within the intergenic regions.[27]

The associations between the methylation of specific CpG loci within *PIWIL4, TAOK3*, and *FYN* in peripheral blood and childhood obesity observed in the genome wide-array were validated by sodium bisulfite pyrosequencing. Validation by pyrosequencing showed a signficant association between the methylation of these CpG loci and obesity with the direction of association, consistent with that observed by the 450K array. There was a difference in the magnitude of the methylation change observed between the case and control groups between the 2 techniques, but this may reflect the fact that additional subjects were analyzed in the validation step when using pyrosequencing and that different methods were used to assess DNA methylation. A number of previous studies have also reported small differences in DNA methylation levels measured by 450K arrays in comparison with pyrosequencing.[28,29]

DNA methylation patterns are often tissue specific; whether and how the methylation of such marks in blood may reflect methylation in other more metabolically relevant tissues is not known. However, there have been studies that show consistency in methylation between tissues. For instance, Murphy et al. have shown that methylation across the DMRs for *H19, MEST*, and *PEG10* did not differ across a range of conceptual tissues (buccal, brain, eye, intestine, liver, lung, muscle, and umbilical cord blood).[30] Talens et al. found that, for a number of non-imprinted genes, DNA methylation levels measured in blood were equivalent in buccal cells, despite the fact that these cell types arise from different germ layers (mesoderm and ectoderm, respectively).[31] But whether altered methyaltion of the CpGs studied here are causally involved in the development of obesity or simply markers of the disease state is unknown. Interestingly,

Table 3. Methylation differences between obese (n = 79) and controls (n = 71) groups at the CpG loci identified within *FYN, IGFBP3, PIWIL4*, and *TAOK3*

CpG Site	Median Methylation Cases	Median Methylation Controls	*P*-value
FYN CpG 1 (Cg26846943) Chr6:112165062	12.2 (10–25.7)	10.8 (9.2 – 18.2)	0.012
FYN CpG 2 Chr6: 112165053	9.5 (8.2–24.2)	8.7 (7.5–17.4)	0.031
FYN CpG 3 Chr6: 112165057	16.6 (13.6–28.9)	14.6 (12.3–21.5)	0.004
PIWIL4 (Cg16436762) Chr11:94307971	26.8 (20.2–931.9	32.3 (25.1–37.9)	0.003
TAOK3 (Cg17627898) Chr12:118782453	23.7 (19.7 – 28.3)	27.2 (23.0 – 32.0)	0.001

Median and interquartile range are shown. *FYN* 112165062, *TAOK3* 118782453, *PIWIL4* 9430797

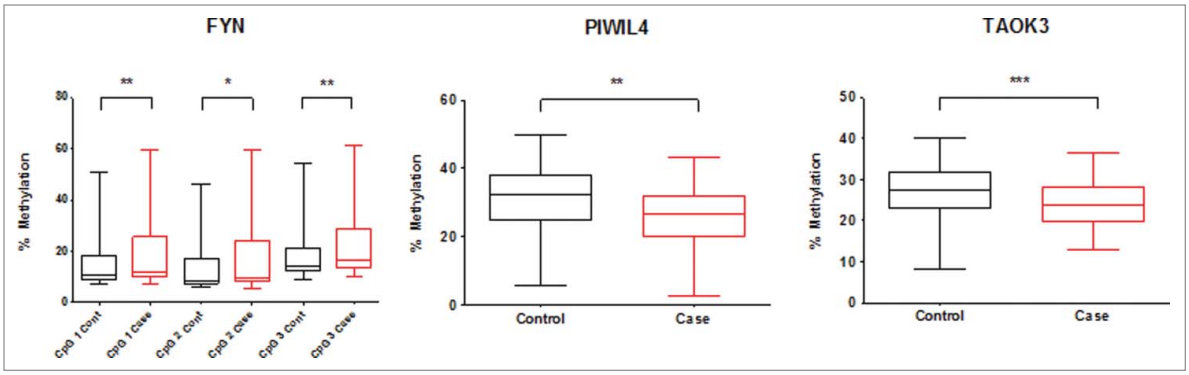

Figure 2. Percentage methylation differences between obese (n=79) and controls (n=71) at (**A**) *FYN*; (**B**) *PIWIL4* (Cg16436); and C) *TAOK3*. * = *P*-value < 0.05, ** = *P*-value <0.01.

FYN has been previously linked to adipocyte development and function. *FYN* is a member of the Src family of non-receptor tyrosine kinases and plays an important role in inflammation,[32,33] adipocyte differentiation,[34] energy expenditure,[35] and insulin signaling.[36] *FYN* knockout mice displayed increased glucose clearance and whole body insulin sensitivity associated with decreased adiposity resulting from increased fatty acid use and energy expenditure,[37] and preferentially laid down adipose tissue in the subcutaneous, rather than the visceral, compartment and showed reduced inflammatory cell infiltration.[38] *FYN* knockout mice also remain fully glucose-tolerant and insulin-sensitive, even on a high-fat diet. These metabolic characteristics have been suggested to result from the regulation of LKB1 and AMPK by FYN in skeletal muscle and adipose tissue.[39] The methylation of the 3 *FYN* CpG sites measured in this study, which lie in the 5′UTR of *FYN*, were highly correlated, suggesting that these CpG sites may be similarly regulated. For all 3 sites, higher methylation was associated with obesity. To date, however, little is known about the role that the 5′ UTR plays in the regulation of *FYN* expression or the effect that methylation in this region may have on transcription.

PIWIL4, which belongs to the Argonaute protein family, exhibits a ubiquitous expression pattern in human tissues and recent studies have shown that over-expression of *PIWIL4* results in a 7-fold increase in the dimethylation state of H3K9 at the first intron of the $p16^{INK4a}$ gene and decreased $p16^{INK4a}$ expression,[40] suggesting that PIWIL4 may be a regulator of $p16^{INK4a}$, a known regulator of adipocyte proliferation, differentiation, and senescence.[41]

TAOK3 activates p38, inhibits cJun NH_2-terminal kinase (JNK) signaling,[42] and is a member of the mitogen activated protein kinase (MAPK) cascade, affecting fundamental cellular signaling pathways. JNK and MAPK signal transduction pathways are activated by obesity and are required for obesity-induced insulin resistance.[43,44] These effects have been seen in adipose, muscle, and hypothalamic tissues.[44] A mouse study has shown that *JNK* expression by macrophages promotes obesity-induced insulin resistance.[45] In humans, indirect evidence of the importance of this locus comes from a study on a French Canadian population that identified a genetic region overlapping *TAOK3* that was associated with anthropometric and metabolic traits.[46] Similarly to the previous 2 loci, *TAOK3* has a strong biological premise for an association with obesity; however, our data provide the first evidence that methylation of a *TAOK3*-related CpG site may be a biomarker of obesity.

Interestingly, the methylation status of identified CpG sites within *PIWIL4* and *TAOK3* were negatively associated with obesity and highly correlative with each other, suggesting that the obese phenotype may be associated with a hypomethylated state. TAOK3 is involved in cellular development and growth and recent studies have also implicated PIWIL4 in regulating the expression of the cell cycle inhibitor $p16^{INK4a}$, suggesting that these genes may work within the same network, which may account for the high correlation observed between the methylation status of their CpG sites.

Pathway analysis revealed an enrichment of DMCpGs in GO processes involved in developmental processes, immune system regulation, regulation of cell signaling, and small GTPase-mediated signal transduction. Enrichment of genes involved in developmental process concurs with the growing body of literature showing that obesity has a strong developmental component.[47] Alterations in pathways associated with immune function are consistent with the low grade chronic inflammation characteristic of the obese state.[48] Small GTPases have also been shown to play a critical role in the inflammatory response as well as cell proliferation, differentiation, and survival.[49] Whether the differential methylation of CpGs within genes associated with inflammation and cell signaling is a consequence or cause of the inflammation associated with obesity is unknown at present. However, these findings do suggest that altered methylation of such sites may act to sustain inflammation associated with obesity. Caution is nevertheless required when interpreting the enrichment of inflammation and immune pathways in whole blood samples.

Other human studies have begun to demonstrate associations between epigenetic changes and obesity.[50-52] Dick et al. (2014) found that methylation in the first intron of the *HIF3A* gene measured in blood strongly correlated with adult BMI and this result was validated across independent cohorts including obese subjects.[53] Milagro et al. found differences in methylation between *CLOCK* and *BMAL1* genes in overweight/obese

patients compared to normal weight women.[50] Xu et al. showed a substantial number of differentially methylated CpG sites between obese and non-obese adolescents (14–20 years old).[51] Interestingly, a number of these differentially methylated CpG sites were found to be associated with genes previously linked to obesity and Type 2 Diabetes, such as *FTO, GCK, HNF1A* and *HNF1B, PPARγ*, *PTEN*, and *TCF7L2*. In a separate study, Wang et al. reported methylation changes in genes associated with inflammatory response, immune response, cytokine production, and antigen binding in obese compared to lean adolescents.[52] In our study, we investigated methylation changes associated with severe obesity in peripheral blood of children aged 6–14 years, an age group not previously examined. Differences in the age of the individuals studied, the characteristics used to define the cases, the use of DNA either from whole blood, as used here, or purified leucocytes, together with differences in the techniques used to measure "epigenome-wide" methylation status, may explain some of the differences in DMCpGs identified in these different studies. Nevertheless, a number of the DMCpGs identified in this study in children 6–14 years old were also identified in the studies by Wang et al. (*TRIM3, HIPK, NOTCH4*) [52] and Xu et al. (*FTO, PTEN*),[51] who examined methylation changes associated with obesity in adolescents, suggesting similar changes in methylation are observed in relation to obesity in both age groups.

Several limitations of this study need to be recognized. First, we used a pooling strategy for the 450K array analysis. Methylation analysis on pooled genomic DNA has previously been shown to provide an accurrate estimate of DNA methylation.[54,55] and effective means for identifying DNA methylation changes associated with a diseased phenotype.[14,56] when DNA availibility is limited. However, pooling does prevent insights on inter-individual methylation differences. Nevertheless, our pooling strategy successfully identify 3 CpG loci that were validated by pyrosequencing in individual samples, thereby practically demonstrating that it may be a useful approach to identify robust changes associated with particular phenotypes. The second limitation to this study was that we used DNA from whole blood, which represents different cell populations with distinct epigenetic profiles. To correct for the differences in methylation resulting from differences in cellular heterogeneity, the Houseman correction was used,[21] and CpGs related to the different cell populations were not taken forward for subsequent validation or pathway analysis. However, we cannot rule out the possibility that some of the differences observed in methylation between case and control groups may result from the presence of cell types not accounted for within the algorithm. Nevertheless, even if these changes do represent changes in cellular composition, our studies suggest that altered methylation of the identified CpG loci and pathways are markers of childhood obesity. The third limitation was that we only excluded the possibility of SNPs within 45 bp of the CpG influencing methylation status of the CpGs of interest, as methylated quantitative trait loci are overwhelmingly found in cis and peak enrichment for SNP to CpG distance is within 45 bp,[57] however, there is a possibility that SNPs further away or in trans may mediate methylation at these sites. A strength of this study is that all the participants studied were young, between 9–14 years old, and therefore the results are less likely to be confounded by obesity comorbidities or medication use, both of which are very common in adult subjects with obesity.

In conclusion, we have identified widespread DNA methylation changes in whole blood associated with childhood obesity, providing evidence that epigenetic dysregulation is associated with obesity in children. Although further studies are required to determine the causality of such changes, the identification of such alterations may provide novel insights into the development of obesity and potential biomarkers to identify those individuals at increased risk of disease.

Materials and Methods

Cohort characteristics

Children seeking treatment for obesity at the tertiary pediatric hospital Princess Margaret Hospital for Children (PMH) outpatient department in Western Australia were recruited as obese cases. The criteria for entry were: a age- and sex-adjusted BMI Z-score greater than 2.5 with 2 additional comorbidities or a BMI Z-score greater than 3.0. BMI Z-scores were individually calculated based on the Center for Disease Control and Prevention (CDC) growth charts.[58] Age- and sex-matched controls were taken from the longitudinal Childhood Growth and Development (GAD) study of Western Australia, which followed children aged 6 to 14 years at recruitment annually for 3 years.[59] In this study, healthy weight controls were recruited from randomly selected primary schools in the Perth metropolitan area. The PMH Ethics Committee approved the study. **Table 1** shows the characteristics of all the samples used for pyrosequencing (validation studies). **Table S1** shows the cohort characteristics of the subjects whose DNA was pooled for the 450K analysis.

Infinium humanMethylation450 beadchip array

DNA was prepared from whole blood cells of 54 case and 54 control subjects using a standard phenol:chloroform extraction and ethanol precipitation, as described previously.[16] Four pools of DNA from obese subjects were used (Pool 1: Males, high-fasting insulin, n = 13; Pool 2: Males, low-fasting insulin, n = 12; Pool 3: Females, high-fasting insulin, n = 14; Pool 4: Females, low-fasting insulin, n = 15). Pools from age- and sex-matched control groups were: Pool 1 Control, Males, n = 13; Pool 2 Control, Males, n = 12; Pool 3 Control, Females, n = 14; Pool 4 Control, Females, n = 15) (**Table 1** and **Table S1**). Genomic DNA (1 ug) from each of the 8 pools was bisulphite-converted using Zymo EZ DNA Methylation-Gold kit (ZymoResearch, Irvine, California, USA, D5007) and the DNA was analyzed using the Infinium HumanMethylation450 platform (Illumina, Inc., CA, USA) by The Genome Center, Barts and London, School of Medicine and Dentistry, John Vane Science Center, Charterhouse Square, London.

Infinium HumanMethylation450 BeadChip array data processing

Signal extraction was performed in GenomeStudio™ Methylation Module on the intensity files (.idat) produced by the Illumina iSCAN system. Raw β-values were extracted from GenomeStudio without further data processing (i.e., no background subtraction or normalization). CpGs with 2 beads or less for either methylated or unmethylated signal, for any sample, or with signal detection *P*-values (calculated from the individual bead intensities) higher than 0.05, for any sample, were discarded for all samples. A total of 479,379 CpGs remained for further analysis. To correct for color imbalance, values were normalized to values from control probes and background subtracted. After these intra-sample normalization procedures, M-values (logit transformation) were calculated. The Type II M-value range was fitted to the Type I range, as suggested by Dedeurwaerder et al.[60] β-values were then calculated. Finally, inter-sample normalization was performed by quantile normalization. This procedure has been described and benchmarked in Pan et al., 2012.[61] No batch or chip effects were possible, as samples were contained within one array and processed together. We performed one pool run as a replicate of the cases and another pool run as a replicate of the controls. Pearson correlation (R) within replicates were 0.9987 and 0.9989 for case and control, respectively. Principal component analysis showed replicates were clustered together. Therefore, final β-values were averaged between replicates. The four pools of obese subjects were compared to the 4 pools of control subjects, using paired t-tests, pairing the pools by gender and insulin resistance status, to determine changes associated with obesity and independent of sex and fasting insulin levels.

Estimation of cell fractions from the methylation data

To assess the potential impact of variation in cell fraction between samples, we used R package minfi.[62] to estimate the fraction of CD8T-, CD4T-, NK- and B-cells, monocytes, and granulocytes in the samples. The R package minfi allows for estimating cell fractions in Illumina 450K methylation data from whole blood (**Table S3**). This method is based on the methylation data published for flow-sorted cells,[63] and algorithms derived from the study by Houseman et al.[21]

Metacore pathway analysis

The Metacore Pathway Analysis software was used to investigate whether the genes mapped to differentially methylated CpGs were enriched for specific functional relationships.

Pyrosequencing

DMCpGs were validated using sodium bisulfite pyrosequencing. Genomic DNA was prepared and bisulphite conversion was carried out using the EZ DNA Methylation-Gold kit (ZymoResearch, Irvine, California, USA; D5007). The pyrosequencing reaction was carried out using primers listed in **Table S5**. Modified DNA was amplified using HotStar Taq Plus DNA polymerase (Qiagen, Germany; 203605). PCR products were immobilized on streptavidin–sepharose beads (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK; GZ17511301), washed, denatured, and released into annealing buffer containing the sequencing primers. Pyrosequencing was carried out using the PyroMark Gold Q96 Reagent kit (Qiagen, Germany; 972824) on a PyroMark Q96 MD machine (Biotage, Uppsala, Sweden) and the % methylation was calculated using the Pyro Q CpG software (Biotage). Within-assay precision was between 0.8 and 1.8% and detection limits were 2–5% methylation. SNP PCR was carried out to exclude the presence of SNPs at the CpGs of interest using primers listed in **Table S7**. DNA was amplified using HotStar Taq Plus DNA polymerase. PCR Products were treated as for pyrosequencing. Genotyping analysis was carried out using PyroMark MD 1.0 software (Biotage). Genotyping analysis in all subjects excluded the presence of SNPs at the cytosine of all 3 DMCpGs within *PIWIL4, FYN*, and *TAOK3*. Primers and sequencing probes were also designed to exclude any known SNP.

Statistical Analysis of Pyrosequencing Data

Statistical analysis was carried out using SPSS version 21.0 (IBM). Histograms of all continuous variables were plotted to check for normality. The distributions of methylation were not normally distributed and differences in methylation were therefore calculated using the non-parametric Mann-Whitney U test. Logistic regression models were built using case control status as the outcome for each CpG. Methylation of the particular CpG and gender were entered into the model as predictors. A separate model was formulated for each CpG of interest.

Chi-Square Analysis

Analysis was carried out to determine if the distribution of hypomethylated (or hypermethylated) CpG sites was the same as the distribution of analyzed probes for each gene location (TSS200, 3′UTR, etc.) and for each gene environment (island, shore, etc.). This analysis was performed using chi-square goodness of fit tests, if appropriate, or exact tests, if 20% or more of the expected cell frequencies were lower than 5 (4 tests in total).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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