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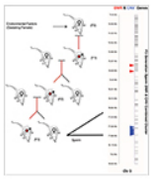

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RESEARCH PAPER

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DNA methylation mediates the impact of exposure to prenatal maternal stress on BMI and central adiposity in children at age 13½ years: Project Ice Storm

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Prenatal maternal stress (PNMS) in animals and humans predicts obesity and metabolic dysfunction in the offspring. Epigenetic modification of gene function is considered one possible mechanism by which PNMS results in poor outcomes in offspring. Our goal was to determine the role of maternal objective exposure and subjective distress on child BMI and central adiposity at 13½ years of age, and to test the hypothesis that DNA methylation mediates the effect of PNMS on growth. Mothers were pregnant during the January 1998 Quebec ice storm. We assessed their objective exposure and subjective distress in June 1998. At age 13½ their children were weighed and measured (n = 66); a subsample provided blood samples for epigenetic studies (n = 31). Objective and subjective PNMS correlated with central adiposity (waist-to-height ratio); only objective PNMS predicted body mass index (BMI). Bootstrapping analyses showed that the methylation level of genes from established Type-1 and -2 diabetes mellitus pathways showed significant mediation of the effect of objective PNMS on both central adiposity and BMI. However, the negative mediating effects indicate that, although greater objective PNMS predicts greater BMI and adiposity, this effect is dampened by the effects of objective PNMS on DNA methylation, suggesting a protective role of the selected genes from Type-1 and -2 diabetes mellitus pathways. We provide data supporting that DNA methylation is a potential mechanism involved in the long-term adaptation and programming of the genome in response to early adverse environmental factors.

Introduction

The developmental origins of adult health and disease hypothesis[1] states that perturbations in early life, including intrauterine exposure to maternal stress, could program metabolic functions and lead to poor health outcomes later in life, such as cardiovascular disease, obesity, and metabolic syndrome. Exposure to high levels of prenatal maternal stress (PNMS) is associated with a perturbation of the hypothalamic-pituitary-adrenal axis,[2] the body's central stress modulating mechanism, which is involved in metabolic pathways.[3]

A growing amount of evidence shows that the induction of stress during pregnancy in animals, or psychological stress during pregnancy in humans, is associated with an increased risk of obesity and metabolic dysfunction in the offspring. In animals, for instance, maternal stress induced a long-lasting disturbance in feeding behavior and dysfunctions related to Type 2 diabetes mellitus in the aged rat.[4] Likewise, prenatal stress increased susceptibility to diet-induced obesity in rat offspring.[5] In another study, prenatal stress has been shown to increase the obesogenic effects of a high-fat-sucrose diet in a sex-specific manner in rat.[6] In mice, exposure to chronic variable stress produced alterations in long-term body weight and energy homeostasis regulation.[7] In a human Dutch famine study, adults who were prenatally exposed were found to have increased body weight, BMI, and waist circumference.[8] Furthermore, in other studies, maternal bereavement during pregnancy was found to predict a high risk

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of Type-1 and -2 diabetes[9,10] in the offspring in late childhood. More recently, maternal stress during pregnancy or during the first year of life was related to increased risk of the infant being overweight.[11] Finally, another study showed that antenatal depression was associated with higher central adiposity in children at the age of 3.[12]

The animal PNMS literature, with its experimental method, can make clear, causal conclusions; we cannot, however, generalize the results to the more complex stress experience in humans.[13] In the human literature, although there is ample evidence of an association between some kinds of prenatal events or conditions and obesity outcomes in children, several issues remain. Retrospective approaches, as in the studies of bereavement, are unable to isolate the active ingredient in the PNMS effect: Are the effects due to the objective hardship experienced as a result of the event, or to the mother's distress? As well, such studies generally fail to assess the severity of the exposure or the severity of the distress, and instead compare exposed and non-exposed groups, a statistical approach that limits power. Next, given that women are not randomly assigned to experience depression or relationship difficulties in pregnancy, studies showing associations between maternal mood or psychosocial stressors and child outcomes cannot disentangle the genetic transmission of maternal traits from effects of the intrauterine environment and from conditions in the postnatal environment. Finally, subjects in retrospective studies may be biased in their reporting of life events since they will be knowledgeable about the outcomes for their child and may be searching for causes.

In order to improve understanding of prenatal stress effects in humans, we need a human model that uses a stressor that is independent of the mother's potential influence, and whose stress severity is randomly distributed. Natural disasters are such events. Project Ice Storm has been following a cohort of children whose mothers were pregnant during the worst natural disaster in Canadian history, the 1998 Quebec ice storm. The strength of this study lies in the independent nature of the stressor, which is not associated with potentially confounding maternal and household characteristics and thereby approximates the random assignment to stress conditions that is possible with laboratory animals. Furthermore, we are able to distinguish between 2 aspects of the maternal stress experience—objective hardship and subjective distress—that is impossible in animal studies and in archive-based or retrospective research. We have previously reported that objective PNMS predicted increased BMI and obesity risk in the Ice Storm cohort at age 5½,[14] as well as greater insulin secretion at age 13½.[15]

The underlying molecular mechanisms responsible for these adverse PNMS effects are still not well understood. Epigenetic modification of gene function is considered to be one mechanism by which PNMS results in poor outcomes in the offspring. DNA methylation is an intensively studied epigenetic mechanism that could be modulated by exposure to a variety of maternal experiences and might participate in processes that "adapt" the genome to stress signals across multiple tissues and explain the broad-ranging effects of early life stress on the fetus.[16,17] Although epigenetic processes, such as DNA methylation, are proposed to be strong candidates as underlying mechanisms through which PNMS could affect metabolic health in offspring, there are few studies that are able to conduct empirical tests of these effects using mediation analyses. Baron and Kenny described a mediating effect as the possible effect of a third variable, intervening in an existing relationship between a predictor and an outcome.[18]

By studying the genome-wide DNA methylation profile in isolated T-cells of the Ice Storm offspring at age 13½, we reported that prenatal maternal objective stress, but not subjective distress, was significantly correlated with methylation of CpG sites on genes related to metabolic function.[19] However, whether and to what extent DNA methylation can mediate PNMS effect on metabolic outcomes, such as BMI and central adiposity, remain to be investigated. The goal of the present study was to determine the impact of PNMS on BMI and central adiposity in adolescence at age 13½, and the potential mediating effect of DNA methylation. We hypothesized that children born to mothers who experienced higher levels of PNMS would exhibit higher body mass index (BMI) and central adiposity [waist-to-height ratio (WHtR)] than those born to mothers who experienced lower levels of PNMS, and that these effects would be mediated by DNA methylation of genes involved in metabolic pathways.

Results

Participants' characteristics

Three children were excluded from analyses because of exposure to maternal gestational diabetes. At the time of anthropometric assessment, the remaining 66 children were on average 13.6 y of age (SD = 0.1) and 95.6% were from households in the middle class and above (lower class, 1.5%; lower-middle class, 2.9%; middle class, 32.4%; upper-middle class, 48.5%; upper class, 14.7%). There were 37 boys and 29 girls. Of these, 9 children (13.6%) were classified as obese (8 boys and 1 girl). The data from a subgroup of 31 subjects, for whom both DNA methylation and body measures were available, were used for further DNA methylation mediation analyses. **Table 1** shows means and standard deviations for maternal variables, child variables, and storm-related variables for both data sets.

Correlations

Objective and subjective PNMS were significantly correlated ($r = 0.338$, $P = 0.005$). Bivariate Pearson product-moment correlations were analyzed between the 2 outcome variables (BMI and WHtR) and the predictors (**Table 2**). Objective PNMS was significantly correlated with WHtR, with a trend-level correlation with BMI ($P = 0.051$), with higher objective PNMS predicting higher scores. Subjective PNMS was not significantly correlated with BMI but was positively associated with WHtR, again with greater PNMS associated with larger waists relative to heights. Sex was marginally significantly related to WHtR ($P = 0.053$) and BMI ($P = 0.081$), with boys having higher scores than girls. More prenatal maternal life events (other than the ice storm) were significantly associated with larger BMI scores and WHtR.

Table 1. Summary of descriptive statistics of study participants

	Subsample (n = 31)		Sample (n = 66)	
	Mean	**SD**	**Mean**	**SD**
Children				
Central adiposity (WHtR)	0.4	0.1	0.4	0.1
Body mass index (BMI)	22.2	4.8	21.1	4.7
Prenatal maternal stress (PNMS)				
Objective	11.2	4.2	11.4	4.6
Subjective (log transformed)	8.9 (1.9)	9.0 (1.0)	10.5 (1.9)	12.5 (1.2)
Timing of exposure (days of pregnancy)	95.7	96.6	101.9	101.8
Mothers				
Hollingshead socioeconomic index (SES)*	28.0	11.4	28.3	11.9
Obstetric complications	4.6	3.2	4.6	2.9
Life events (pregnancy)	5.8	2.9	5.8	3.5
Life events (13½ y)	3.4	2.3	3.5	2.6
GHQ anxiety (June 1998)	0.2	0.3	0.3	0.3
GHQ anxiety (13½ y)	0.1	0.2	0.2	0.3
Children's birth characteristics				
Birth weight (g)	3419.8	664.4	3470.4	586.5
Birth length (cm)	50.7	3.2	50.5	3.0
Birth ponderal index	26.4	3.6	27.2	3.7

Note: BMI = Body Mass Index; SES = Household socioeconomic status,
*higher numbers indicate lower SES; Obstetric complications = number of moderate-severe complications; GHQ = General Health Questionnaire; SD = standard deviation

Hierarchical Linear Regression models (n = 66)

Final, trimmed results of the hierarchical multiple regression of WHtR are presented in **Table 3**. In Step 1, objective PNMS was significantly related to WHtR and explained 10.6% of the variance: higher objective PNMS levels predicted larger waists relative to heights. In Step 2, subjective PNMS was also related to the children's WHtRs, accounting for an additional 5.2% of the variance: higher subjective PNMS levels predicted larger waists relative to heights. Finally, in Step 3, the number of life events in pregnancy was also related to WHtR and accounted for an additional 5.2% of the variance: women who experienced more prenatal life events (excluding the ice storm) had children with higher WHtRs. Together, objective PNMS, subjective PNMS, and prenatal life events accounted for 21.0% of the variance in the children's WHtRs. Neither the child's sex nor timing of exposure significantly explained any additional variance in the children's WHtRs.

Final, trimmed results of the hierarchical multiple regression of BMI are presented in **Table 4**. Objective PNMS predicted BMI at a strong trend level ($P = 0.051$) in Step 1, explaining 5.8% of the variance: higher objective PNMS levels predicted higher BMI levels. Life events in pregnancy accounted for an additional 9.7% of the variance in the children's BMI. Together, objective PNMS and prenatal life events accounted for 12.5% of the variance in the children's BMI levels. None of the other predictors (subjective PNMS, child's sex, timing of exposure) significantly explained additional variance in the children's BMI.

Table 2. Pearson correlations between outcome measures and predictors (n = 66)

	Central adiposity (WHtR)		Body Mass Index (BMI)	
Predictors	**r**	***P***	**r**	***P***
Objective PNMS (Storm32)	**0.326**	**0.012**	**0.241**	**0.051**
Subjective PNMS (IES-R)	**0.307**	**0.018**	0.157	0.207
Sex of child (males = 1, girls = 2)	**−0.253**	**0.053**	**−0.217**	**0.081**
Timing of exposure (days of pregnancy)	−0.102	0.444	−0.040	0.748
Socioeconomic status	0.014	0.917	0.042	0.739
Obstetric complications	0.067	0.615	0.060	0.632
Life events (pregnancy)	**0.370**	**0.004**	**0.317**	**0.009**
Life events (13½ y)	0.217	0.108	0.149	0.248
Anxiety (June 1998)	−0.108	0.417	−0.140	0.262
Anxiety (13½ y)	−0.072	0.594	−0.079	0.537
Birth weight (g)	0.035	0.793	0.067	0.593
Birth length (cm)	0.049	0.713	0.075	0.551
Birth ponderal index	−0.028	0.835	−0.056	0.659

Table 3. Hierarchical multiple regression of central adiposity (WHtR) (n = 66)

Predictor variable	β	R	R^2	ΔR^2	F	ΔF
STEP 1		0.326	0.106		6.761*	
Objective PNMS	0.326*					
STEP 2		0.397	0.158	0.052	5.249**	3.446#
Objective PNMS	0.262*					
Subjective PNMS	0.236#					
STEP 3		0.458	.210	0.052	4.873***	3.630#
Objective PNMS	0.210					
Subjective PNMS	0.160					
Life events (pregnancy)	0.251#					

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, #$P < 0.1$.

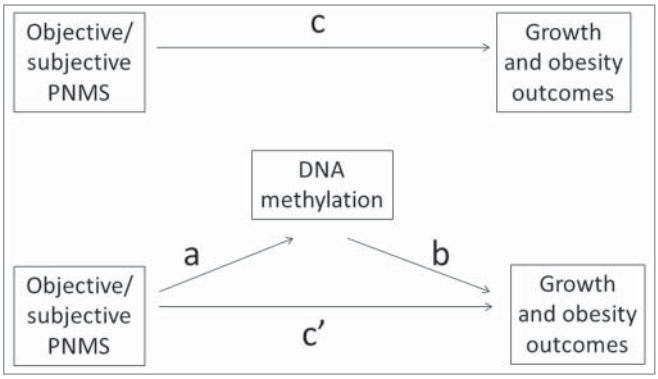

Figure 1. Mediation analysis on the relationship between exposure to objective/subjective PNMS and growth outcomes. The path coefficients are known as the direct effects, indirect effects, and total effects. The direct effect indicates the effect of a risk factor on an outcome controlling for the mediators; the indirect effect (mediating effect) indicates the effect of the risk factor on an outcome variable through an intervening variable; the total effect is the full effect of risk factor on the outcome. It represents the sum of direct and indirect effects of the path. Path a is the effect of the objective/subjective PNMS (predictor variable) on the DNA methylation (mediator), path b is the effect of the DNA methylation on growth outcomes (outcome variable) controlling for the objective/subjective PNMS, and path c′ is the direct effect of the objective/subjective PNMS on growth outcomes controlling for the DNA methylation. The path a*b indicates the indirect effect/mediating effect of objective/subjective PNMS on growth outcomes through DNA methylation (mediator). Path c is the total effect of objective/subjective PNMS on growth and obesity outcomes. This model can be represented by the following equation: c = c′+ a*b.

Mediation analysis (n = 31)

The bootstrapping analyses for the selected genes from the Type 1 diabetes pathway revealed 17 genes (57 CpG sites) for which there was a negative mediating effect (path a*b) (**Fig. 1**) between objective PNMS and BMI (**Table 5**) and 15 genes (51 CpG sites) negatively mediating the effect on central adiposity (**Table 6**). The average effect size (ES) of the mediation analyses for CpG sites in the Type 1 pathway was 0.18 (range: 0.09–0.31) for BMI and 0.18 (range: 0.11–0.31) for central adiposity. Among the selected genes from the Type 2 diabetes pathway, we found 10 genes (16 CpG sites) negatively mediating the effect of objective PNMS on BMI (**Table 5**) and 7 genes (11 CpG sites) negatively mediating the effect on central adiposity (**Table 6**). The average ES of the mediation analyses for CpG sites in the Type 2 pathway was 0.16 (range: 0.09–0.26) for BMI and 0.16 (range: 0.12–0.22) for central adiposity. All above CpGs showing significant mediating effects from both Type-1 and -2 diabetes mellitus were arranged into groups (**Table 7**). Track on the screenshot of Integrative Genomics Viewer (IGV) window marked their location (**Fig. S1**). **Table S2** lists the effects of objective PNMS on DNA methylation (path a), the effects of the DNA methylation on BMI and central adiposity controlling for objective PNMS (path b), the direct effects (path c'), and mediating effects (path a*b) for each CpG site. From that table, we observe that, in nearly all cases of mediation, the higher the objective PNMS (Storm32 score), the greater the methylation of the CpG; the greater the methylation of the CpG, the lower the BMI and central adiposity in the adolescent. In other words, although the total and direct effects of objective stress on BMI and WHtRs are positive (greater stress, greater BMI and central adiposity), the mediation via DNA methylation results in limiting the effect of the objective stress (greater stress, greater methylation; greater methylation, lower BMI and central adiposity), suggesting that the DNA methylation of these selected CpGs, at least, protects from the adverse metabolic outcomes. Remarkably, none of the genes we tested were found to mediate the effect of subjective PNMS on BMI or central adiposity.

Table 4. Hierarchical multiple regression of body mass index (BMI) (n = 66)

Predictor variable	β	R	R^2	ΔR^2	F	ΔF
STEP 1		0.241	0.058		3.943#	
Objective PNMS	0.241#					
STEP 2		0.353	0.125	0.097	4.498*	4.871*
Objective PNMS	0.238#					
Life events (pregnancy)	0.300*					

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, #$P < 0.1$

Discussion

The first aim of this study was to test the effects of objective and subjective PNMS and other risk factors on BMI and central adiposity in 13½ year-old adolescents. The hierarchical multiple regression analyses revealed that objective and subjective PNMS were both independently related to central adiposity, while only objective PNMS was related to BMI. Life events in pregnancy other than the ice storm explained additional, unique variance in central adiposity beyond that explained by objective and subjective ice storm stress. The second aim was to determine the extent to which DNA methylation could mediate the impact of PNMS on these outcomes. The methylation levels of genes selected from established diabetes genes in both Type-1 and -2 diabetes mellitus pathways showed significant negative mediation of the effect of objective PNMS on both central adiposity and BMI,

Table 5. Genes and their mediating effects on the impact of objective PNMS on BMI (n = 31)

			Mediation effect					
Diabetes Mellitus Pathways	**Gene**	**CpG Site**	**Effect (a*b)**	**SE[a] Boot**	**Boot LLCI[b]**	**Boot ULCI[c]**	**Significant Indication**	**Effect Size**
Type 1	LTA	cg09736959	**−0.347260**	0.155926	−0.715874	−0.088977	TRUE	0.277368
	LTA	cg11586857	**−0.322884**	0.136606	−0.665665	−0.110514	TRUE	0.306886
	LTA	cg24216966	**−0.318009**	0.145291	−0.688993	−0.092503	TRUE	0.257337
	LTA	cg16280132	**−0.306870**	0.137828	−0.614909	−0.066532	TRUE	0.234269
	LTA	cg10476003	**−0.305826**	0.129363	−0.663911	−0.110133	TRUE	0.264344
	LTA	cg02402436	**−0.284806**	0.126782	−0.626809	−0.093500	TRUE	0.247945
	LTA	cg26348243	**−0.282170**	0.142773	−0.578100	−0.022185	TRUE	0.216861
	NFKBIA	cg00689225	**−0.281652**	0.129977	−0.622969	−0.094709	TRUE	0.256262
	LTA	cg21999229	**−0.277587**	0.129483	−0.604441	−0.074913	TRUE	0.227873
	LTA	cg01157951	**−0.277478**	0.125938	−0.595722	−0.078583	TRUE	0.226326
	LTA	cg13815684	**−0.272675**	0.120631	−0.585075	−0.089894	TRUE	0.225331
	LTA	cg17169196	**−0.263933**	0.135148	−0.633542	−0.072668	TRUE	0.221260
	LTA	cg14597739	**−0.251420**	0.131776	−0.592555	−0.047685	TRUE	0.204156
	LTA	cg09621572	**−0.245711**	0.136112	−0.639000	−0.059165	TRUE	0.205793
	CD3D	cg03074244	**−0.245089**	0.127708	−0.578766	−0.060561	TRUE	0.199088
	LTA	cg16219283	**−0.237365**	0.131275	−0.584865	−0.046882	TRUE	0.192078
	TNFRSF1B	cg22677556	**−0.228751**	0.138247	−0.595856	−0.047750	TRUE	0.188050
	CD3D; CD3G	cg13750061	**−0.228036**	0.118148	−0.530580	−0.056249	TRUE	0.182519
	LTA	cg14441276	**−0.226895**	0.120870	−0.568164	−0.056637	TRUE	0.194485
	IL1RAP	cg16588163	**−0.226822**	0.122115	−0.555198	−0.047381	TRUE	0.184506
	LTA	cg14437551	**−0.226212**	0.109446	−0.565216	−0.075606	TRUE	0.201670
	CD3D	cg07728874	**−0.222742**	0.119104	−0.532439	−0.048312	TRUE	0.181321
	CD3D	cg24841244	**−0.219280**	0.121170	−0.529188	−0.043758	TRUE	0.179665
	CD247	cg09473725	**−0.215190**	0.113564	−0.518957	−0.052847	TRUE	0.183492
	CD3D; CD3G	cg07545925	**−0.214677**	0.116189	−0.527428	−0.054572	TRUE	0.182083
	NFKBIA	cg16518861	**−0.214289**	0.108513	−0.523565	−0.059897	TRUE	0.209952
	CD3G	cg15880738	**−0.208559**	0.135924	−0.576549	−0.032014	TRUE	0.167712
	CD247	cg14278300	**−0.206255**	0.131950	−0.561283	−0.024637	TRUE	0.167662
	CD3E	cg24612198	**−0.201783**	0.103936	−0.484214	−0.054387	TRUE	0.173863
	CD3D; CD3G	cg03254928	**−0.197920**	0.120710	−0.526256	−0.035334	TRUE	0.160146
	CD3D; CD3G	cg05160234	**−0.197702**	0.114469	−0.491153	−0.031189	TRUE	0.161086
	FASLG	cg00071250	**−0.196267**	0.113635	−0.508255	−0.042060	TRUE	0.171230
	CD247	cg09032544	**−0.195888**	0.116160	−0.505789	−0.031706	TRUE	0.169786
	CD3D	cg25643644	**−0.195828**	0.111152	−0.498899	−0.042313	TRUE	0.172657
	FASLG	cg10161121	**−0.190920**	0.109249	−0.479510	−0.033093	TRUE	0.160139
	BCL2	cg08223235	**−0.188597**	0.093623	−0.449632	−0.056644	TRUE	0.170653
	HLA-E	cg27486585	**−0.187989**	0.119293	−0.507821	−0.019188	TRUE	0.157915
	FASLG	cg06983746	**−0.187111**	0.107178	−0.483365	−0.041451	TRUE	0.160957
	MAP3K14	cg16826777	**−0.186886**	0.104196	−0.472710	−0.039611	TRUE	0.164964
	CD3E	cg06164961	**−0.185807**	0.117338	−0.494745	−0.016541	TRUE	0.152406
	CD247	cg07786657	**−0.185502**	0.113439	−0.500028	−0.034892	TRUE	0.162032
	IRF1	cg15375424	**−0.180211**	0.107431	−0.462510	−0.025189	TRUE	0.154448
	HLA-DMB	cg10453850	**−0.173834**	0.111797	−0.481024	−0.026027	TRUE	0.149093
	HLA-DMB	cg01374870	**−0.165360**	0.123216	−0.515515	−0.019282	TRUE	0.139271
	HLA-E	cg21366673	**−0.165179**	0.110690	−0.467698	−0.013897	TRUE	0.141012
	HLA-E	cg17615629	**−0.160185**	0.095097	−0.410258	−0.022332	TRUE	0.130362
	HLA-E	cg05201185	**−0.159688**	0.109593	−0.473055	−0.018627	TRUE	0.136209
	CD247	cg13210595	**−0.155554**	0.082519	−0.388346	−0.038534	TRUE	0.139662
	HLA-DOB	cg27246453	**−0.153887**	0.101444	−0.458761	−0.019558	TRUE	0.130435
	SOCS1	cg03014241	**−0.153199**	0.103512	−0.437169	−0.010281	TRUE	0.130685
	TNFRSF1B	cg05599723	**−0.142988**	0.094263	−0.404934	−0.019966	TRUE	0.127840
	HLA-DMB	cg13524037	**−0.132227**	0.090252	−0.398518	−0.008246	TRUE	0.116794
	CD28	cg24336674	**−0.129244**	0.095151	−0.414118	−0.000531	TRUE	0.111746
	HLA-E	cg16535080	**−0.125698**	0.097292	−0.409652	−0.007357	TRUE	0.108237
	CD247	cg03727968	**−0.122704**	0.085253	−0.365787	−0.014490	TRUE	0.108399
	HLA-E	cg19109457	**−0.115851**	0.081092	−0.347117	−0.006804	TRUE	0.099030
	TNFRSF1B	cg15526535	**−0.099339**	0.065544	−0.274318	−0.001141	TRUE	0.086658
Type 2	PIK3CD	cg01320698	**−0.295446**	0.134437	−0.638886	−0.090645	TRUE	0.235502
	NFKBIA	cg00689225	**−0.281652**	0.129977	−0.622969	−0.094709	TRUE	0.256262

(*Continued on next page*)

Table 5. Genes and their mediating effects on the impact of objective PNMS on BMI (n = 31) *(Continued)*

			Mediation effect					
Diabetes Mellitus Pathways	**Gene**	**CpG Site**	**Effect (a*b)**	**SE[a] Boot**	**Boot LLCI[b]**	**Boot ULCI[c]**	**Significant Indication**	**Effect Size**
	PIK3CD	cg07970040	**−0.237896**	0.128254	−0.571491	−0.048734	TRUE	0.196559
	TNFRSF1B	cg22677556	**−0.228751**	0.138247	−0.595856	−0.047750	TRUE	0.188050
	NFKBIA	cg16518861	**−0.214289**	0.108513	−0.523565	−0.059897	TRUE	0.209952
	ACSL6	cg14841483	**−0.202179**	0.129974	−0.547472	−0.031100	TRUE	0.162682
	MAP3K14	cg16826777	**−0.186886**	0.104196	−0.472710	−0.039611	TRUE	0.164964
	PRKCH	cg14001486	**−0.178253**	0.114396	−0.489453	−0.018176	TRUE	0.150961
	PRKCH	cg17306848	**−0.177050**	0.095500	−0.445493	−0.043842	TRUE	0.164329
	PIK3CD	cg07499142	**−0.156684**	0.098014	−0.424145	−0.017737	TRUE	0.136892
	SOCS1	cg03014241	**−0.153199**	0.103512	−0.437169	−0.010281	TRUE	0.130685
	PRKCZ	cg02481000	**−0.146903**	0.096539	−0.430894	−0.023558	TRUE	0.132116
	PIK3R3	cg04610450	**−0.146696**	0.101894	−0.439181	−0.014170	TRUE	0.126629
	TNFRSF1B	cg05599723	**−0.142988**	0.094263	−0.404934	−0.019966	TRUE	0.127840
	CD36	cg14479884	**−0.127679**	0.084681	−0.364939	−0.005608	TRUE	0.111040
	TNFRSF1B	cg15526535	**−0.099339**	0.065544	−0.274318	−0.001141	TRUE	0.086658

a: SE (Standard Error)
b: LLCI (Lower limit confidence interval)
c: ULCI (Upper limit confidence interval)

suggesting a protective role of the selected CpGs. This is the first study to determine that DNA methylation mediates the relationship between PNMS due to a natural disaster and growth and adiposity outcomes in human offspring.

A growing body of evidence from human and nonhuman studies shows that PNMS may have a long term effect on prenatal development and on growth and metabolism in offspring.[20] Project Ice Storm, a prospective longitudinal study, provides a human model of prenatal stress. Our current analyses, based on 66 children at age 13½, indicated that objective PNMS predicts greater central adiposity and BMI, while subjective PNMS predicted central adiposity. This finding is in accord with our previous studies that observed a significant predictive association between objective PNMS and BMI in the Ice Storm cohort at age 5½,[14] and that at age 8½ and 11½ (unpublished data). Together, these results suggest that it is the severity of the women's objective exposure to the ice storm that plays a greater role in predicting BMI than their subjective reactions to the crisis, and that children of mothers experiencing severe hardship in pregnancy could be at greater risk of developing obesity. Moreover, these findings highlight the longevity of the predictive role of PNMS on offspring adiposity in humans.

In our previous report with the same sample, we investigated the genome-wide DNA methylation profile and observed that only objective PNMS was related to methylation, and that the associated methylation patterns correspond to biological pathways that have been linked to metabolic outcomes.[19] Therefore, based on these findings, in the current study we were able to analyze the potential mediation of exposure to ice storm and growth outcome in later life by altered DNA methylation. Given that we have shown in this same subgroup of Project Ice Storm participants who agreed to give blood at age 13½, that objective PNMS was associated with greater insulin secretion, which is a feature of insulin resistance, increasing risk for Type 2 diabetes,[15] and, given the important role of Type-1 and -2 diabetes mellitus pathways on outcomes such as obesity, insulin resistance and diabetes, we selected genes associated with these pathways to conduct the mediation analyses.

Although both the total effect (c) (data not shown) and the direct effect (c') of objective PNMS on BMI and central adiposity were positive (higher stress, BMI, and central adiposity), the direction of all of the mediation effects were, however, negative (e.g., higher PNMS, higher methylation level; higher methylation level, lower BMI and central adiposity), suggesting that the positive effects of PNMS on child growth outcomes were dampened by DNA methylation. Thus, it seems that the methylation of these CpGs from Type-1 and -2 diabetes mellitus pathways protects the offspring from at-risk metabolic health, rather than explaining how PNMS predicts increased risk of adiposity in offspring. As social and physical environmental factors experienced during the early life period could have a long-lasting effect on offspring's health, DNA methylation might participate in sculpting genome function in response to these environmental signals.[16] As such, our data provide preliminary evidence of epigenomic programming of adaptation to the social environment. The fact that the adverse outcomes due to the objective PNMS cannot be explained by the negative mediating effect of DNA methylation is likely due, in part, to the lack of data on DNA methylation in other genes, which make up the Type-1 and -2 diabetes mellitus pathways. For example, the Type 1 diabetes mellitus pathway consists of 120 genes, besides the 19 genes analyzed in the current study; the remaining 101 genes might have positive mediating effects that could explain the positive effects of PNMS on offspring adiposity. A second explanation for the counterintuitive mediation results might be the lack of biological material at birth. As the blood samples were collected at age 13½, we cannot rule out the possibility that postnatal environmental factors could also have modified the offspring's DNA methylation profile in

Table 6. Genes and their mediating effects on the impact of objective PNMS on central adiposity (n = 31)

			Mediation effect					
Diabetes Mellitus Pathways	**Gene**	**CpG Site**	**Effect (a*b)**	**SE[a] Boot**	**Boot LLCI[b]**	**Boot ULCI[c]**	**Significant Indication**	**Effect Size**
Type 1	LTA	cg09736959	**−0.003893**	0.001754	−0.008056	−0.000945	TRUE	0.266215
	LTA	cg24216966	**−0.003947**	0.001731	−0.008288	−0.001256	TRUE	0.277915
	LTA	cg11586857	**−0.003810**	0.001642	−0.007872	−0.001224	TRUE	0.312677
	LTA	cg10476003	**−0.003673**	0.001568	−0.007869	−0.001250	TRUE	0.274902
	LTA	cg01157951	**−0.003433**	0.001549	−0.007410	−0.000969	TRUE	0.243191
	LTA	cg16280132	**−0.003369**	0.001800	−0.007183	−0.000106	TRUE	0.220256
	LTA	cg26348243	**−0.003380**	0.001837	−0.007126	−0.000026	TRUE	0.224411
	LTA	cg02402436	**−0.003373**	0.001516	−0.007446	−0.001107	TRUE	0.253475
	LTA	cg21999229	**−0.003165**	0.001520	−0.007044	−0.000804	TRUE	0.222994
	LTA	cg13815684	**−0.003140**	0.001486	−0.007038	−0.000873	TRUE	0.22299
	LTA	cg14597739	**−0.003141**	0.001508	−0.007122	−0.000883	TRUE	0.221497
	LTA	cg17169196	**−0.003096**	0.001548	−0.007444	−0.000917	TRUE	0.223607
	NFKBIA	cg00689225	**−0.002854**	0.001546	−0.007021	−0.000695	TRUE	0.21673
	LTA	cg09621572	**−0.002938**	0.001582	−0.007483	−0.000729	TRUE	0.212545
	LTA	cg16219283	**−0.002821**	0.001510	−0.006900	−0.000616	TRUE	0.196972
	LTA	cg00501919	**−0.002720**	0.001669	−0.007002	−0.000139	TRUE	0.209267
	CD3D	cg03074244	**−0.002591**	0.001526	−0.006636	−0.000384	TRUE	0.179409
	LTA	cg14441276	**−0.002634**	0.001405	−0.006770	−0.000688	TRUE	0.194113
	IL1RAP	cg16588163	**−0.002574**	0.001381	−0.006426	−0.000649	TRUE	0.179813
	CD247	cg14278300	**−0.002458**	0.001595	−0.006647	−0.000204	TRUE	0.172361
	LTA	cg14437551	**−0.002499**	0.001341	−0.006717	−0.000698	TRUE	0.1898
	CD3D	cg07728874	**−0.002389**	0.001471	−0.006194	−0.000243	TRUE	0.166154
	TNFRSF1B	cg22677556	**−0.002363**	0.001775	−0.007175	−0.000088	TRUE	0.165286
	CD3D	cg24841244	**−0.002285**	0.001450	−0.005960	−0.000162	TRUE	0.159508
	CD3D; CD3G	cg13750061	**−0.002343**	0.001429	−0.006016	−0.000262	TRUE	0.15981
	CD3G	cg15880738	**−0.002321**	0.001604	−0.006719	−0.000170	TRUE	0.160133
	NFKBIA	cg16518861	**−0.002313**	0.001267	−0.006051	−0.000580	TRUE	0.190529
	CD247	cg09032544	**−0.002159**	0.001362	−0.005752	−0.000202	TRUE	0.159846
	CD3E	cg06164961	**−0.002181**	0.001412	−0.005926	−0.000114	TRUE	0.154138
	CD3D; CD3G	cg07545925	**−0.002183**	0.001346	−0.005723	−0.000301	TRUE	0.156965
	FASLG	cg00071250	**−0.002209**	0.001307	−0.005778	−0.000396	TRUE	0.164945
	CD247	cg09473725	**−0.002134**	0.001293	−0.005601	−0.000304	TRUE	0.15379
	CD3E	cg24612198	**−0.002139**	0.001203	−0.005435	−0.000436	TRUE	0.156765
	IRF1	cg15375424	**−0.002063**	0.001221	−0.005336	−0.000310	TRUE	0.151819
	FASLG	cg10161121	**−0.002077**	0.001262	−0.005468	−0.000252	TRUE	0.148999
	SOCS1	cg07786657	**−0.001990**	0.001215	−0.005371	−0.000344	TRUE	0.148014
	HLA-DMB	cg10453850	**−0.002033**	0.001277	−0.005439	−0.000283	TRUE	0.150048
	CD3D	cg25643644	**−0.002007**	0.001283	−0.005603	−0.000260	TRUE	0.149703
	CD3D; CD3G	cg05160234	**−0.001987**	0.001290	−0.005306	−0.000088	TRUE	0.137851
	CD3D; CD3G	cg03254928	**−0.002020**	0.001443	−0.005874	−0.000034	TRUE	0.139368
	BCL2	cg08223235	**−0.001992**	0.001130	−0.005271	−0.000475	TRUE	0.152627
	HLA-E	cg27486585	**−0.002026**	0.001374	−0.005838	−0.000131	TRUE	0.14539
	MAP3K14	cg16826777	**−0.001882**	0.001163	−0.005074	−0.000267	TRUE	0.140401
	HLA-DMB	cg01374870	**−0.001898**	0.001233	−0.005382	−0.000352	TRUE	0.137375
	FASLG	cg06983746	**−0.001900**	0.001196	−0.005242	−0.000258	TRUE	0.138648
	HLA-E	cg21366673	**−0.001823**	0.001219	−0.005229	−0.000146	TRUE	0.13321
	CD28	cg24336674	**−0.001748**	0.001156	−0.005257	−0.000196	TRUE	0.13178
	CD247	cg13210595	**−0.001575**	0.001001	−0.004600	−0.000276	TRUE	0.119626
	HLA-DMB	cg13524037	**−0.001506**	0.000981	−0.004439	−0.000196	TRUE	0.114081
	HLA-E	cg05201185	**−0.001492**	0.001114	−0.004718	−0.000034	TRUE	0.107684
	TNFRSF1B	cg05599723	**−0.001542**	0.001046	−0.004485	−0.000185	TRUE	0.117536
Type 2	PIK3CD	cg01320698	**−0.003024**	0.001613	−0.007285	−0.000660	TRUE	0.204551
	NFKBIA	cg00689225	**−0.002854**	0.001546	−0.007021	−0.000700	TRUE	0.216730
	PIK3CD	cg07970040	**−0.002604**	0.001656	−0.006768	−0.000150	TRUE	0.183871
	TNFRSF1B	cg22677556	**−0.002363**	0.001775	−0.007175	−0.000090	TRUE	0.165286
	NFKBIA	cg16518861	**−0.002313**	0.001267	−0.006051	−0.000580	TRUE	0.190529
	PRKCH	cg14001486	**−0.001963**	0.001296	−0.005601	−0.000160	TRUE	0.142278
	MAP3K14	cg16826777	**−0.001882**	0.001163	−0.005074	−0.000270	TRUE	0.140401
	PRKCH	cg17306848	**−0.001871**	0.001056	−0.004895	−0.000390	TRUE	0.146720

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Table 6. Genes and their mediating effects on the impact of objective PNMS on central adiposity (n = 31) *(Continued)*

			Mediation effect					
Diabetes Mellitus Pathways	**Gene**	**CpG Site**	**Effect (a*b)**	**SE[a] Boot**	**Boot LLCI[b]**	**Boot ULCI[c]**	**Significant Indication**	**Effect Size**
	PRKAG2	cg22528270	**−0.001842**	0.001215	−0.005228	−0.000150	TRUE	0.131101
	PIK3R3	cg04610450	**−0.001572**	0.001109	−0.004687	−0.000090	TRUE	0.115888
	PRKCZ	cg02481000	**−0.001643**	0.000995	−0.004462	−0.000290	TRUE	0.126360
	TNFRSF1B	cg05599723	**−0.001542**	0.001046	−0.004485	−0.000190	TRUE	0.117536

a: SE (Standard Error)
b: LLCI (Lower limit confidence interval)
c: ULCI (Upper limit confidence interval)

order to adapt to the postnatal environment, which differed from the stress of the prenatal environment. Therefore, future longitudinal studies are needed to differentiate between different timing of DNA methylation changes, and their relative effects on long-term outcomes.

Given that there is a well-established, complex partnership between immune and metabolic regulation, it is not surprising to observe that the majority of the genes whose methylation levels were correlated with both PNMS and child adiposity are involved in the immune system. Three genes, *LTA, NFKBIA*, and *PIK3CD*, which showed the greatest mediating effects, are of particular interest. Among all the genes investigated, *LTA* had the greatest number of significant CpGs, which are involved in both Type-1 and -2 diabetes mellitus pathways. *LTA*'s 17 CpGs are distributed within TSS1500/200, 5′UTR, 1st exon and gene body (**Table** 7). *LTA* encodes lymphotoxin-α protein, a member of the tumor necrosis factor family, which is a well-identified pro-inflammatory cytokine produced by lymphocytes. Associations between variations in the *LTA* and metabolic traits have previously been reported. For example, the polymorphism of the *LTA* was associated with Type 2 diabetes[21,22] and other phenotypes of the metabolic syndrome.[23] *LTA* has been reported to be associated with insulin-dependent diabetes mellitus.[24] Two CpGs located within gene body in *NFKBIA* were found to have significant mediation effects. *NFKBIA* is one member of a family of cellular proteins that function to inhibit the NF-kappa-B. It is well known that, together with its inhibitor (NFKBIA), NF-kappa-B is an important transcription factor that participates in the activation of genes involved in immune responses. It has been shown that one polymorphism, A/G in the 3′UTR region of *NFKBIA*, could influence the pathogenesis of diabetes mellitus and affect its complications.[25] In addition, SNP rs1951276 in the 3′ region of *NFKBIA* was reported to be associated with obesity-induced insulin resistance.[26] Results also involved *PIK3CD*, which encodes p110delta; this enzyme belongs to the class I PI3K (Phosphatidylinositol 3-kinases), which are key mediators of insulin action. Three CpGs located within 3′UTR in *PIK3CD* were found to have significant mediation effects in the current study. PIK3CD was reported to be essential to the increased vascular contractile response in a mouse model of Type 1 diabetes.[27] Recently, it was observed that the mRNA level of PIK3CD in leukocyte was overexpressed in women with gestational diabetes mellitus.[28] To our knowledge, no prior PNMS studies have investigated DNA methylation in these genes. As such, *LTA, NFKBIA*, and *PIK3CD* could be considered potential candidate genes for follow-up PNMS studies. Our data highlight the important role of the immune system on obesity outcomes.

The present results are somewhat limited by the small sample size. To approximate the power of our bootstrapping method to detect the methylation mediation effect, we performed a bootstrapping power analysis method similar to the one explained in a recent publication[29] and found that our results are actually underestimates of the number of moderate-to-strong mediations that we would have found with a larger sample. Furthermore, as our original design is not a case-control study design, but rather focused on exploration of "dose-response" relationships, our finding lacks data for the methylation profile of youth who were not exposed (i.e., a control group). In regard to the tissue-specificity, we would have studied DNA methylation in adipose tissue taken from the children, but obtaining blood is obviously a much less invasive procedure. As a viable alternative, blood has been used successfully in epigenetic research in obesity. Additionally, blood serves as crucial material for studying the role of inflammation and immune dysfunction in obesity.[30,31] Still, the heterogeneity between blood and adipose tissue is a limitation of our study. Moreover, we were not able to obtain the RNA samples from the subjects due to the low amount of blood collected. As such, the gene expression levels of *LTA, NFKBIA*, and *PIK3CD*, which showed greatest mediating effects, need to be carefully examined in further studies. As the DNA methylation and BMI data were taken at the same age (13½), our results do not allow any definitive conclusions about the direction of cause between DNA methylation and BMI. In order to further explore the potential for reverse causality we tested 2 alternative models. Model 1 posited an indirect effect of PNMS on DNA methylation at age 13½ through BMI at age 13½, thus reversing the causal direction. No significant mediation analyses were found in this alternative model, and statistical comparisons of the fit indices showed that our original model was a significantly

Table 7. The location of analyzed CpGs that mediates the effect of PNMS on BMI and central adiposity

Chr	UCSC_REFGENE_NAME	Probe ID	UCSC_REFGENE_GROUP
1	PRKCZ	cg02481000	Body
	PIK3CD	cg01320698	3′UTR
		cg07499142	
		cg07970040	
	TNFRSF1B	cg22677556	Body
		cg15526535	
		cg05599723	
	CD247	cg03727968	Body
		cg13210595	
		cg09473725	
		cg14278300	
		cg09032544	
		cg07786657	
	FASLG	cg10161121	TSS200
		cg06983746	
		cg00071250	1stExon;5′UTR
2	CD28	cg24336674	3′UTR
3	IL1RAP	cg16588163	5′UTR
5	ACSL6	cg14841483	Body
	IRF1	cg15375424	Body
6	HLA-E	cg05201185	Body
		cg21366673	
		cg27486585	
		cg17615629	
		cg19109457	3′UTR
		cg16535080	
	LTA	cg14441276	TSS1500;TSS200
		cg09621572	TSS200;1stExon;5′UTR
		cg14437551	
		cg14597739	
		cg16219283	
		cg21999229	
		cg17169196	
		cg02402436	
		cg09736959	1stExon;5′UTR
		cg24216966	
		cg11586857	
		cg10476003	
		cg01157951	5′UTR
		cg13815684	
		cg16280132	
		cg26348243	
		cg00501919	Body
	HLA-DOB	cg27246453	Body
	HLA-DMB	cg13524037	Body
		cg01374870	
		cg10453850	
7	CD36	cg14479884	5′UTR;1stExon
	PRKAG2	cg22528270	5′UTR;Body
11	CD3E	cg24612198	5′UTR
		cg06164961	Body
	CD3D	cg25643644	Body
		cg07728874	1stExon
		cg24841244	5′UTR;1stExon
		cg03074244	
	CD3D; CD3G	cg05160234	TSS1500
		cg03254928	
		cg13750061	
		cg07545925	TSS200;TSS1500
	CD3G	cg15880738	5′UTR;1stExon
14	NFKBIA	cg16518861	Body
		cg00689225	
	PRKCH	cg14001486	Body
		cg17306848	
16	SOCS1	cg03014241	3′UTR
17	MAP3K14	cg16826777	5′UTR
18	BCL2	cg08223235	Body
19	PIK3R3	cg04610450	3′UTR

better fit than the reverse causation model. Our Model 2 posited an indirect effect of PNMS on BMI at the later age of 15½ through DNA methylation at 13½. Despite testing the model with a smaller sample due to attrition, many significant mediation effects were detected. Taken together, we believe these findings offer preliminary support for our causal chain hypothesis.

The present study is strengthened by its prospective design using a unique human model, a natural disaster, through which we can isolate objective and subjective aspects of PNMS and their associations with offspring phenotypes. This is because the objective degree of ice storm exposure was quasi-randomly distributed in the population, and completely independent of the women's control or influence (unlike many common life events), and, therefore, objective PNMS was not biased by genetic or socioeconomic confounding. Based on the high-throughput investigation of DNA methylation across 480,000 individual CpG sites throughout the genome, we were able to conduct a mediation analysis and provided evidence that objective PNMS affects growth outcomes via DNA methylation of genes involved in Type 1 and Type 2 diabetes mellitus pathways. These results support the contention that PNMS has a significant effect on growth outcomes in offspring. Additionally, our study implicates the potential role of DNA methylation as a protective factor for growth outcomes. Taking together, this study contributes new data suggesting DNA methylation could act as an intervening variable between PNMS and growth outcomes, and could help to understand how risk factors such as genetic, environmental, social and biological factors work together to alter risk for some disorders.[32]

In conclusion, this study has shown that objective PNMS resulting from the 1998 Quebec ice storm predicted BMI and central adiposity in the adolescent offspring. Although we cannot explain the above findings through our observation of the protective mediating effect of selected genes from 2 metabolic pathways, we provide data supporting the hypothesis that DNA methylation is a potential mechanism involved in the long-term adaptation and programming of the genome programs in response to early adverse environmental factors.

Materials and Methods

Participants

Participants were involved in the longitudinal Project Ice Storm,[33] which focuses on disaster-related PNMS and child development. Assessments of the women and their children have been conducted several occasions since the families were first contacted in June 1998, 5 months after the storm.[34] All mothers were 18 y of age or older at recruitment, and all participants were Francophone Caucasians. At age 13½ (M = 13.6, SD = 0.09), 69 families agreed to a comprehensive assessment of the children's physical, cognitive, motor and behavioral development. The children (38 boys and 31 girls) had been in their first (n = 17), second (n = 18), or third (n = 17) trimester of pregnancy on January 9, 1998 (the peak of the ice storm), or were conceived within 3 months of the storm (n = 17), when maternal stress hormones could still be elevated. As part of a separate wave of data collection, we invited subjects to give a fasting blood sample in October-November 2011. The 31 adolescents who accepted (19 boys, 12 girls) were, on average, 13.3 (SD = 0.29) years of age at the time of blood sampling. All phases of this study were approved by the Research Ethics Board of the Douglas Hospital Research Center. We obtained informed consent from parents at every assessment, and informed assent from children at the age 13½ assessment.

Predictors

Storm-related variables

The degree of objective and subjective PNMS, as well as family demographic variables, was assessed in a postal questionnaire mailed to participants on June 1, 1998. Objective PNMS was calculated using the mothers' responses to questionnaire items tapping into categories of exposure used in other disaster studies: Threat, Loss, Scope, and Change.[35] Because each natural disaster presents unique experiences to the exposed population, questions pertaining to each of the 4 categories must be tailor-made. Each of the 4 dimensions was scored on a scale of 0–8, ranging from no exposure to high exposure. A total objective hardship score (Storm32) was computed by summing scores from all 4 dimensions using McFarlane's approach.[36] Details of the Storm32 items and scoring are presented elsewhere.[37]

Subjective PNMS was assessed using the validated French version[38] of the widely used Impact of Event Scale – Revised (IES-R).[39] The 22-item scale describes symptoms from 3 categories relevant to post-traumatic stress disorder: Intrusive Thoughts, Hyperarousal, and Avoidance. The IES-R instructions for respondents allow investigators or clinicians to "write in" the traumatic event in question. Participants responded on a 5-point Likert scale, from "Not at all" to "Extremely," the extent to which each item described how they felt over the preceding 7 d in response to the ice storm crisis. We used the total score in all analyses.

Timing of exposure to the ice storm *in utero* was calculated using the child's birth date, and the gestational age at birth (as recorded in the birth records) to estimate conception date, then calculating the number of days between conception and January 9, 1998 as the date of the peak of the ice storm. As such, higher numbers indicate exposure later in pregnancy.

Maternal variables

The level of maternal psychological functioning was assessed with a validated French version of the widely-used General Health Questionnaire-28 (GHQ).[40] The GHQ is a self-report screening tool for psychiatric symptoms and includes 7 items in each of the depression, anxiety, dysfunction, and somatization sub-scales. Items are scored on a 4-point Likert scale indicating the degree to which each symptom was experienced in the preceding 2 weeks. In the present study, each item was re-scored as either 0 (a rating of 0 or 1) or 1 (a rating of 2 or 3), according to the Goldberg method,[40] resulting in a minimum possible score of 0 and a maximum possible score of 28. The total score was used in analyses. The GHQ was included in the June 1998 questionnaire, and also when their children were 13½ years of age.

Exposure to potentially stressful maternal life events was assessed in a questionnaire sent 6 months after each woman's due date, and again at the 13½-year assessment. Women answered the Life Experiences Survey (LES),[41] a self-report measure that lists 57 life changes, such as death of a spouse or a promotion at work. To keep the questionnaire length reasonable, we reduced this to 29 events by eliminating items not likely to have occurred in this sample (e.g., combat experience). At the 6-month questionnaire, women indicated events (except the ice storm) that occurred during the 6 months since the baby's due date, the 9 months of pregnancy, and the 3 months before conception. At 13½ years, they reported on events that occurred during the previous 2 y Women gave the approximate date of each event experienced, and rated the impact of each event on a 7-point Likert scale ranging from "extremely negative" to "extremely positive." The numbers of life events at each assessment were used in analyses.

Data on maternal and paternal age and education, and parental job classification, were collected in June 1998. Socioeconomic status (SES) was computed using Hollingshead Index criteria.[42]

The number of obstetrical complications, including flu with fever, was determined by maternal recall using an adaptation of the scale used by Kinney[43] in our 6-month postpartum questionnaire, and verified using hospital records. We used the total number of obstetrical complications experienced by the women that were rated as moderate-to-severe using the McNeil-Sjöström Scale for Obstetric Complications.[44]

Children's birth weight, birth length, and gestational age were obtained from maternal reports (transcribed from Quebec birth records given at discharge) in the 6-month postpartum questionnaire, and from hospital records. Birth ponderal index was calculated ($100 \times$ (birth weight (g)/birth lenght^3 (cm)).

Blood samples and T-cell isolation at 13½ years of age

Blood was collected from 31 subjects for T-cell isolation and DNA extraction using methods which have been described previously.[19,45] Briefly, T-cells were isolated from PBMCs by

immunomagnetic separation with Dynabeads CD3 (Dynal, Invitrogen). DNA extraction from T-cells was performed using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions.

Infinium Human Methylation 450 BeadChip Array and data analysis

Infinium HumanMethylation450 BeadChip, an array containing 485,577 probes covering 99% RefSeq genes and 96% of CpG islands, was used to determine DNA methylation levels in T-cells. We then correlated the levels of methylation with objective/subjective PNMS. Probes on chromosomes X and Y were excluded. To avoid artifacts due to hybridization bias, probes with minor allele frequency (MAF) $\geq$ 5% in the HapMap CEU population were removed. Furthermore, CpGs with an interquartile range (IQR) less than 0.10 (i.e., 10% methylation difference) were not analyzed. The remaining 10,553 probes were tested for association with the objective/subjective PNMS. The Benjamini-Hochberg algorithm was used to correct for multiple testing by computing the false discovery rate (FDR), which was set at $<$0.2. Illumina 450K Methylation BeadChip analyses were completed using standard procedures and described previously.[19]

Child outcome measures at age 13½

During the face-to-face assessment of the child, height, weight and waist measurements were collected following standard guidelines,[46] repeating each measure twice. Standing height was measured without shoes to the nearest 0.1 cm and weight to the nearest 0.1 kg. Waist circumference was measured twice at the level of the umbilicus, to the nearest 0.1 cm. The mean of the 2 measurements was used for analysis. Body Mass Index (BMI, kg/m^2) Z-Scores were computed based on World Health Organization growth references.[47] Central adiposity was calculated as the ratio of waist circumference (cm) by height (cm).

Selection of candidate genes for testing mediation by methylation

In order to determine the extent to which gene methylation mediates the association between objective hardship or subjective distress and BMI and central adiposity at 13½ years, we tested genes that we have previously shown to have their methylation signatures from isolated T-cells associated with objective hardship levels in this sample[19]; subjective PNMS had not correlated with methylation of any CpG sites. We then matched the genes whose methylation had been significantly correlated with PNMS to the Type-1 and -2 diabetes mellitus pathways as classified by IPA software (www.ingenuity.com). In total, 19 genes (64 CpGs) in the Type 1 diabetes mellitus pathway, and 12 genes (19 CpGs) in the Type 2 diabetes mellitus pathway, were selected. Because there were 6 CpGs overlapping between Type-1 and -2 diabetes mellitus pathways, there were 75 unique CpGs which were used for further analysis (**Table S1**). Additionally, to reduce the risk of missing potentially significant CpG site mediations we tested the associations between outcomes and all remaining CpG sites and none were significant.

Statistical analysis

Pearson product-moment correlations were conducted between the outcome measures and all predictors. Hierarchical linear regression analysis was conducted on the children's WHtR and BMI scores. Because of the relatively small sample size, all non-significant main effects were then trimmed from the equation. Only the final models are presented. All analyses were completed with SPSS 20.0 (SPSS, Chicago, IL).

Mediation analysis and bootstrapping methods

To investigate whether the genes we selected mediated the relationship between exposure to objective hardship, subjective distress and growth outcomes in the subsample of 31 subjects, we conducted a mediation analysis using state-of-the-art bootstrapping.[48] Mediation models hypothesize a causal chain in which the independent variable affects the mediator variable, which, in turn, affects the dependent variable. The current mediation analysis included one of the 2 body measurements as the outcome, and either objective or subjective PNMS as the predictor, and DNA methylation levels of CpGs as mediators. The theoretical model of the mediation analysis is presented in **Figure 1**.

We used bootstrap methods, one of the routinely used approaches, to determine the significance of mediation effects in the mediation analyses.[49] Bootstrapping is a powerful approach because it takes into account that the sampling distribution of the mediated effect is skewed away from 0[50] and this approach can be applied for small-to-moderate samples (i.e., sample sizes ranging from 20–80 cases).[51] Bootstrapping involves randomly resampling with replacement from the dataset to compute the desired statistic in each resample, providing confidence intervals by which the significance of a mediation effect can be accessed. We tested the indirect effects of objective and subjective PNMS on the WHtR and BMI through each CpG site associated with either the Type 1 diabetes pathway (64 CpG sites in 19 genes) or the Type 2 diabetes pathway (19 CpG sites in 12 genes). To that end, 95% bias-corrected bootstrap confidence intervals were computed, as explained by Hayes.[52] The PROCESS procedure for SPSS[52] was used to conduct the analyses. Each bootstrap resampled the initial sample 10,000 times. In order to be able to recover the same results, the random seed for all bootstraps was fixed at 1509805407, an integer randomly generated between 1 and 2000000000 prior to the first bootstrap. A mediating effect was considered significant if 0 was not included in the bootstrap confidence interval.

The effect size of each CpG site was measured using the method described by Preacher and Kelley.[53] According to this approach, effect sizes of mediation analyses can be interpreted in a manner similar to that using the Pearson correlation coefficient r-value.

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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