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ORIGINAL ARTICLE

Pregnant women's cognitive appraisal of a natural disaster affects DNA methylation in their children 13 years later: Project Ice Storm

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Prenatal maternal stress (PNMS) can impact a variety of outcomes in the offspring throughout childhood and persisting into adulthood as shown in human and animal studies. Many of the effects of PNMS on offspring outcomes likely reflect the effects of epigenetic changes, such as DNA methylation, to the fetal genome. However, no animal or human research can determine the extent to which the effects of PNMS on DNA methylation in human offspring is the result of the objective severity of the stressor to the pregnant mother, or her negative appraisal of the stressor or her resulting degree of negative stress. We examined the genome-wide DNA methylation profile in T cells from 34 adolescents whose mothers had rated the 1998 Québec ice storm's consequences as positive or negative (that is, cognitive appraisal). The methylation levels of 2872 CGs differed significantly between adolescents in the positive and negative maternal cognitive appraisal groups. These CGs are affiliated with 1564 different genes and with 408 different biological pathways, which are prominently featured in immune function. Importantly, there was a significant overlap in the differentially methylated CGs or genes and biological pathways that are associated with cognitive appraisal and those associated with objective PNMS as we reported previously. Our study suggests that pregnant women's cognitive appraisals of an independent stressor may have widespread effects on DNA methylation across the entire genome of their unborn children, detectable during adolescence. Therefore, cognitive appraisals could be an important predictor variable to explore in PNMS research.

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INTRODUCTION

Prenatal maternal stress (PNMS) can impact a variety of outcomes in the offspring throughout childhood and persisting into adulthood as shown in both human and animal studies.[1,2] As an agent of fetal programming,[3] it is widely believed that PNMS affects the fetus via maternal stress hormones which, at high levels, are known to readily cross the placenta and thereby impact the developing fetus.[4] This might affect fetal physiology *in utero* directly, or indirectly, and be manifest across the lifespan.

Many of the effects of PNMS on offspring outcomes likely reflect the effects of epigenetic changes to the fetal genome.[5] DNA methylation is the epigenetic modification that has been the best characterized to date. Many animal and human studies provide evidence about PNMS effects on DNA methylation profile. In rats, global DNA methylation was reported to be altered in offspring brain using prenatal maternal bystander stress.[6] Also, chronic restraint stress to the pregnant dam was found to affect methylation levels of candidate genes such as *11βHSD2*, *DNMT3a* and *DNMT1* in placenta and offspring brain.[7] In human PNMS studies, *NR3C1* is the best-investigated candidate gene and its methylation level was reported to be associated with different types of PNMS such as maternal depressed mood,[8] prenatal maternal war-related stress,[9] partner violence during pregnancy[10] and prenatal maternal emotional stress.[11] Likewise, infant methylation profiles of other candidate genes, such as *SLC6A4*,[12] and imprinted genes such as *IGF2*, *MEG3* and *PLAGL1*,[13] were also correlated with maternal depressed mood in pregnancy.

The current state of animal and human research on PNMS and DNA methylation suggests that there are significant and important changes to the human epigenome resulting from maternal stress exposure in pregnancy. Yet, the research to date cannot elucidate the active psychosocial ingredients of these effects. Unlike the random assignment of animals to PNMS conditions, human research on maternal mood or partner violence in pregnancy cannot use random assignment to stress groups to assure the internal validity of their findings; that is, they cannot isolate the effects of the 'stress' in pregnancy from the mother's own propensity to experience environmental stressors or personal distress, which may be passed on genetically. Even the research on independent stressors, such as war stress, is unable to determine which aspect of the stress experience is the active ingredient in the PNMS effects: the objective severity of the stressor, the woman's cognitive appraisal of the stressor, or her subjective degree of distress.

The term 'stress' was borrowed from physics where it refers to an external force applied to a material body resulting in strain. Hans Selye,[14] adopting the term for biology, defined stress as 'the non-specific response of the body to any demand placed upon it'. In 1984, Lazarus and Folkman[15] published their Transactional Model proposing that 'negative stress' results from an imbalance

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between the demands of the stressor and the individual's perceived ability to cope with those demands. Briefly, the model suggests that, in the presence of an event, the individual engages in a primary appraisal to determine its threat level: if no threat is perceived then there is no stress. If threat is perceived, then the secondary appraisal involves comparing the degree of threat with one's perception of their ability to cope: if the appraisal results in the conclusion that one's resources are sufficient, then it will result in 'positive stress' (eustress); on the other hand, if one concludes that their resources are insufficient to meet the demands of the stressor, then it will result in negative stress. The stress resulting from a negative appraisal of an event is an important determinant of the cortisol stress response.[16] By modulating the stress response, as well as other physiological processes, negative stress can be transmitted to a developing fetus via the placenta and alter its development. To date, however, no animal or human research can determine the extent to which the effects of PNMS on DNA methylation in human offspring is the result of (a) the objective severity of the stressor to the pregnant mother, (b) her negative appraisal of the stressor or (c) her resulting degree of negative stress (that is, her subjective distress).

To control for genetic bias and to disentangle the objective degree of exposure to an event from the cognitive appraisal and subjective degree of distress, a major independent stressor affecting a large population is required. In January 1998, a series of ice storms caused power outages for more than 1.4 million Québec households during the coldest period of the year for periods ranging from a few hours to more than 6 weeks. The ice storm has been described as Canada's most costly natural disaster in history.[17,18] Project Ice Storm provides an opportunity to examine the effects of an independent stressor on a number of developmental outcomes prospectively. In June 1998, we recruited women who had been pregnant during the ice storm. Our questionnaires assessed the severity of each woman's objective exposure (that is, the stressor) and subjective distress (that is, negative stress). Results over the first 12 years of Project Ice Storm show that higher levels of maternal PNMS are associated with poorer physical, behavioral, motor and cognitive measures among the offspring from those pregnancies.[19–26]

Although the results of Project Ice Storm show significant and wide-ranging effects of prenatal maternal objective and/or subjective stress, the molecular mechanisms underlying the potential effects of maternal exposures are only emerging slowly. At the age of 13 years, a subset of Project Ice Storm teens agreed to provide blood samples. Analyses of these samples have demonstrated the power of maternal objective exposure, but not subjective distress, to impact immune function,[27] and insulin secretion and glucose tolerance.[28] In our first examination of the effects of PNMS on genome-wide DNA methylation in this subgroup we found, once again, that maternal objective stress, but not subjective distress, was significantly correlated with 1675 CG sites affiliated with 957 genes predominantly related to immune function and metabolism. DNA methylation changes in *SCG5* and *LTA*, both highly correlated with maternal objective stress, were comparable in T cells, peripheral blood mononuclear cells and saliva cells.[29]

To expand our consideration of the elements of the stress model, we returned to our initial data set to find a reflection of the women's cognitive appraisals of the ice storm. One item asked women about the overall consequences of the ice storm on them and their families, and to provide a rating on a five-point scale from very negative to neutral to very positive.

Thus, the objectives of this study were to determine (a) the level of cognitive appraisal (that is, rating of consequences from the ice storm) in women exposed to the 1998 Québec ice storm, and the associations between cognitive appraisal and objective/subjective PNMS; (b) the extent to which mothers' cognitive appraisal could predict the epigenetic profiles in their children, that is, by distinct global DNA methylation signatures in offspring; and (c) the similarities and differences between the effects of cognitive appraisal and of Objective PNMS on DNA methylation patterns in the children.

MATERIALS AND METHODS

Participants and assessments

We recruited 224 women in regions affected by the ice storm southeast of Montreal, who were pregnant during the January 1998 Quebec ice storm or who became pregnant within 3 months of the storm.[22] In June 1998, a postal survey was mailed to them and included scales of storm-related PNMS. Using a large number of questions related to objective PNMS from the ice storm, we created a scheme that provided scores on four categories of exposure: threat, loss, scope and change. Each category had a maximum possible score of eight points. We summed these to create the Storm32 scale.[24] To assess subjective distress, the postal survey included a validated French version[30] of the Impact of Events Scale-Revised.[31] The Impact of Events Scale-Revised assessed the current severity of the women's posttraumatic stress-like symptoms relative to the storm in three categories: hyperarousal, intrusion and avoidance. To assess the mothers' cognitive appraisal about the ice storm, we included the following item: 'Overall, what were the consequences of the ice storm on you and your family?'; response options were on a five-point scale of 'Very negative' (1), 'Negative' (2), 'Neutral' (3), 'Positive' (4) and 'Very positive' (5). There were missing data for six women, therefore, 218 mothers' cognitive appraisal scores were analyzed here.

In 2011, we invited adolescents from Project Ice Storm to participate in a blood draw that included the epigenetic study: 34 (20 boys and 14 girls) agreed. These adolescents had a mean age of 13.3 years (s.d. = 0.3). The adolescents' health status and medication use were screened before the blood draw.

Ethics statement

All the phases of this study were approved by the Research Ethics Board of the Douglas Hospital Research Center in Montreal, QC, Canada. We obtained written informed consent from the parents and written assent from the adolescents.

DNA specimens

To analyze the effect of maternal cognitive appraisal on the epigenome, we focused on methylation levels of DNA extracted from T cells. Blood was collected from 34 subjects for T cell isolation and DNA extraction using methods which have been described previously.[29] Briefly, T cells were isolated from peripheral blood mononuclear cells by immunomagnetic separation with Dynabeads CD3 (Dynal, Invitrogen, Carlsbad, CA, USA). DNA extraction from T cells and peripheral blood mononuclear cells was performed using Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Infinium Human Methylation 450 BeadChip Array and data analysis

Illumina 450 K Methylation BeadChip analyses were completed at McGill University and Génome Québec Innovation Centre in Montreal using standard procedures as described previously.[29] Briefly, we used Illumina Infinium Human Methylation 450 K BeadChip Array to determine DNA methylation levels in T cells at 480 000 CGs across the genome and then correlated the levels of methylation with cognitive appraisal. Probes on chromosomes X and Y were excluded. To avoid artifacts due to hybridization bias, probes with minor allele frequency $\geqslant 5\%$ in the HapMap CEU population were removed. Furthermore, CGs with an interquartile range < 0.10 (that is, 10% methylation difference) were not analyzed. The remaining 10 553 probes were tested for association with the cognitive appraisal using *t*-tests. The Benjamini–Hochberg algorithm was used to correct for multiple testing by computing the false discovery rate (FDR), which was set at < 0.2.

Ingenuity pathway analysis

Significant probe accession names were input into the Ingenuity Pathway Analysis software (www.ingenuity.com) analysis, and differentially methylated genes were classified. A right-tailed Fisher's exact test was used to

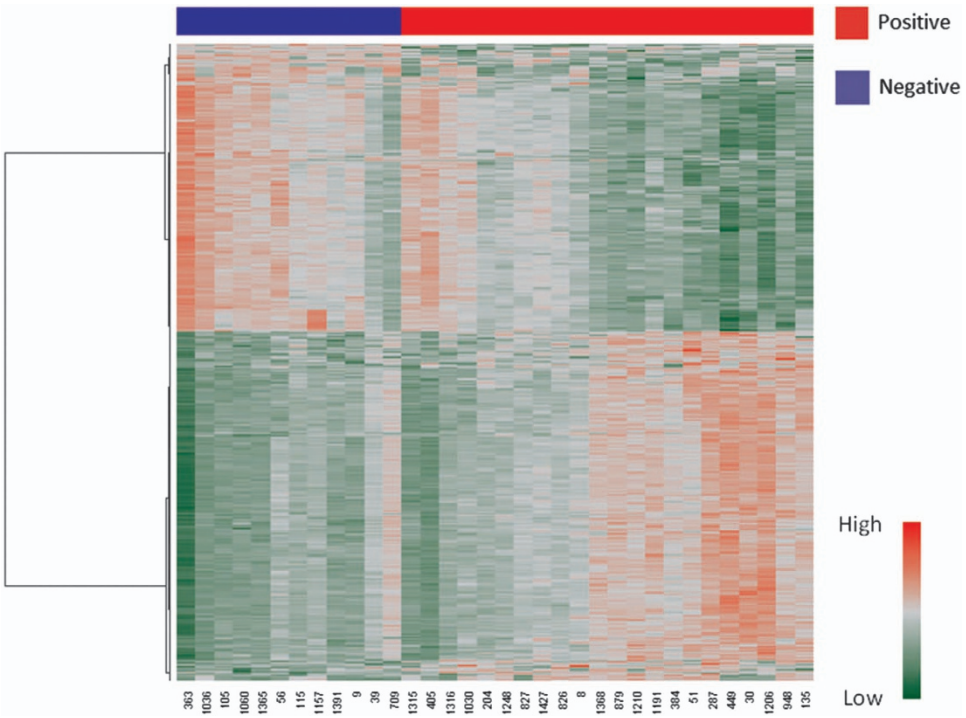

Figure 1. Differentially methylated CGs responding to cognitive appraisal level. Heatmap showing methylation of the 500 most differentially methylated CGs ($P < 0.003$, FDR < 0.055) across all 34 individuals. Each column represents an individual and each row a single CG. A color gradient intensity scale at the lower right-hand corner of the Heatmap expresses methylation changes. The darkest green indicates the lowest methylation level and the darkest red indicates the highest methylation level. The color bar above the Heatmap indicates subjects categorized by their mother's cognitive appraisal: blue indicates negative cognitive appraisal level, red indicates positive cognitive appraisal level. FDR, false discovery rate.

calculate the gene enrichment. Biological functions with a cutoff *P*-value < 0.05 were considered statistically significant.

Statistical analysis

The *t*-tests and multiple testing corrections were performed using R packages. All other analyses were performed using SPSS (Version 20, SPSS, Chicago, IL, USA). To test if the distribution of cognitive appraisal in the epigenetics sample differed significantly from its initial distribution, Mann–Whitney *U* and Kolmogorov–Smirnov nonparametric tests were conducted. Associations between objective PNMS, subjective PNMS and cognitive appraisal were calculated using Pearson's correlation coefficient, which was corrected according to Bonferroni. All *P*-values reported are two-sided.

RESULTS

Cognitive appraisal and associations with objective PNMS and subjective PNMS

Among the 218 women who completed the initial assessment in 1998, 10 (4.6%) women rated the consequences of the ice storm on them and their families as 'Very negative', and 65 (29.8%) gave a rating of 'Negative'; 69 (31.7%) women rated the consequences as 'Neutral'. In contrast, 70 (32.1%) women rated the consequences as 'Positive', and 4 (1.8%) rated as 'Very positive'. Thus, 35% of the women in the initial sample rated the consequences of the storm as negative, while 65% rated them as neutral or positive. In the subgroup of 34 mothers whose children participated in the epigenetic study, none had rated the consequences of the storm as 'Very negative' in 1998, while 12 (35.3%) had given a rating of 'Negative', 4 (11.8%) had given a 'Neutral' rating, 17 (50.0%) had given a rating of 'Positive' and 1 (2.9%) had considered the consequences 'Very positive'. As such, the current sample is similar to the full, initial sample in that in both 35% of the women rated the storm as either very negative or negative. The distribution of the scores did not differ significantly between the initial sample and the current subsample after analyzing using Mann–Whitney *U* and Kolmogorov–Smirnov nonparametric tests ($P = 0.183$ and $P = 0.239$, respectively). In this study, our interest was the negative cognitive appraisal about the ice storm. Thus, in the subgroup cohort for epigenetic study, we combined the 22 women who had rated the storm's consequences as either positive, very positive or neutral into one group (the positive appraisal group; 65% of the subsample) and compared them with the 12 who had rated the consequences as negative (the negative appraisal group; 35% of the subsample).

Results show that the three aspects of the mothers' stress experience are relatively independent. In the initial sample ($n = 218$) and in the current subsample ($n = 34$), cognitive appraisal levels had small, negative correlations with not only objective PNMS ($r = -0.262$, $P < 0.001$; $r = -0.296$, $P = 0.089$), but also subjective PNMS ($r = -0.210$, $P = 0.002$; $r = -0.002$, $P = 0.990$). As such, these three components of the stress experience can be considered to be fairly independent factors, with cognitive appraisal explaining $< 9\%$ of the variance in objective and subjective PNMS.

Associations between cognitive appraisal and genome-wide DNA methylation profiling

We observed that 2872 CGs were significantly differentially methylated ($P < 0.05$, FDR < 0.2) between the negative and positive appraisal groups (Supplementary Table S1). Of these, 1375 CGs were hypermethylated in the negative appraisal group, and 1497 CGs were hypermethylated in the positive appraisal group. The profile of the 500 most significantly differentially methylated CGs is presented in the Heatmap (Figure 1). Hierarchical cluster analysis of individual methylation patterns was performed; the results are represented in a dendrogram as shown on the left of the Heatmap. The methylation profiles of the

Table 1. Top 50 most significantly differentially methylated CGs sorted by *P*-value and FDR

N	*Target ID*	P	*FDR*	*Chr*	*UCSC_REFGENE_NAME*	*UCSC_REFGENE_GROUP*	*RELATION_TO_UCSC_CPG_ISLAND*	*REGULATORY_FEATURE_GROUP*
1	cg19851816	1.18004E – 06	0.014848438	22	TUBGCP6	Body	Island	Gene_Associated_Cell_type_specific
2	cg17901382	1.65517E – 05	0.054104691	17	TSEN54	Body	S_Shore	Unclassified
3	cg17477806	1.95976E – 05	0.054104691	19	SEMA6B	Body	Island	
4	cg00815832	2.11272E – 05	0.054104691	1			Island	
5	cg03549146	3.38685E – 05	0.054104691	16	MIR140;WWP2	TSS200;Body;Body		
6	cg27400313	4.05176E – 05	0.054104691	18	MBP	5'UTR;5'UTR	Island	
7	cg15401418	4.79229E – 05	0.054104691	17	SEPT9	5'UTR;5'UTR;Body;1stExon		Promoter_Associated
8	cg25945642	6.77093E – 05	0.054104691	3	SLC9A9	Body		
9	cg19235893	7.8799E – 05	0.054104691	2			S_Shelf	
10	cg11586857	8.50692E – 05	0.054104691	6	LTA	1stExon;5'UTR;5'UTR		Promoter_Associated
11	cg03020863	9.10217E – 05	0.054104691	11	PDGFD	Body;Body		
12	cg10365563	0.000101568	0.054104691	7	SP4	Body	S_Shore	
13	cg09736959	0.000107516	0.054104691	6	LTA	1stExon;5'UTR;5'UTR		Promoter_Associated
14	cg00500359	0.000111231	0.054104691	11	OSBPL5	5'UTR;5'UTR;5'UTR		
15	cg00158530	0.000126894	0.054104691	16	MIR140;WWP2	TSS200;Body;Body		
16	cg24216966	0.000159003	0.054104691	6	LTA	1stExon;5'UTR;5'UTR		Promoter_Associated
17	cg17226602	0.000192559	0.054104691	5	KIF4B	1stExon		
18	cg10476003	0.000196802	0.054104691	6	LTA	1stExon;5'UTR;5'UTR		Promoter_Associated
19	cg22872349	0.000201684	0.054104691	15			Island	
20	cg21203569	0.000203548	0.054104691	11	NR1H3	TSS200;TSS200;5'UTR		
21	cg11975397	0.000218701	0.054104691	1				
22	cg09452568	0.000219239	0.054104691	5	ESM1	Body;Body		
23	cg19809575	0.00022118	0.054104691	16	BANP	Body;Body	Island	
24	cg01937809	0.000229471	0.054104691	1	ZC3H12A	Body	S_Shore	Promoter_Associated
25	cg14597739	0.000234966	0.054104691	6	LTA	TSS200;1stExon;5'UTR		Promoter_Associated
26	cg18446110	0.000235149	0.054104691	5	SLC23A1	Body;Body	S_Shore	
27	cg07197230	0.000252202	0.054104691	22	CECR2	1stExon		
28	cg04057219	0.000253947	0.054104691	16	ABCC1	Body;Body;Body;Body;Body		Unclassified
29	cg21999229	0.000254014	0.054104691	6	LTA	TSS200;1stExon;5'UTR		Promoter_Associated
30	cg05413628	0.000257521	0.054104691	16	CLCN7	Body;Body	N_Shelf	
31	cg15719903	0.000266739	0.054104691	12	TAPBPL	Body		
32	cg27136994	0.000288238	0.054104691	15	C15orf26	TSS200	Island	Unclassified_Cell_type_specific
33	cg12810837	0.000291021	0.054104691	12	CLEC2D	TSS200;TSS200		Unclassified
34	cg26412374	0.000291637	0.054104691	11	ATL3	Body	N_Shore	Promoter_Associated
35	cg20184271	0.000301168	0.054104691	12				
36	cg13549277	0.000304238	0.054104691	12	IFFO1	Body;TSS1500;Body	S_Shore	
37	cg16219283	0.000304864	0.054104691	6	LTA	TSS200;1stExon;5'UTR		Promoter_Associated
38	cg04339360	0.000309036	0.054104691	13	KLF5	Body	S_Shore	Unclassified_Cell_type_specific
39	cg05350315	0.000311807	0.054104691	1	LCK	5'UTR;1stExon	S_Shelf	Promoter_Associated
40	cg04757806	0.000312067	0.054104691	11	FUT4	1stExon	Island	
41	cg13462957	0.000326466	0.054104691	7			N_Shore	Unclassified_Cell_type_specific
42	cg01823958	0.000333899	0.054104691	1	SLC1A7	Body	N_Shore	
43	cg22662844	0.000335662	0.054104691	7	MAD1L1	Body;Body;Body	Island	
44	cg03391657	0.000345581	0.054104691	1	LOC647121	TSS1500	N_Shore	Unclassified_Cell_type_specific
45	cg01554529	0.000346945	0.054104691	1	FBXO6;FBXO44	TSS1500;3'UTR;3'UTR;3'UTR;3'UTR	N_Shore	
46	cg26305174	0.000358436	0.054104691	7	SLC12A9;TRIP6	Body;TSS1500	N_Shore	
47	cg06513247	0.000369945	0.054104691	17	SEPT9	1stExon;Body;5'UTR;Body;Body;Body;Body	N_Shore	
48	cg11186344	0.000372696	0.054104691	1	IFFO2	Body		Promoter_Associated
49	cg03022510	0.000375216	0.054104691	2				Unclassified_Cell_type_specific
50	cg04261496	0.000381463	0.054104691	7	FOXK1	Body		Unclassified_Cell_type_specific

Abbreviations: FDR, false discovery rate; TSS, transcription start site; UTR, untranslated region.

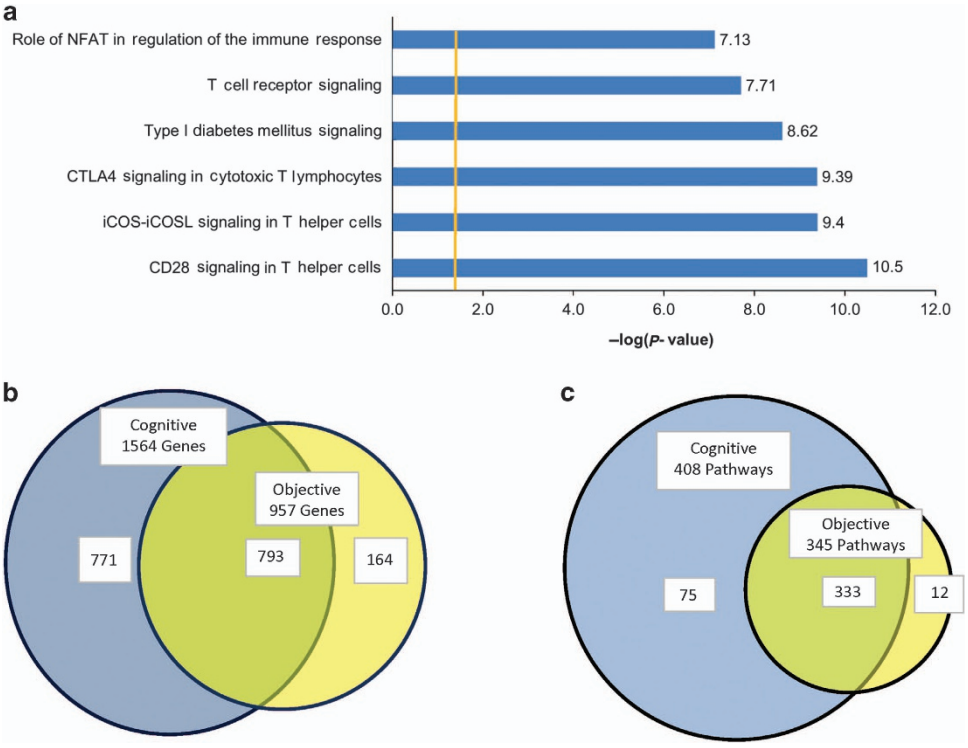

Figure 2. Venn diagram showing overlapping between cognitive appraisal-related and objective PNMS-related genes. (**a**) Top six functions of the 2872 differentially methylated genes. The y axis shows functions while the x axis shows $-\log(P\text{-value})$. The yellow line indicates the threshold value of $P < 0.05$. (**b**) For the overlap search all genes with significant change were used from cognitive appraisal versus objective PNMS data set (957 genes). The central region corresponds to genes with changed DNA methylation in both data sets. (**c**) For the overlap search, all biological pathways from cognitive appraisal versus that from objective PNMS data set (345 biological pathways). The central region corresponds to common biological pathways. A Fisher's exact test was used for calculating *P*-values for the significance of the overlaps. PNMS, prenatal maternal stress.

negative and positive appraisal groups are well distinguished, although those of several individuals on the boundary of the Heatmap are not distinct due to the sample size. Significant CGs were identified in 22 chromosomes (probes for chromosomes X and Y were excluded), revealing that cognitive appraisal levels triggered a broad signature in the genome. Table 1 provides a list of the top 50 ($P < 0.0004$, FDR < 0.055) most significantly differentially methylated CGs sorted by *P*-value and FDR.

We observed that 284 (9.9%) of the CGs were located in CpG island, 177 (6.2%) and 180 (6.3%) were located in N-shelf and S-shelf, 390 (13.6%) and 293 (10.2%) in N-shore and S-shore, respectively, and the remaining 1548 (53.9%) were located in the open sea. One hundred and seventy-eight differentially methylated CGs were in immediate proximity (200 bp) of transcription start sites (TSS), 352 were 1500 bp away from TSS, 429 were in the 5′-UTR and 123 are in the first exon. A total of 1249 CGs were in gene bodies and 146 were located in 3′-UTR. The rest of the 755 CGs were in intergenic regions. A total of 1564 genes were associated with the differentially methylated 2872 CGs; among them 2000 CGs were affiliated with only one gene, whereas 117 CGs were associated with more than one gene. Although the majority of genes (72.3%) had significant methylation differences in only one CG, 206 genes had significant methylation differences in two CG sites and 80 genes had significant methylation differences in three CGs; 63 genes had significant methylation differences in four or more CGs. Interestingly, *LTA* (lymphotoxin alpha), which is involved in regulating the innate and adaptive immune system, had the greatest number of differentially methylated CGs (19 differentially methylated CGs).

Gene Pathways involved in the immune system are prominently affected by changes in DNA methylation in response to cognitive appraisal

The Ingenuity Pathway Analysis database (www.ingenuity.com) was used to identify biological functions or diseases affiliated with the gene sets whose degree of methylation differ significantly between the negative and positive appraisal groups. Figure 2a charts the top six canonical pathways and a detailed summary of the pathway analysis is presented in Supplementary Table S2. Interestingly, pathways involved in the immune system are prominent. For example, the top pathway is CD28 signaling in T helper cells: 32 of the 136 genes included in this pathway were found to be associated with cognitive appraisal in the present study ($P = 3.03\text{E}10^{-11}$). Furthermore, highly significant enrichments in biological functions have been observed to be related to the immune system; for example, inflammatory response ($P < 3.84\text{E}10^{-20}$–$1.53\text{E}10^{-5}$), immunological disease ($P < 8.93\text{E}10^{-16}$–$1.65\text{E}10^{-5}$), hematopoiesis ($P < 3.09\text{E}10^{-14}$–$4.98\text{E}10^{-6}$) were frequently encountered. Moreover, the potential upstream regulators of the differentially methylated genes such as TNF ($P = 2.10\text{E}10^{-19}$), TGFB1 ($P = 1.44\text{E}10^{-15}$) and TCR ($P = 5.00\text{E}10^{-14}$) have been observed.

Although the biological functions of the significantly differentiated genes in the present study were predominantly involved in immune system, genes involved in metabolic functioning were also associated with the mother's cognitive appraisal of their ice storm experience. For example, the methylation patterns of 28 of the 121 genes involved in the type I diabetes mellitus signaling pathway ($P = 2.42\text{E}10^{-9}$), and significant enrichments in disease and disorders such as cardiovascular disease ($P < 5.30\text{E}10^{-14}$–$1.73\text{E}10^{-5}$) was encountered.

As we noticed that the magnitude of the results based on cognitive appraisal is quite similar to that reported in our previous study on objective PNMS, we then investigated the association between the two sets of findings. A Venn diagram was used to represent the overlap between genes that were significantly differentially methylated according to objective PNMS or cognitive appraisal. Of the 1564 genes with CGs whose methylation levels differed significantly between the positive and negative appraisal groups, 793 (50.7%) had also been found to be significantly correlated with objective PNMS; a Fisher's exact test demonstrates a significant tendency for genes that are associated with one type of stress to also be associated with the other ($P < 0.001$): of the 957 genes associated with objective PNMS, 83% were also significantly associated with cognitive appraisal (Figure 2b). A second Venn diagram represents the overlap of biological pathways significantly associated with objective PNMS and maternal cognitive appraisal (Figure 2c). Of the 345 pathways associated with objective PNMS, only 12 (3%) were not associated with cognitive appraisal as well. Of the 408 pathways related to cognitive appraisal, there were 333 (81.6%) that were also correlated with objective PNMS ($P < 0.001$, Fisher's exact test). Seventy-five pathways, which were unique to cognitive appraisal, are presented in Table 2. The top pathway was the Signal Transducers and Activators of Transcription 3 (STAT3) Pathway.

DISCUSSION

The main goal of this study was to determine the extent to which genome-wide DNA methylation levels collected from a cohort of adolescents in 2011 could be related to the cognitive appraisals their mothers had made in 1998 about the Québec ice storm which they had experienced during their pregnancies. We reported previously from this data set that the teenagers' DNA methylation levels were significantly correlated with their mothers' degree of objective PNMS from the ice storm, but not with their mothers' degree of subjective distress about the disaster. In the current analyses, we aimed to determine the extent to which another aspect of the women's stress experience, their cognitive appraisal of the consequences of the ice storm on them and their families, would also predict methylation levels. We contrasted the 35% of subjects whose mothers had rated the ice storm consequences as negative with the 65% whose mothers had rated the consequences as either neutral, positive, or very positive.

We observed that the methylation levels of 2872 CGs differed significantly between adolescents in the positive and negative maternal appraisal groups. These CGs are affiliated with 1564 different genes, and with 408 different biological pathways, which are prominently featured in immune function. As our current study is based on T cells from blood samples, it is not surprising that the genes showing differential methylation between the negative and positive cognitive appraisal groups are involved in biological processes relevant in immune function (Figure 2a and Supplementary Table S2), such as, CD28 signaling in T helper cells and CTLA4 signaling in cytotoxic T lymphocytes. Moreover, the inflammatory and immune response categories, with specific signaling pathway, such as cytokine signaling (for example, interleukin 4 (IL-4), IL-6 and IL-8 signaling), were also identified in our pathway analysis (Supplementary Table S2). As illustrated on the Heatmap, which shows a well-distinguished methylation pattern, among the top 500 significantly methylated CGs, around half of the CGs was hypermethylated, and the other half hypomethylated, in the negative compared with the positive cognitive appraisal group. Of the genes with the greatest number of significant CGs were genes linked to immune function, such as, *LTA* that is involved in regulating the innate and adaptive immune systems.[32] The consequences of these DNA methylation changes on immune and HPA axis functioning, which may alter downstream gene expressions, will be explored in future analyses.

Importantly, there was a significant overlap in the differentially methylated CGs or genes and biological pathways that are associated with cognitive appraisal and those associated with objective PNMS as reported in our previous study. On the other hand, we can also see that a certain number of results were uniquely associated with maternal cognitive appraisal of the ice storm, and not with their objective exposure: 49% of the genes and 19% of the pathways implicated in the differentially methylated CGs were unique to cognitive appraisal, while 17% of genes and 3% of pathways were unique to objective stress. STAT3 pathway was the top pathway which was uniquely associated with maternal cognitive appraisal. STAT3 is known to have a key role in many cellular processes, such as cell growth and apoptosis, by mediating the expression of a variety of genes in response to cell stimuli. Recently, STAT3 was reported to be involved in the stress response. For instance, in a study investigating the HPA axis response to chronic stress, STAT3 signaling cascade was activated by IL-6 stimuli in rat hypothalami.[33] Likewise, a study in mice showed that IL-10/STAT3 signaling cascade mediated chronic stress-induced immune suppression and was involved in the disequilibrium of Th1/Th2 cytokine balance caused by chronic stress.[34] Interestingly, pathways such as PDGF signaling, DNA methylation and transcriptional repression signaling and role of JAK1, JAK2 and TYK2 in interferon signaling were also uniquely related to cognitive appraisal.

The mechanisms by which a woman's cognitive appraisal of her ability to cope with a potentially threatening stressor might be transmitted to her unborn child, if not by way of her subsequent subjective level of distress which could cascade onto her physiological stress response and pass through the placenta, remain unknown. Clues may be found in research with non-pregnant subjects. In a study reporting the psychological determinants of the cortisol stress response,[16] it was shown that anticipatory cognitive appraisal predicted the HPA axis responses to acute stress from the Trier Social Stress Test when measured by salivary cortisol. The same researchers further demonstrated that cognitive appraisal affected stress hormone release, which modulated the expression of inflammatory cytokines such as TNFα (tumor necrosis factor alpha) and IL-6.[35] Therefore, we could hypothesize that pregnant women's cognitive appraisal of the ice storm might have induced elevated cortisol levels, which could pass through the placenta and alter the fetus's HPA axis function and immune system. Together, it reflects the importance of understanding the underlying mechanisms of cognitive appraisal in relation to the immune system in the offspring. The fact that the common genes and biological pathways associated with the women's cognitive appraisal of the ice storm are predominantly involved in immune functions provides evidence that PNMS could affect immune function in human offspring in a similar manner that has been observed in laboratory animals (reviewed by Veru *et al.*[27]). These results suggest that the methylome of the immune system could serve as an important target for studying behavioral and psychosocial epigenetics.

In Project Ice Storm, we have found the three aspects of the stress experience (the objective hardship, the cognitive appraisal of the storm's consequences and the women's enduring subjective distress) to be relatively independent of each other (the correlation between objective and subjective PNMS in the present subsample is $r = 0.161$). In addition, we have shown in other Project Ice Storm analyses that the objective and subjective PNMS scores predict different outcomes in the children: although objective PNMS predicts cognitive outcomes,[23] insulin secretion[28] and obesity,[26] subjective PNMS predicts fingerprint asymmetry[36] and asthma risk;[37] some outcomes, such as motor function[38] and autistic-like symptoms,[39] are predicted by a combination of both. When it comes to the effects of PNMS on epigenetic signatures, it

Table 2. Seventy-five pathways which were uniquely associated with maternal cognitive appraisal of the ice storm

Ingenuity Canonical Pathways	*– log(P-value)*	*Molecules*
STAT3 pathway	3.14E+00	SOCS1,MAP3K11,FLT1,RAC1,STAT3,JAK2,DDR1,TGFBR2,MAPK14,BMPR1A, CDKN1A,MAPK10,IGF1R,MAP2K1
γ-Linolenate biosynthesis II (Animals)	2.21E+00	ACSL3,ACSBG1,ACSL6,CYB5R3,SLC27A1
PDGF signaling	2.01E+00	SYNJ2,PIK3R6,PIK3R5,PLCG1,PIK3CD,STAT3,PIK3R2,JAK2,PDGFD,STAT1, MAP2K1,INPP5D
Agrin interactions at neuromuscular junction	2.00E+00	ITGB1,ITGB2,PXN,NRG2,ACTA2,MAPK10,RAC1,ITGA6,ERBB3,ITGAL,CTTN
DNA methylation and transcriptional repression signaling	1.89E+00	DNMT3B,CHD3,HDAC1,MTA2,ARID4B
Wnt/β-catenin signaling	1.60E+00	LRP5,PPARD,CSNK1G3,HDAC1,TLE1,WNT6,PPP2R5A,TGFBR2,PPP2R4, GNAO1,PPP2R2B,RARB,TLE4,CD44,PPP2R5C,LEF1,PPP2R5E,TCF7L2, WNT5A,SOX5
Role of JAK1, JAK2 and TYK2 in interferon signaling	1.56E+00	SOCS1,IFNGR2,STAT3,JAK2,STAT1
Biotin-carboxyl carrier protein assembly	1.54E+00	HLCS,ACACA
Stearate biosynthesis I (Animals)	1.40E+00	ACSL3,ACSBG1,PPT1,ACSL6,SLC27A1,ACOT7
Renal cell carcinoma signaling	1.17E+00	ETS1,SLC2A1,PIK3R6,RAC1,PIK3R5,PIK3CD,HIF1A,PIK3R2,MAP2K1
Semaphorin signaling in neurons	1.08E+00	ITGB1,FYN,CRMP1,RAC1,LIMK2,RHOH,FNBP1
Tumoricidal function of hepatic natural killer cells	1.03E+00	PRF1,ITGAL,CASP7,FASLG
Oxidative ethanol degradation III	9.70E – 01	ALDH4A1,ACSL3,ALDH1A2
Remodeling of epithelial adherens junctions	9.12E – 01	ACTR3,RAB5C,ACTA2,ZYX,TUBB4A,TUBB,IQGAP1,ARPC4
Leucine degradation I	8.64E – 01	BCAT1,HMGCL
Ethanol degradation IV	8.53E – 01	ALDH4A1,ACSL3,ALDH1A2
Spliceosomal cycle	8.51E – 01	U2AF1
Proline degradation	8.51E – 01	ALDH4A1
4-Hydroxyproline degradation I	8.51E – 01	ALDH4A1
GDP-L-fucose biosynthesis I (from GDP-D-mannose)	8.51E – 01	GMDS
Glutamate biosynthesis II	8.51E – 01	GLUD1
Glutamate degradation X	8.51E – 01	GLUD1
Fatty acid β-oxidation I	7.61E – 01	ACSL3,ACSBG1,ACSL6,SLC27A1
Regulation of the epithelial–mesenchymal transition pathway	7.61E – 01	ETS1,PIK3R5,mir-155,WNT6,STAT3,HIF1A,JAK2,SMURF1,TGFBR2,PIK3R6, PIK3CD,LEF1,PIK3R2,PDGFD,MAP2K1,TCF7L2,WNT5A
Human embryonic stem cell pluripotency	7.24E – 01	TGFBR2,BMPR1A,PIK3R6,TDGF1,PIK3R5,PIK3CD,LEF1,WNT6,PIK3R2, PDGFD,LEFTY2,TCF7L2,WNT5A
Ketogenesis	7.21E – 01	ACAT2,HMGCL
Role of PI3K/AKT signaling in the pathogenesis of influenza	7.08E – 01	NFKBIA,PIK3R6,GNAI1,PIK3R5,PIK3CD,PIK3R2,MAP2K1
Role of NANOG in mammalian embryonic stem cell pluripotency	7.08E – 01	IL6ST,BMPR1A,PIK3R6,PIK3R5,WNT6,PIK3CD,STAT3,PIK3R2,JAK2,MAP2K1, WNT5A
Trehalose degradation II (Trehalase)	6.91E – 01	GCK
Glycerol-3-phosphate shuttle	6.91E – 01	GPD2
Maturity onset diabetes of young (MODY) signaling	6.29E – 01	CACNA1D,GCK,CACNA1C
Choline biosynthesis III	6.10E – 01	PLD3,PCYT1A
Histamine degradation	6.10E – 01	ALDH4A1,ALDH1A2
Arginine degradation I (Arginase Pathway)	5.82E – 01	ALDH4A1
Lactose degradation III	5.82E – 01	GLB1
Androgen biosynthesis	5.63E – 01	SRD5A2,HSD17B14
Urate biosynthesis/Inosine 5'-phosphate degradation	5.63E – 01	IMPDH1,NT5C2
Colanic acid building blocks biosynthesis	5.63E – 01	GMDS,MPI
Isoleucine degradation I	5.21E – 01	BCAT1,ACAT2
CDP-diacylglycerol biosynthesis I	5.21E – 01	ABHD5,LCLAT1
Leukotriene biosynthesis	5.21E – 01	DPEP1,LTC4S
Mevalonate pathway I	5.21E – 01	ACAT2,HMGCR
tRNA charging	5.21E – 01	FARS2,CARS2,LARS2,AARS
Pentose phosphate pathway (Oxidative Branch)	5.00E – 01	H6PD
Glycerol degradation I	5.00E – 01	GPD2
Acetate conversion to acetyl-CoA	5.00E – 01	ACSL3
Phosphatidylglycerol biosynthesis II (Non-plastidic)	4.48E – 01	ABHD5,LCLAT1
Arginine biosynthesis IV	4.36E – 01	GLUD1
Acetyl-CoA biosynthesis I (Pyruvate Dehydrogenase Complex)	4.36E – 01	PDHA2
Superoxide radicals degradation	4.36E – 01	SOD3
Glycogen biosynthesis II (from UDP-D-Glucose)	4.36E – 01	GBE1
Chondroitin sulfate biosynthesis	4.25E – 01	CHST2,SULT1C4,XYLT1,CHSY1,CSGALNACT1
Tryptophan degradation X (Mammalian, via Tryptamine)	4.16E – 01	ALDH4A1,ALDH1A2
Endoplasmic reticulum stress pathway	4.16E – 01	CASP7,TAOK3
Dermatan sulfate biosynthesis	3.92E – 01	CHST2,SULT1C4,XYLT1,CHSY1,CSGALNACT1
Superpathway of geranylgeranyldiphosphate biosynthesis I (via Mevalonate)	3.87E – 01	ACAT2,HMGCR
Phosphatidylcholine biosynthesis I	3.85E – 01	PCYT1A
Thyroid hormone metabolism II (via Conjugation and/or Degradation)	3.78E – 01	DIO1,CSGALNACT1,UGT3A1
Cardiomyocyte differentiation via BMP receptors	3.61E – 01	NPPB,BMPR1A

Table 2. (Continued)

Ingenuity Canonical Pathways	*– log(P-value)*	*Molecules*
Purine nucleotides degradation II (Aerobic)	3.61E – 01	IMPDH1,NT5C2
Chondroitin sulfate biosynthesis (Late Stages)	3.59E – 01	CHST2,SULT1C4,CHSY1,CSGALNACT1
Ethanol degradation II	3.58E – 01	ALDH4A1,ACSL3,ALDH1A2
Glycoaminoglycan-protein linkage region biosynthesis	3.41E – 01	XYLT1
Polyamine regulation in colon cancer	3.14E – 01	AZIN1,SAT2
Phosphatidylethanolamine biosynthesis II	3.05E – 01	ETNK1
Calcium transport I	2.74E – 01	ATP2B2
Ketolysis	2.74E – 01	ACAT2
UDP-N-acetyl-D-galactosamine biosynthesis II	2.74E – 01	GCK
Folate transformations I	2.74E – 01	MTHFD1L
Dopamine degradation	2.73E – 01	ALDH4A1,ALDH1A2
Pentose phosphate pathway	2.47E – 01	H6PD
Triacylglycerol degradation	2.39E – 01	ABHD5,PNPLA2
Glutaryl-CoA degradation	2.23E – 01	ACAT2
Purine nucleotides *de novo* biosynthesis II	2.23E – 01	IMPDH1
Guanosine nucleotides degradation III	2.02E –01	NT5C2

Abbreviation: STAT3, signal transducers and activators of transcription 3.

appears that the objective degree of hardship to which the pregnant woman was exposed has a strong effect, but that her cognitive appraisal of the consequences of the storm on her and her family have an even greater effect; the mothers' subjective reactions to the ice storm, in the form of PTSD symptoms 5–6 months after the storm, appear to have no effect on offspring DNA methylation at age 13 years. Furthermore, we performed several analyses to investigate whether the methylation profile was involved in the offspring's metabolic outcomes and found significant mediating effects of genes from the type I diabetes mellitus signaling pathway on a variety of metabolic measures such as BMI, central adiposity, C-peptide secretion and insulin secretion.[40]

The current paper is the first time that we have included the cognitive appraisal rating in our analyses; as such, we are unable to rule out the possibility that this aspect of the stress experience may also have influenced other aspects of child development. This study is limited by the modest sample size for an epigenetic study which also meant that we did not have the statistical power to conduct sex-specific analyses. In addition, the distribution of cognitive appraisal ratings in this subsample meant that we could not use the full range of five ratings (very negative through very positive), and could not adequately analyze a neutral appraisal group. Among the subsample for epigenetic study, only four individuals (12%) rated cognitive appraisal as 'Neutral', their methylation profiles cannot be distinguished from those of the combined negative group. Also, we cannot know the extent to which our results on DNA methylation could be related to gene expression since no mRNA was obtained from this cohort. Thus, confirmation of our results in an independent replication with a larger sample will be needed to gain greater confidence in the validity of our results. Finally, although brain tissues are the best for determining DNA methylation changes in response to psychosocial stress, we have no access to this tissue in a living cohort. Given this limitation, data are emerging supporting the utility of peripheral DNA methylation measures for mental health research.[41–44]

Despite these limitations, to the best of our knowledge, this is the first human PNMS study investigating the effect of maternal cognitive appraisal from an independent stressor such as a natural disaster that detects DNA methylation differences throughout the genome using a genome-wide array in the offspring during adolescence. As epigenetic modification is cell- or tissue-specific, we only focused on the methylation profile of T cells, which are responsive to both stress[45] and HPA axis functioning,[46] and avoided the heterogeneous mixture of white blood cell types.

In conclusion, our study suggests that pregnant women's cognitive appraisals of an independent stressor may have widespread effects on DNA methylation across the entire genome of their unborn children, detectable during adolescence. Therefore, cognitive appraisals could be an important predictor variable to explore in PNMS research. These effects may have important implications for the immune functioning in children later in life, and are consistent with the fetal programming hypothesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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