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Original Article

ORIGINAL ARTICLE

High-dose folic acid supplementation alters the human sperm methylome and is influenced by the *MTHFR* C677T polymorphism

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Abstract

Dietary folate is a major source of methyl groups required for DNA methylation, an epigenetic modification that is actively maintained and remodeled during spermatogenesis. While high-dose folic acid supplementation (up to 10 times the daily recommended dose) has been shown to improve sperm parameters in infertile men, the effects of supplementation on the sperm epigenome are unknown. To assess the impact of 6 months of high-dose folic acid supplementation on the sperm epigenome, we studied 30 men with idiopathic infertility. Blood folate concentrations increased significantly after supplementation with no significant improvements in sperm parameters. Methylation levels of the differentially methylated regions of several imprinted loci (*H19*, *DLK1/GTL2*, *MEST*, *SNRPN*, *PLAGL1*, *KCNQ1OT1*) were normal both before and after supplementation. Reduced representation bisulfite sequencing (RRBS) revealed a significant global loss of methylation across different regions of the sperm genome. The most marked loss of DNA methylation was found in sperm from patients homozygous for the methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism, a common polymorphism in a key enzyme required for folate metabolism. RRBS analysis also showed that most of the differentially methylated tiles were located in DNA repeats, low CpG-density and intergenic regions. Ingenuity Pathway Analysis revealed that methylation of promoter regions was altered in several genes involved in cancer and neurobehavioral disorders including *CBFA2T3*, *PTPN6*, *COL18A1*, *ALDH2*, *UBE4B*, *ERBB2*, *GABRB3*, *CNTNAP4* and *NIPA1*. Our data reveal alterations of the human sperm epigenome associated with high-dose folic acid supplementation, effects that were exacerbated by a common polymorphism in *MTHFR*.

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Introduction

The impact of the environment on the human genome has recently attracted the interest of scientists, clinicians and public health officials. Increasing numbers of studies show that factors such as diet, environment and stress may not only affect the health of individuals but also may be transmitted to their offspring, predisposing them to various diseases (1,2). Epigenetics provides a mechanism by which our genes can sense and respond to the environment, through heritable modifications of the genome, that in turn control gene activity without altering DNA sequence (3,4). Such modifications may occur at the levels of post-translational modifications of histones, coding and non-coding RNAs and DNA methylation; the latter has been widely studied and well characterized in recent years.

DNA methylation takes place at the 5-position of cytosine residues mainly within CpG dinucleotide sequences that occur at about 30 million sites throughout the human genome, with ~60–80% of CpG dinucleotides being methylated (5). Methylation of DNA is recognized as a mechanism for the epigenetic modification of genes and is essential for normal developmental programs. It is catalyzed by a family of DNA methyltransferase enzymes (DNMTs) that use methyl groups mainly provided by S-adenosyl methionine (SAM) as a substrate (6,7). SAM, in turn, is a major product of folate-dependent one carbon metabolism.

One carbon folate metabolism is required for a number of biochemical processes including methionine production and *de novo* purine and pyrimidine synthesis, and is integral to the production of methyl groups necessary for DNA methylation. The reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methylTHF, via 5,10-methyleneTHF reductase (MTHFR), and the subsequent transfer of the methyl group of 5-methylTHF to homocysteine result in the production of methionine. Methionine then provides the methyl groups required for SAM production and subsequently for DNA methylation (8,9). Folates are essential and must be provided by diet or supplementation. Folic acid, the synthetic form of folate, is used for the fortification of foods and in supplements. If folate intake is decreased or if folate metabolism is disrupted, the balance of SAM can be altered and methylation reactions disturbed with possible health consequences (10,11). A common polymorphism in the gene coding the MTHFR enzyme (C677T) results in lower activity of the enzyme, lower folate status and higher concentrations of circulating homocysteine (12,13). Individuals with the variant enzyme are predisposed to several diseases and health problems including male infertility (14,15).

A male factor is detected in ~50% of infertile couples and most male infertility cases are categorized as idiopathic. Some studies have suggested a higher frequency of homozygosity for the *MTHFR* C677T polymorphism (TT genotype) among infertile men (14). Folate is thought to be essential for spermatogenesis given its important role in the synthesis, repair and methylation of DNA. This premise is supported by randomized studies showing that folic acid supplementation at high doses (5 mg daily intake; >10 times the 400 µg recommended daily allowance) results in improved sperm concentration in infertile men (16,17). However, folic acid supplementation in infertile men occurs during adult spermatogenesis, a time when DNA methylation patterns are being actively maintained and remodeled in male germ cells to prepare the epigenome for embryogenesis (18). It is therefore crucial to understand the possible impact of supplementation of infertile men with high-dose folic acid on the sperm epigenome. The aim of this study was to use sensitive next generation sequencing-based approaches to investigate the impact of high-dose folic acid supplementation on the sperm DNA methylome in men presenting with infertility.

Results

High-dose folic acid supplementation significantly increases blood folate concentrations

Men presenting with infertility ($n = 30$; mean age: 37.9 ± 1.3 years) were recruited from the McGill University Reproductive Centre and the Clinique OVO, Montreal, Canada, between 2009 and 2011. Participants were given supplements of folic acid (5 mg/day) for 6 months as part of their infertility treatment protocols. Blood and sperm samples were obtained prior to and after the supplementation period. Serum folate concentrations increased significantly after 6 months of high-dose folic acid supplementation. Red blood cell (RBC) folate concentration, an indicator of long-term exposure to folate, also increased (*t*-test $P < 0.0001$, Fig. 1A and B). Further blood analysis showed no other changes in blood hormones and biochemistry (Table 1). We also analyzed the samples for the common C677T polymorphism in the gene coding the folate pathway enzyme, MTHFR. Interestingly, 7 participants (23.3%) were homozygous for the *MTHFR* TT variant while 13 (43.3%) and 10 (33.3%) were CC and CT genotypes, respectively. A higher than expected frequency (~10–15%) (19) of the *MTHFR* TT genotype among the participants led us to investigate genotype differences in response to supplementation. There were no differences in baseline blood folate concentrations and blood folate increased significantly after supplementation regardless of *MTHFR* genotype (Fig. 1A and B). Thus, high-dose folic acid supplementation successfully increased blood folate concentrations and no *MTHFR* 677 genotype effect was detected.

Semen parameters remain unchanged after high-dose folic acid supplementation

We observed a non-significant increase in sperm concentration after folic acid supplementation in *MTHFR*677 CC and CT participants (Fig. 1C). Similarly, changes in semen volume, sperm motility and sperm chromatin integrity (assessed by sperm chromatin structure assay or SCSA, and reported as % high DNA stainability or %HDS) were not significant (Table 1). Sperm DNA integrity, assessed by SCSA and reported as % DNA fragmentation index (% DFI), demonstrated a trend toward improvement (*t*-test $P = 0.057$, Fig. 1D). As with blood folate concentrations, there were no differences between the three *MTHFR* genotypes for any of the semen parameters. Therefore, contrary to previous studies (16,17), we did not observe a significant improvement in semen parameters in our cohort of patients following folic acid supplementation although the improvement in sperm DNA integrity approached significance.

Methylation of imprinted loci in sperm remain normal before and after folic acid supplementation

To study the epigenetic impact of high-dose folic acid supplementation on the sperm of infertile men, we investigated the methylation status of imprinted genes in sperm DNA. Bisulfite pyrosequencing was utilized to assess the germline differentially methylated regions (DMRs) of two paternally methylated (*H19* and *DLK1/GTL2* IG-DMR) and four maternally methylated (*KCNQ1OT1, PLAGL1, MEST, SNRPN*) imprinted loci. Details of chromosomal locations and primers used are shown in Supplementary Material, Table S1. Assays were designed to analyze

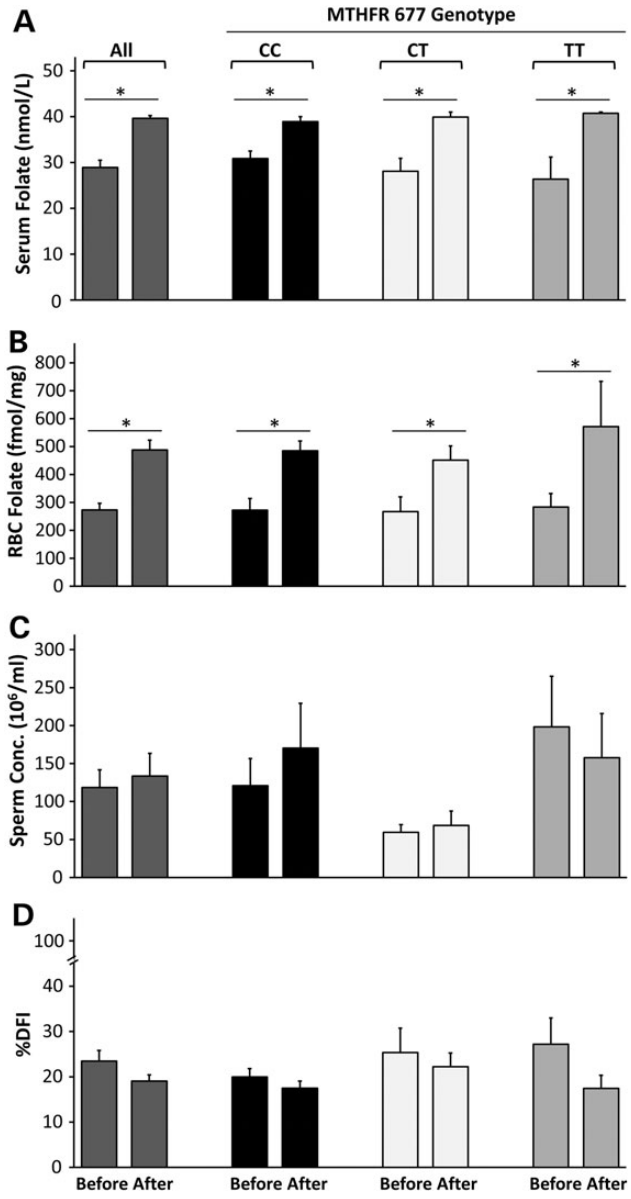

Figure 1. Supplementation of infertile men with high-dose folic acid improved folate status but not sperm parameters. Serum (**A**) and RBC (**B**) folate concentrations increased significantly after a course of 6-month supplementation with high-dose folic acid. However, sperm concentration (**C**) and DNA integrity (**D**) did not change significantly. No differences were detected among *MTHFR* C677T genotypes. Bars represent the mean ± Standard Error of mean (SEM). Asterisks indicate significant differences between groups (*t*-test; $P < 0.0001$). RBC: Red Blood Cell, DFI: DNA Fragmentation Index.

the methylation status of at least 5 and 10 CpGs for each paternally and maternally methylated imprinted locus, respectively. In somatic tissues, either the paternal or maternal allele of imprinted genes is completely methylated while the other parental allele is not methylated. Consequently, the average methylation of imprinted genes in somatic tissues is around 50%. Conversely in sperm DNA, where only one parental allele is present, methylation levels of paternally and maternally methylated loci are expected to be 100 and 0%, respectively. Analysis of the sperm DNA before and after high-dose folic acid supplementation showed that the methylation status of imprinted loci was within the normal range for all loci tested and was not altered in response to high-dose folic acid supplementation (Fig. 2A). Moreover, average methylation of imprinted loci was similar among different *MTHFR* C677T genotypes (Fig. 2B). Overall, our findings show that neither infertility nor high-dose folic acid supplementation for 6 months affected the methylation status of several imprinted genes.

Table 1. Blood folate and sperm parameters after supplementation with high-dose folic acid

	Before supplementation (Mean ± SEM)	After supplementation (Mean ± SEM)	P-value
Sperm parameters:			
Semen volume (ml)	2.86 ± 0.22	2.80 ± 0.25	0.773
Normal motile sperm (%)	36.1 ± 4.8	37.2 ± 4.1	0.788
Sperm high DNA stainability (HDS; %)	2.13 ± 0.30	2.28 ± 0.30	0.188
Blood parameters:			
Homocysteine (µmol/l)	9.5 ± 0.44	9 ± 0.34	0.190
Vitamin B12 (pmol/l)	255.6 ± 20.2	240.8 ± 20.51	0.158
FSH (IU/l)	5.82 ± 0.77	5.95 ± 0.72	0.460
LH (IU/l)	3.78 ± 0.45	4.16 ± 0.55	0.155
Prolactin (µg/l)	6.62 ± 0.50	7.16 ± 2.90	0.194
Testosterone (nmol/l)	14.46 ± 1.36	14.58 ± 1.14	0.912
TSH (mIU/l)	1.78 ± 0.17	1.74 ± 0.18	0.719

SEM, Standard Error of Mean.

Folic acid supplementation results in global loss of methylation across the sperm methylome and is exacerbated in *MTHFR* 677TT individuals

To assess the global impact of high-dose folic acid on the sperm methylome, we used a next generation sequencing-based method, reduced representation bisulfite sequencing (RRBS). Analysis of methylation status of ~1.8 million CpGs, in 100 base pair tiles, across the sperm epigenome was performed before and after supplementation in 28 participants (Supplementary Material, Table S2); samples from two individuals were excluded from RRBS analysis due to contamination with patients' somatic cells—see Methods. We initially hypothesized that providing high-dose folic acid as a substrate for the folate cycle would increase the availability of methyl groups and consequently increase genomic methylation levels. However, RRBS revealed the opposite; a slight but significant loss of methylation of all tiles analyzed. Significant loss of methylation was detectable in intergenic regions, introns and exons and, interestingly, was different among the *MTHFR* genotypes. While the global loss of methylation in sperm from *MTHFR* 677 CC men was only significant in intergenic regions, having at least one T allele (CT or TT) was associated with a significant loss of methylation across all sequenced tiles in promoters, exons, introns and intergenic regions (Fig. 3A and Supplementary Material, Fig. S1).

While the majority of CpGs in the genome are either highly methylated (>80%) or unmethylated (<20%), a significant number of loci do not fit into these categories and are categorized as

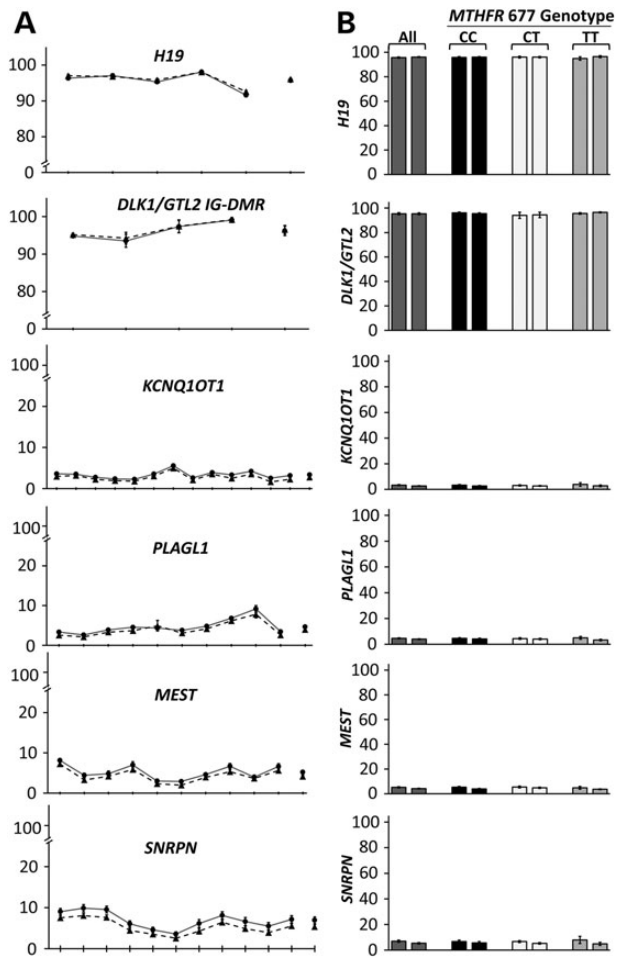

Figure 2. Methylation status of germline DMRs of imprinted loci was normal and did not change after folic acid supplementation. (**A**) Methylation levels of individual CpGs (% mean of all samples ± SEM) are demonstrated on the vertical axis before (round dots and straight lines) and after (triangle dots and dashed lines) supplementation as assessed by bisulfite pyrosequencing. Mean methylation levels (±SEM) of DMRs are shown separately on the right before (round) and after (triangle) supplementation. (**B**) % Mean methylation levels (± SEM; vertical axis) of DMRs did not differ among the *MTHFR* C677T genotypes.

having intermediate methylation. Recent evidence indicates that regions of intermediate methylation include those that exhibit allelic methylation (e.g. imprinting), intercellular variability and enhancer function and thus may have specific roles in gene activity (20). To better understand the methylation changes in our subjects, we categorized all the sequenced CGs in each sample into low (<20%), intermediate (20–80%) or high (>80%) levels of methylation, and compared the fractions of CpGs in each category before and after supplementation. As depicted in Figure 3B, there were trends of a decrease in the number of CGs with high methylation and an increase of CpGs with low methylation across all sperm samples after supplementation (*t*-test $P = 0.13$ and 0.08, respectively); the number of CpGs with intermediate methylation levels decreased significantly (*t*-test $P < 0.05$). Interestingly, having more CGs with low methylation levels after supplementation was more prominent in *MTHFR* 677 TT participants (Fig. 3B). Finally, differentially methylated tiles (DMTs) after supplementation that showed significant >10% change in methylation at a minimum of 10-fold sequencing coverage were analyzed (adjusted False Discovery Rate *q*-value = 0.01, see Methods for details). In line with our findings for global methylation levels, sperm DNA from individuals with the *MTHFR* 677TT genotype had higher numbers of DMTs with loss of methylation in comparison to those with the CC and CT genotypes (Fisher's exact test $P < 0.0001$; Fig. 3C). These findings show that contrary to expectations, high-dose folic acid supplementation resulted in a global loss of methylation across the sperm epigenome. Loss of methylation was more prominent in the sperm of individuals with *MTHFR* 677TT homozygosity.

Individual CpGs in intergenic regions are more susceptible to methylation changes after high-dose folic acid supplementation

To better understand the epigenomic impact of high-dose folic acid supplementation on the sperm of infertile men, we next analyzed the distribution of DMTs among different genomic regions. Distributions of all sequenced tiles, regardless of their methylation levels, among genomic regions were then compared with DMTs. Specific genomic regions were considered potentially more susceptible to supplementation when larger fractions of DMTs were observed within these regions. Distributions of sequenced tiles were first analyzed among different intergenic and genic (exons, introns, promoters, 3′ and 5′ untranslated regions and transcription termination sites) regions and revealed higher numbers of DMTs in intergenic regions. Moreover, lower numbers of DMTs were localized to promoters in comparison with sperm genome-wide sequenced tiles (Fig. 4A). In all of the genomic regions, numbers of DMTs with loss of methylation were higher than the numbers of DMTs with gain of methylation (Supplementary Material, Fig. S2 and Table S3). To validate the RRBS findings, we selected 14 DMTs representing different genomic regions with loss/gain of methylation and analyzed their methylation status before and after supplementation by bisulfite pyrosequencing. As shown in Supplementary Material, Table S4, 11 of these DMTs had at least two CpGs showing significant differential methylation in the same direction as detected by RRBS.

Analysis of DMT distributions among CG islands (CGI), shore, shelf or open sea regions revealed that most of the DMTs were located in open seas which represent the low CpG-density regions of the genome (Fig. 4B). Similar to genomic regions, DMTs with loss of methylation on CGIs, shores and open seas were significantly more frequent than DMTs with gain of methylation (Supplementary Material, Fig. S2 and Table S3). Moreover, distributions of DMTs across different genic and CpG regions were similar among *MTHFR* C677T genotypes (data not shown). These data suggested that the impact of high-dose folic acid supplementation was most frequently observed in intergenic and low CG-density regions of the sperm genome.

Most genomic methylation changes associated with high-dose folic acid supplementation occur in sperm DNA repeat regions and transposable elements

Since a large number of DNA repeats and transposable elements are found within the DNA intergenic regions, we hypothesized that repeat regions were affected by changes in DNA methylation levels. Analysis of DNA repeat regions revealed that ~70% of DMTs were located in such regions while only half of all sequenced tiles were within the repeat regions. In fact, the fractions of DMTs on short interspersed nuclear elements (SINEs), SINE-variable number of tandem repeats-Alu elements (SVAs) and long terminal repeats (LTRs) were significantly higher when compared with all sequenced tiles, while similar fractions were located in long interspersed nuclear elements (LINEs). On the

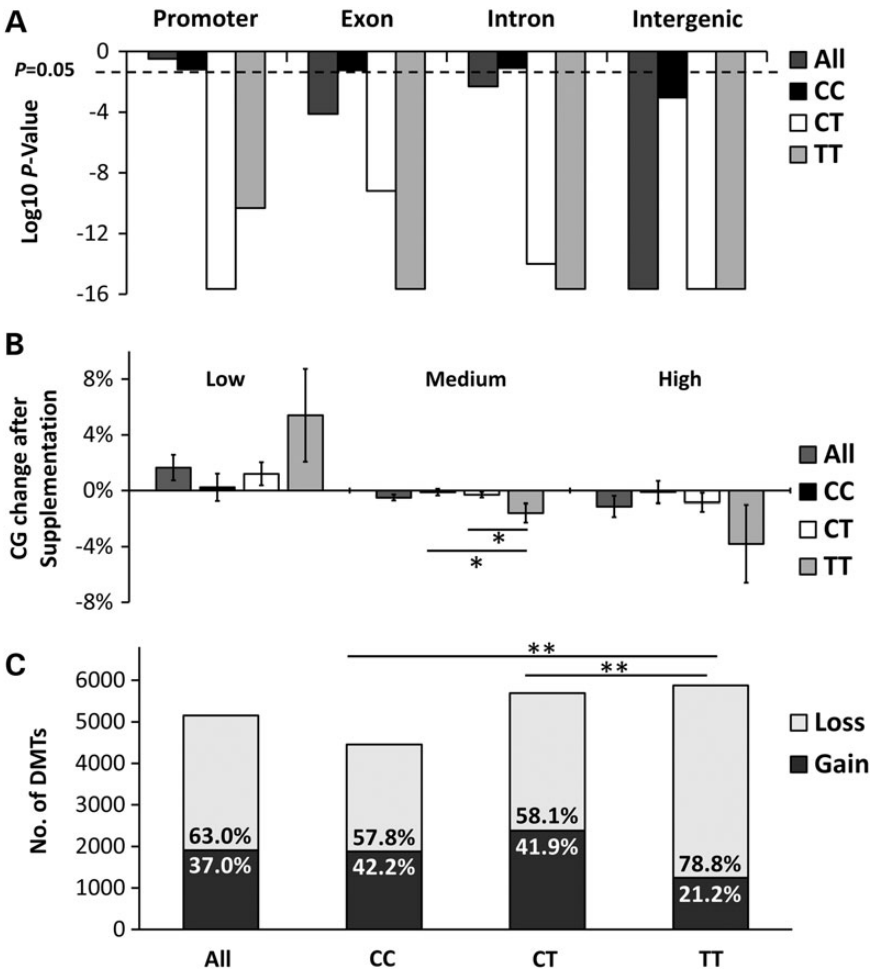

Figure 3. Global loss of methylation was detected by RRBS among different genomic regions after supplementation with high-dose folic acid. (**A**) Bars indicate the log10 of the *P*-value for the significant global loss of methylation across all RRBS sequenced tiles. Data for all participants and the *MTHFR* C677T genotypes are presented. Patients with *MTHFR* 677TT genotype demonstrated the most significant loss of methylation. (**B**) RRBS sequenced CGs were calculated and classified as low (<20% methylation), medium or intermediate (20–80% methylation) and high (>80% methylation). Bars indicate the relative change in class after supplementation for all participants and the *MTHFR* C677T genotypes. *MTHFR* 677TT participants demonstrated the most diverse and greatest changes in terms of an increase in CGs with low methylation and decrease in CGs with medium and high methylation levels. (**C**) The mean number of DMTs with greater than 10% change of methylation levels is depicted for all participants and the *MTHFR* C677T genotypes. The percentage of tiles with loss or gain of methylation is shown. Asterisks indicate significant differences between groups (ANOVA for B and Fisher's exact test for C; $P < 0.05$ for one asterisk and $P < 0.0001$ for two asterisks).

other hand, smaller fractions of DMTs were found in other types of DNA repeat regions as well as non-repeat areas of the sperm genome (Fig. 4C). As predicted, most of the tiles in repeat regions demonstrated loss of methylation (Supplementary Material, Fig. S2 and Table S3). Therefore, analysis of sperm DNA repeat regions showed that these regions were susceptible to high-dose folic acid supplementation and that changes mostly included loss of methylation regardless of the *MTHFR* C677T genotype.

High-dose folic acid supplementation alters methylation of genes related to cancer and neurobehavioral disorders

While most methylation changes due to high-dose folic acid supplementation occurred in intergenic regions, we postulated that the affected genic regions may represent disease-related genes. Ingenuity Pathway Analysis (IPA) was utilized to test this hypothesis; only the genic (near or within genes) DMTs were included to avoid possible bias towards some intergenic regions with altered methylation which were far from any of the identified genes. DMTs from genic regions were divided, prior to IPA, based on the direction of methylation changes to either gain or loss of methylation. Interestingly, both groups of genic DMTs consisted of genes related to cancers and neurobehavioral/developmental disorders. As shown in Figure 5A cancer-related genes were detected in almost all of the 28 participants analyzed. Genes related to gastrointestinal (GI) cancers mostly demonstrated gain of methylation whereas endocrine cancer-related genes were more frequently detected in the group of DMTs with loss of methylation. Reproductive cancer-related genes demonstrated both loss and gain of methylation; some patients had alterations in methylation of genes related to neurobehavioral/developmental disorders and obesity (Fig. 5A).

To narrow the list of disease-related genes and possibly target the most meaningful alterations in methylation after high-dose folic acid supplementation, we selected the list of DMTs for each participant that had significant alterations in the promoter regions. This approach was based on evidence for an inverse relationship between methylation levels of gene promoters and their expression. The list was then screened by IPA to search more specifically which, interestingly, revealed several genes

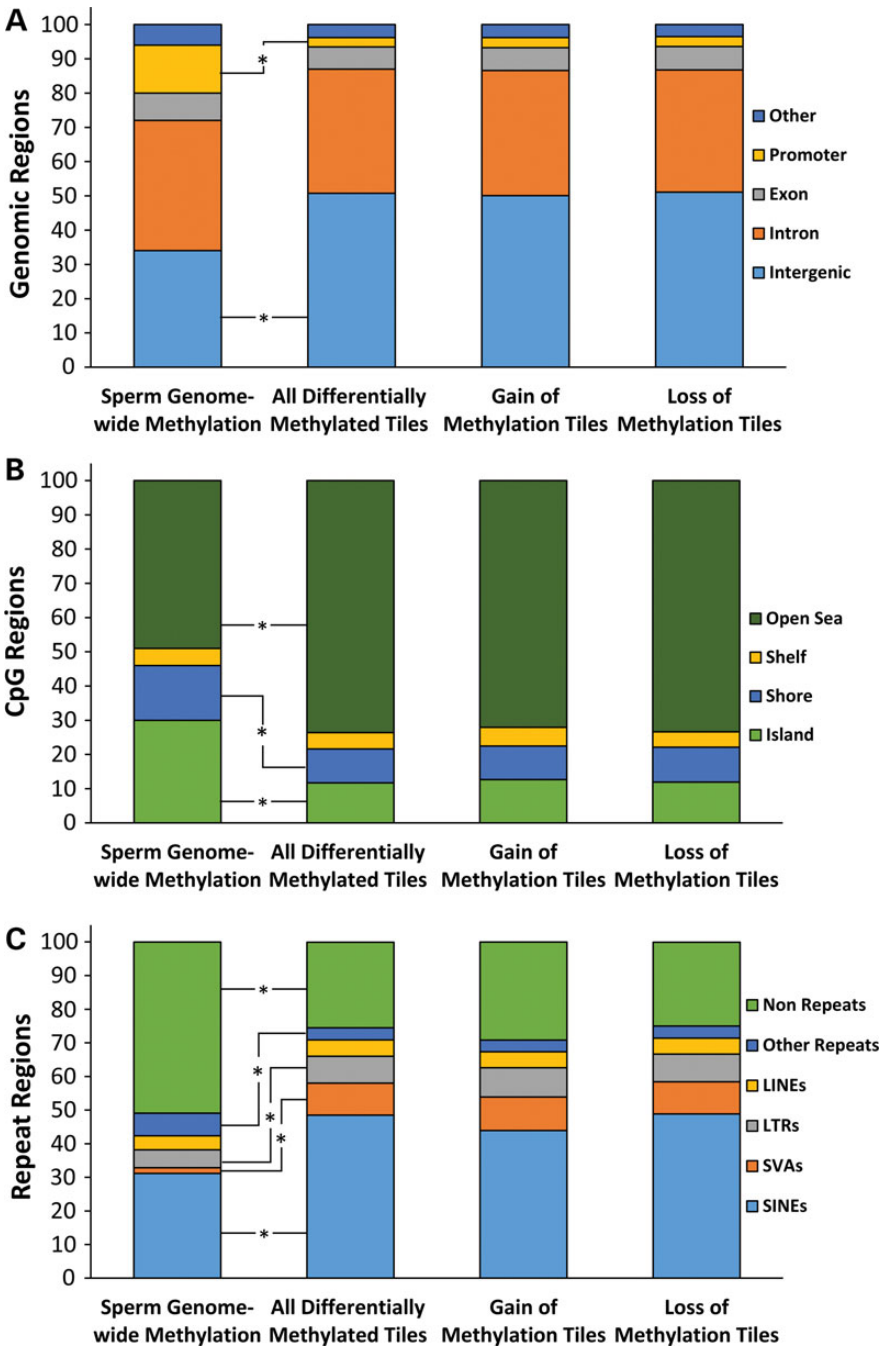

Figure 4. Supplementation with high-dose folic acid resulted in DMTs within intergenic regions, DNA repeats and Open Sea regions (>4kb distance from CpG islands). Distributions of all sequenced tiles, regardless of their methylation changes, and proportion of DMTs (DMTs with >10% gain/loss of methylation) among all participants are presented for different genomic (**A**), CpG-rich (**B**) and repeat (**C**) regions. DMTs were further classified as tiles with significant gain or loss of methylation. Smaller proportions of tiles on promoters, CGI and shores as well as non-repeat regions were affected than expected. Asterisks indicate significant differences between the distributions of all sequenced tiles (sperm genome-wide methylation) and DMTs (ANOVA $P < 0.0001$).

related to various types of cancers and developmental, neurobehavioral, cardiovascular and infertility disorders (Fig. 5B). We further selected 15 DMTs for validation of RRBS methylation changes by bisulfite pyrosequencing. DMTs selected for validation included tumor suppressor genes (TSGs) with gain of promoter methylation and hence possible lower expression, tumor growth factors (TGFs) with loss of promoter methylation and possible higher expression and finally a group of genes related to neurobehavioral disorders including autism and schizophrenia. Pyrosequencing confirmed the RRBS findings; 11 out of 15 selected promoter DMTs had at least 50% of their CpGs with significant differential methylation in the same direction as detected by RRBS (Table 2 and Supplementary Material, Table S5). Considering the proposed roles of long non-coding RNAs (lncRNAs) in cancer and normal development (21,22), we also examined the enrichment for sequences coding lncRNAs among the DMTs. A slightly higher percentage of lncRNA sequences was observed in DMTs in comparison with all sequenced tiles (13.8 versus12.1%,

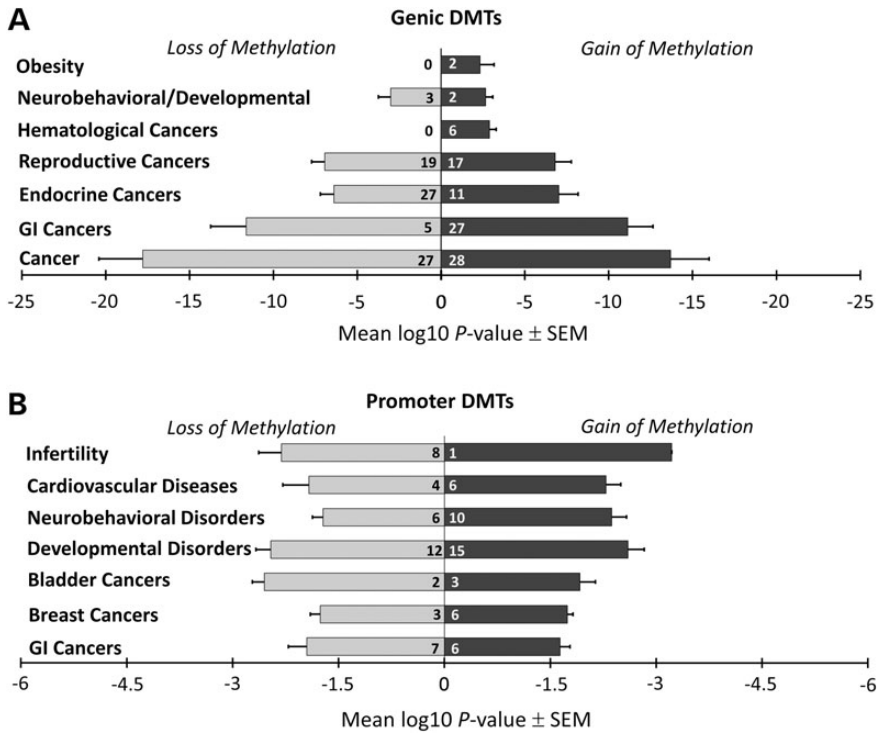

Figure 5. Ingenuity Pathway Analysis (IPA) on human sperm DMTs (all participants) showed that high-dose folic acid supplementation altered the methylation of genes related to cancer and neurobehavioral disorders. DMTs from genic (**A**) or promoter (**B**) regions were classified based on their significant gain or loss of methylation. Bars demonstrate the mean log10 of *P*-value (±SEM) for the significance of affecting the genes related to specific corresponding diseases/disorders, as indicated by IPA. The numbers on each bar represent the number of participants who had DMTs with significant correlations to the disease-related genes.

Table 2. Validation of RRBS results in representative disease-related genes

	Promoter tile coordinates	Number of CGs	RRBS methylation change	Pyrosequencing methylation change (number of validated CGs)
Tumor suppressor genes				
PTPN6	Chr12:7060101-200	4	↑	↑ (4)
COL18A1	Chr21:46875401-500	5	↑	↑ (4)
CBFA2T3	Chr16:89043501-600	5	↑	↑ (3)
Tumor growth factor genes				
UBE4B	Chr1:10092101–200	5	↓	↓ (5)
ALDH2	Chr12:112203801–900	4	↓	↓ (4)
ERBB2	Chr17:37856001–100	2	↓	↓ (1)
Neurobehavioral disorders				
GABRB3	Chr15:26874201-300	3	↓	↓ (3)
CNTNAP4	Chr16:76343601-700	4	↓	↓ (4)
NIPA1	Chr15:23087601-700	5	↓	↓ (5)

respectively; *t*-test $P < 0.0001$). Together, our data show that the methylation status of a number of disease-related genes, especially cancer genes, was altered after supplementation of infertile men with high-dose folic acid for 6 months irrespective of their *MTHFR* C677T genotypes.

Discussion

This study provides evidence that high-dose folic acid supplementation leads to genome-wide alterations in sperm DNA methylation, in particular, loss of methylation mostly in intergenic and low-density CpG regions. Genic regions were also affected, some of which may be linked to diseases. Homozygosity for the *MTHFR* C677T polymorphism was demonstrated as a factor that can modify the response to high-dose folic acid supplementation. Reduced representation bisulfite sequencing was used in the current study to examine genome-wide effects of folic acid supplementation on the sperm epigenome. RRBS enriches for GC-rich genomic loci and is cost-effective when compared with whole genome bisulfite sequencing. However, data on some intergenic regions with low CpG-density and possible biological relevance may not be covered by RRBS. Yet, higher numbers of DNA methylation sites are covered by RRBS in contrast to array-based methods such as the Illumina 450 K Infinium

arrays that assess methylation status of ~450 000 CGs from selected genomic regions. Using RRBS, we analyzed sperm DNA methylation for ~1.8 million CpGs, over one-third of which were located in intergenic regions or in CGI.

In our cohort of infertile men, we did not observe a significant improvement in sperm parameters after 6 months of high-dose folic acid supplementation although we noted a trend towards improved sperm DNA integrity after supplementation. Two research groups have studied the effects of high-dose folic acid supplementation (5 mg daily) for 26 weeks in two cohorts of infertile men from the Netherlands and South Africa (16,17). Both randomized, placebo-controlled studies reported a small but significant increase in sperm concentration (from an average of 7.5–9 million/ml) in the treatment arm. A limitation of the current study was that it was not placebo-controlled. While we analyzed each individual patient before and after supplementation, the relatively small sample size may be one of the reasons we did not observe significant changes in any of the semen parameters. It is also important to note that we studied normozoospermic men with idiopathic male infertility while a significant improvement in semen parameters was only noted in oligozoospermic men in the prior studies. It is possible that high-dose folic acid supplementation only slightly increases the sperm concentration in patients with oligozoospermia and not all men presenting with infertility. Also, the baseline folate status of subjects must be considered. In Canada where there is mandatory folic acid fortification of white flour, it would be expected that most men have an adequate folate status, which was confirmed in our analysis. However, in men from regions where fortification is not mandatory baseline folate status may be low, allowing for a more robust response to supplementation. Therefore, more individualized approaches to supplementation with high-dose folic acid are suggested for infertile men, likely a very heterogeneous and genetically diverse population for which a single therapeutic regimen may not benefit all.

Methylation levels of germline DMRs of imprinted loci were within the normal range for two paternally methylated genes (*H19* and *DLK1/GTL2* IG-DMR) and four maternally methylated genes (*MEST*, *SNRPN*, *KCNQ1OT1*, *PLAGL1*) in this study. No changes were detected after 6 months of high-dose folic acid supplementation. Concerns about aberrant methylation of imprinted loci in the sperm of infertile men are related to studies that suggest a higher prevalence of imprinting disorders such as Beckwith–Wiedemann Syndrome in the offspring of infertile couples using assisted reproductive technologies (ART) (23,24). Whether such effects are due to altered methylation of imprinted loci in the gametes or deleterious effects of ART remains unclear (25,26). While abnormal methylation levels of imprinted loci, especially *H19* and *MEST*, have been reported in the sperm of infertile men in some studies, others have reported only subtle differences between infertile and fertile men (27–29). Two recent studies utilized Illumina 450 K arrays to study the sperm epigenome of infertile men and did not find evidence of alterations at imprinted loci (30,31). Factors such as different inclusion criteria, possible contamination of sperm DNA with DNA from somatic cells and use of different techniques for detection of DNA methylation levels, may explain discrepancies between reports. Thus, additional genome-wide studies are still needed to assess the sperm epigenome, including imprinted loci, in well-characterized patient populations.

An unexpected finding in this study was the global loss of methylation across the sperm methylome in response to folic acid supplementation. Sequencing of ~0.5 million 100-base tiles in each patient showed that the observed loss of methylation was significant in most intergenic and genic regions. The findings suggest that methylation capacity and/or homeostasis may be affected by high-dose folic acid supplementation. Evidence for reduction of neural tube defects in pregnancies after folic acid supplementation led to mandatory fortification of white flour in North America in the late 1990s (32,33). As a result, daily folic acid intake increased, blood folate concentrations increased and the incidence of neural tube defects decreased significantly (34,35). Meanwhile, consumption of a multivitamin supplement containing 400–1000 μg/day of folic acid has been recommended to women considering a pregnancy. In addition, high-dose folic acid supplements (up to 5 mg/day) are used in women with high-risk pregnancies and men presenting with infertility (16,34,36,37). While the epigenomic consequences of folate deficiency and supplementation during pregnancy have been examined (10,38,39), little is known about their impact on the sperm epigenome. In the current study, the participants demonstrated normal serum and RBC folate concentrations before supplementation. Therefore, providing participants with excess folic acid may have altered folate metabolism and consequently cellular methylation capacity and DNA methylation patterns. Recently, treatment of two colon cancer cell lines with excess folic acid for 1 week resulted in an increase in the SAM:SAH ratio which was unexpectedly accompanied with a decrease in global DNA methylation. The authors suggested that folic acid-induced epigenetic modifications in the cells may be associated with SAM/SAH independent pathways (40). A limitation of our study is that we did not measure SAM, SAH and other folate-related metabolites. More in-depth studies are required to investigate whether alterations of folate and other metabolic pathways occur after high-dose folic acid supplementation in men.

We observed a higher than expected frequency of men that were homozygous for the *MTHFR* C677T polymorphism (~23%) in our study population. This is roughly twice the expected frequency of the TT genotype (~10%) in the Canadian population (19). The global loss of methylation was pronounced in TT patients compared with CC patients after folic acid supplementation. The *MTHFR* C677T polymorphism leads to substitution of valine for alanine in the MTHFR protein which in turn results in a 50–60% reduction in enzyme activity, due to increased thermolability (13). A higher frequency of individuals with the *MTHFR* 677TT genotype in our cohort of infertile men is in line with several studies that suggested this polymorphism as a risk factor for male infertility (14). Utilizing RRBS to investigate DNA methylation at a genome-wide level, we uncovered a different response to high-dose folic acid supplementation in the sperm methylome of patients with different genotypes but similar sperm parameters, blood folate levels and methylation patterns of imprinted loci. Recently, the effects of high-dose folic acid (10 times daily recommended doses) on folate pathway enzymes have been examined in mice. High-dose folic acid resulted in a state of MTHFR deficiency that affected DNA methylation capacity as well as choline metabolites. The effects were exacerbated in *Mthfr*$^{+/-}$ male mice, a model for individuals with the *MTHFR* 677TT genotype (41). While more studies are required to investigate whether similar metabolic perturbations are found in men with the *MTHFR* 677TT genotype, we postulate that DNA methylation differences observed in these patients in response to high-dose folic acid supplementation are possibly because of further alteration or down regulation of the MTHFR enzyme which may in turn limit the methyl group availability for DNA methylation.

The most significant effects of high-dose folic acid supplementation were visible in DMTs with greater than 10% methylation changes and at least 10-fold sequencing coverage. Interestingly,

DMTs were mostly located in the intergenic and low CpG-density regions of the sperm genome. DNA repeats and transposable elements were also among the genomic regions with the highest number of DMTs; 70% of DMTs were located in repeat regions. To further exclude random distributions of DMTs, we compared them with all sequenced RRBS tiles regardless of methylation changes. This approach confirmed that loci with higher changes in methylation levels after supplementation were differentially distributed across the sperm genome and located less frequently within the promoters, CGI and non-repeat regions. CG islands and promoters coincide in most of the genome and remain hypomethylated to permit gene expression (42,43). DNA repeat regions and open seas (with over 4 kb distance from CGI) are mostly located in the intergenic regions. The tendencies of tiles with higher changes of methylation to be DNA repeats, open seas and intergenic regions suggest that the impact of high-dose folic acid supplementation is most obvious in areas of the sperm genome with lower CG density. Many gene regulatory regions such as enhancers are also located within intergenic areas often far from the genes they regulate. On the other hand, most DNA repeat regions are usually hypermethylated to avoid their unintended expression and possible activation (44). The fact that most of the DMTs in repeat regions demonstrated significant loss of methylation suggests the possibility of alteration of physiological silencing of repeat regions and transposable elements. More studies are required to identify the altered regions in the sperm genome, determine whether these changes are specific or of a stochastic nature and to discover possible mechanisms which may determine the impact of high-dose folic acid on different genomic loci.

Despite the lower number of DMTs in genic regions and promoters, IPA showed that some affected loci may locate within important disease-related genes. Further analysis provided a list of disease-related genes which had significant changes in methylation of their promoters, validated by bisulfite pyrosequencing. *CBFA2T3*, a transcriptional co-repressor also known as *MTG16*, is a TSG with gain of methylation on its promoter after high-dose folic acid supplementation. Hypermethylation of the *CBFA2T3* promoter has been correlated to lower gene expression and been associated with leukemia and breast cancer (45,46). Other TSGs with confirmed gain of promoter methylation included *PTPN6* and *COL18A1*, the promoters of which were hypermethylated and have been associated with lymphoma and astroglioma, respectively (47–49). On the other hand, some TGFs were detected with loss of methylation in their promoters. *ALDH2*, for instance, has high expression and lower promoter methylation in gastric tumors (50). *UBE4B* is another example with high expression in hepatocellular carcinoma, neuroblastoma and breast cancer (51–53). *ERBB2* is also highly expressed specifically in otherwise oncogene-negative lung adenocarcinomas (54,55). Finally, several genes related to autism spectrum disorders and other neurobehavioral disorders such as *CNTNAP4, GABRB3* and *NIPA1* were detected with aberrant promoter methylation (56–59).

Altered methylation of gene promoters in sperm DNA is important as it may be transmitted to the offspring. This is of interest for children conceived using ART where some studies suggest a higher frequency of neurobehavioral disorders (23). The global erasure of parental DNA methylation marks during early stages of embryogenesis is a mechanism for reducing the risk of such transmission (60,61). However, some loci may elude the global erasure mechanisms and progress to the next stages of embryonic development (62). Thus, recommendations for infertile men to receive high-dose folic acid supplementation should be taken with caution to allow for more extensive research, including that in animal models. Such studies should also determine the probability of transmission of altered sperm DNA methylation patterns to the offspring including longer term impacts on health in adulthood. We suggest that a more personalized approach to high-dose folic acid supplementation may be needed, based on factors such as genetic background of the recipients.

Materials and Methods

Patients and sample analysis

A total of 30 healthy normozoospermic men presenting with idiopathic infertility who were recommended by their andrologists, at the McGill University Reproductive Centre or the Clinique OVO, to receive high-dose folic acid supplementation were recruited between 2009 and 2011. Infertile men with known etiology such as varicocele or with known female factor infertility in their partners were excluded. Informed consent was obtained from all participants and the study was approved by the McGill University Health Centre Research Ethics Board. Semen and blood samples were collected prior to and within 1 week after 6 months of high-dose folic acid supplementation. Semen samples were collected by masturbation after 3 days of abstinence and after 30–40 min liquefaction, routine semen analysis was performed according to the World Health Organization guidelines (63) using a computer-aided semen analysis (CASA) system (Sperm Vision HR software v1.01; Penetrating Innovations, Ingersoll, Canada). Sperm DNA and chromatin integrity were assessed by the sperm chromatin structure assay (SCSA) and reported as % DNA fragmentation index (%DFI) and % high DNA stainability (%HDS), respectively, as previously described (64,65).

Blood folate analysis

Serum and RBC folate were measured using the *Lactobacillus casei* microbiological assay (66). RBC folate content was normalized to total protein, which was determined by the modified Lowry assay (67).

Sperm DNA extraction

Sperm DNA was extracted using the QIAamp DNA Mini kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's protocols with minor modifications. Prior to proceeding with the DNA extraction kit, sperm samples were incubated for 2 h at 55°C in lysis buffer containing EDTA, Tris and dithiothreitol (DTT). Genotyping for the *MTHFR* C677T polymorphism was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), as previously described (68,69).

Bisulfite pyrosequencing

Quantitative measurement of the DNA methylation levels was performed by bisulfite pyrosequencing on CpG dinucleotides located on the germline DMRs of imprinted loci as well as the DMTs selected for RRBS validation. Isolated sperm genomic DNA was subjected to bisulfite conversion treatment using the EpiTect Bisulfite kit (Qiagen #59104). Pyrosequencing was performed as described previously (62). Amplified sequences were sequenced using the PyroMark Q24 kit (Qiagen #970802) and the PyroMarkR Q24 Vacuum Workstation (Qiagen, Valencia, CA, USA) using the manufacturer's protocol. Designed primers for assessment of *H19*, *DLK1/GTL2* IG-DMR, *MEST*, *SNRPN*, *KCNQ1OT1* and *PLAGL1* are listed in Supplementary Material, Table S1. DMTs for RRBS validation are listed in Supplementary Material, Table S4.

Pyrosequencing analysis of germline DMRs of imprinted loci resulted in exclusion of sperm samples from two participants for RRBS because of high levels of contamination with somatic cells, as marked by aberrant methylation detected in all of the studied imprinted regions.

Reduced representation bisulfite sequencing (RRBS)

Reduced representation bisulfite sequencing libraries were generated using previously published protocols and the gel-free technique (62,70,71). Briefly, 500 ng of sperm DNA was digested using the methylation-sensitive restriction enzyme, *MspI*, followed by end repair and A-tailing. Small fragments of DNA were then removed utilizing AMPure XP beads (Beckman Coulter, Brea, CA, USA). After ligation to methylated adapters (Illumina), DNA samples underwent two rounds of bisulfite conversion followed by bead clean-up. RRBS libraries were prepared by large scale PCR and assessed for quality prior to sequencing (62). Eight samples of sperm DNA libraries were then multiplexed for paired end sequencing in one lane of a HiSeq 2000 sequencer (Illumina) followed by initial data processing and alignment of reads by the software pipeline bsmap version 2.6 (72). MethylKit software (version 0.5.3) was used for determination of DMTs after folic acid supplementation. This software implements the Benjamini-Hochberg false discovery (FDR)-based method for *P*-value correction and only DMTs passing the q-value threshold (q = 0.01) were considered (73). Analysis was based on 100 bp step-wise tiling windows, ≥2 CpG per tile as well as ≥10 × CpG coverage of each tile per sample. The methylation level of a 100-bp tile was the average of all CpGs within the tile. If significant changes of DNA methylation after high-dose folic acid supplementation exceeded 10%, the tile was designated as a DMT; further annotation of DMTs was performed by the HOMER software version 3.51. Repeat region and CpG island analysis used genomic coordinates obtained from RepeatMasker and CpG Island for human genome assembly GRCh37/hg19 through the UCSC Genome Bioinformatics browser. Coordinates for CpG shores were identified by determining 2 kb regions flanking either side of CpG Islands, whereas CpG shelves were flanked 2k outwards from CpG shores. Coordinates of 21 630 sequences representing lncRNAs were obtained from UCSC genome browser tracks for human genome assembly GRCh37/hg19. Intersection of DMTs and additional tiles to these tracks was performed with bedtools (v2.23.0) utilities.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 16 and the Microsoft Excel 365 ProPlus spreadsheet were used for data entry and analysis. Significant changes of sperm and blood parameters after high-dose folic acid supplementation were detected by *t*-test. Loss of methylation among all RRBS tiles was detected by Wilcoxon signed-rank test. Differences among *MTHFR* C677T genotypes as well as RRBS genomic regions were assessed by analysis of variance (ANOVA) and Fisher's exact tests. $P < 0.05$ was considered statistically significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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