Increased *MTHFR* promoter methylation in mothers of Down syndrome individuals

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Abstract

Despite that advanced maternal age at conception represents the major risk factor for the birth of a child with Down syndrome (DS), most of DS babies are born from women aging less than 35 years. Studies performed in peripheral lymphocytes of those women revealed several markers of global genome instability, including an increased frequency of micronuclei, shorter telomeres and impaired global DNA methylation. Furthermore, young mothers of DS individuals (MDS) are at increased risk to develop dementia later in life, suggesting that they might be "biologically older" than mothers of euploid babies of similar age.

Mutations in folate pathway genes, and particularly in the methylenetetrahydrofolate reductase (*MTHFR*) one, have been often associated with maternal risk for a DS birth as well as with risk of dementia in the elderly. Recent studies pointed out that also changes in *MTHFR* methylation levels can contribute to human disease, but nothing is known about *MTHFR* methylation in MDS tissues.

We investigated *MTHFR* promoter methylation in DNA extracted from perypheral lymphocytes of 40 MDS and 44 matched control women that coinceived their children before 35 years of age, observing a significantly increased *MTHFR* promoter methylation in the first group (33.3 ± 8.1% vs. 28.3 ± 5.8%; $p = 0.001$). In addition, the frequency of micronucleated lymphocytes was available from the women included in the study, was higher in MDS than control mothers (16.1 ± 8.6‰ vs. 10.5 ± 4.3‰; $p = 0.0004$), and correlated with *MTHFR* promoter methylation levels ($r = 0.33$; $p = 0.006$).

Present data suggest that *MTHFR* epimutations are likely to contribute to the increased genomic instability observed in cells from MDS, and could play a role in the risk of birth of a child with DS as well as in the onset of age related diseases in those women.

Keywords: methylenetetrahydrofolate reductase; MTHFR; Down syndrome; mothers; epigenetics; folate; methylation; micronuclei.

1. Introduction

Primary trisomy 21 leading to Down syndrome (DS) originates, in the majority of the cases, from the failure of normal chromosome segregation during maternal meiosis (meiotic nondisjunction), and the major risk factor is advanced maternal age at conception [1]. Indeed, after maternal age 35 years, the risk for a DS pregnancy increases for several years proportionally to increasing maternal age [1]. However, most of DS babies are born from women aging less than 35 years at conception, and this has led to an intense investigation of factors that could contribute to DS risk in young women [2].

The micronucleus assay revealed that young mothers of DS individuals (MDS) have an increased susceptibility to chromosome damage and malsegregation events in peripheral lymphocytes than control mothers [3-6]. Additionally, the analysis of telomere lenght in peripheral lymphocytes showed that women who conceived a DS child before 35 years of age have shorter telomeres than matched control mothers, suggesting that they might be "biologically older" than mothers of euploid babies in the same age group [7]. Interestingly, young MDS were also found to have a five-fold increased risk to develop Alzheimer's disease later in life [8,9]. More recently, altered global DNA methylation levels, evaluated as Long Interspersed Nucleotide Element-1 (LINE1) methylation, were observed in the DNA extracted from peripheral blood lymphocytes of young MDS compared to control mothers, suggesting that DNA methylation reactions are impaired in those women and that this might contribute to chromosome 21 malsegregation events [10]. Collectively, those studies revealed that peripheral lymphocytes of women who conceived a child with DS in young age show several markers of global genome instability.

In 1999 a case-control study conducted in North America suggested that genes encoding enzymes involved in folate metabolism could act as maternal risk factors for the birth of a child with DS [11]. In particular, the authors observed an increased frequency of the methylenetetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism (rs1801133) in MDS than in

control mothers [11]. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is required for the remethylation of homocysteine (Hcy) to methionine in the trans-methylation pathway. Methionine can then be converted to S-adenosylmethionine (SAM), the intracellular donor of methyl groups for methylation reactions [12]. The *MTHFR* 677T allele results in decreased protein activity leading to increased Hcy levels and impaired DNA methylation [12]. Therefore, James and coworkers [11] hypothesized that impairments of folate metabolism, resulting from the presence of polymorphisms in metabolic genes, could lead to aberrant methylation of peri-centromeric regions of chromosome 21, favouring its abnormal segregation during maternal meiosis and leading to the formation of eggs with two copies of chromosome 21 which, if fertilized, would result in a zygote with full trisomy for chromosome 21 [11].

That paper was followed by over 50 case-control studies investigating the potential contribution of folate-pathway gene polymorphisms as maternal risk factors for the birth of a child with DS (reviewed in [13]). Systematic reviews and meta-analyses of those papers revealed that the *MTHFR* 677C>T polymorphism is likely to be a maternal risk factor for a DS birth, particularly in women subjected to nutritional and/or environmental factors leading to reduced folate bioavailability [2,13-16].

Accumulating evidence is revealing that the *MTHFR* gene is regulated by promoter methylation, and increased *MTHFR* promoter methylation has been observed in semen DNA of infertile men [17-19], in DNA extracted from cancerous tissues [20,21], in blood DNA of patients with cardiovascular pathology or renal disease [22,23], as well as in blood and placenta DNA of women with pre-eclampsia [24]. Those studies revealed that increased promoter methylation levels of this gene result in reduced MTHFR protein activity, thus increasing the risk of various human illnesses [17-24]. Several metabolic genes show inter-individual variability in promoter methylation levels, resulting in inter-individual changes in protein activity similar to those conferred by genetic

polymorphisms [25]. However, to the best of our knowledge, there is no available data on *MTHFR* promoter methylation levels in MDS tissues.

Therefore, in the present study, we investigated *MTHFR* promoter methylation levels searching for difference between DNA extracted from peripheral lymphocytes of MDS and matched control mothers. We also searched for correlation between *MTHFR* promoter methylation and micronucleus frequency, an established biomarker of genome instability.

2. Materials and Methods

2.1. Study Population

Peripheral blood samples were available from 40 women who had a DS child with karyotipically confirmed trisomy 21 and 44 healthy women matched with the case mothers for age at sampling. Control mothers had at least one healthy child and no experience of miscarriages, abnormal pregnancies, or children affected by genetic disorders in their life. All women were aged less than 35 years when they conceived (mean age at conception 28.4 ± 4.9 years). Blood samples were collected by the medical personnel of either the Pisa University Hospital (Pisa, Italy) or the paediatric Hospital ‘‘IRCCS Stella Maris Foundation’’ (Pisa, Italy), and control mothers were recruited among people working at the above Hospitals, at the University of Pisa, or among healthy volunteers. Blood samples were not collected at the time of birth but often some years later when women brought their children to the recruiting Hospitals for medical checks. Therefore, we paid extreme caution to match case and control mothers for age at sampling (Table 1). Peripheral blood samples from all the women included in the present study were originally collected in the frame of a previous study aimed at evaluating chromosome damage events in peripheral lymphocytes [4]. Therefore, data on the frequency of binucleated micronucleated (BNMN) lymphocytes were collected at the time of blood drawing by means of the cytokinesis-block micronucleus assay according to the procedure previously described by us [3] and were already available from our cohort [4,26]. Table 1 presents the mean frequency of BNMN lymphocytes in MDS and control mothers.

The individuals included in the study have been selected after the administration of a validated questionnaire [3] designed to document their previous conditions in order to apply the adopted exclusion criteria. Particularly, all women included in the study were healthy at the time of blood collection and had no documented medical or occupational history of exposure to physical,

biological or chemical agents known or suspected to interfere with DNA methylation or with the micronucleus frequency in the three months preceding blood drawing. For instance, alcohol consumption, viral infection, or the current use of pharmacological products known or suspected to interfere with DNA methylation, such as for example folate or B vitamin supplements or epigenetic drugs, were used as exclusion criteria. Case and control mothers were matched for smoking habits (Table 1).

All mothers (MDS and controls) were white Caucasians and residents of central Italy at interview. Written informed consent for inclusion in the study was obtained from each subject. The Ethics Committee of the Scientific Institute IRCCS Stella Maris Foundation approved the study, and all the samples were processed blind, in accordance with the Declaration of Helsinki.

2.2. Extraction of genomic DNA and bisulfite modification:

Peripheral blood samples were collected from each subject in EDTA tubes, and stored at -20°C until assayed. DNA samples were extracted from peripheral blood cells of each subject using the QIAmp DNA Blood Mini Kit (Qiagen, Milan, Italy, Catalogue N° 51106) following the manufacturer's protocol. The extracted DNA was quantified using a Nano Drop ND 2000c spectrophotometer (NanoDrop Thermo Scientific). 200 ng of DNA from each sample were treated with sodium bisulfite in order to convert all unmethylated cytosines into uracil. The EpiTect Bisulfite Kit (Qiagen, Milan, Italy, Catalogue N° 59104) was used for this purpose, following the manufacturer's instructions. All the analyses were performed simultaneously at the Medical Genetics laboratory of the University of Pisa.

2.3. Methylation analysis

Methylation sensitive-high resolution melting (MS-HRM) technique was applied to evaluate *MTHFR* methylation levels using a CFX96 Real-Time PCR detection system (Bio-Rad, Milan, Italy). Particularly, we studied a CpG island in the promoter/5'-untranslated (UTR) region of the *MTHFR* gene spanning from +30 to +184 from the transcription start site and containing 7 CpG sites whose methylation levels were found to be inversely correlated with gene expression levels in human lung cancer cells [20]. Table 2 shows the sequence of the primers, the annealing temperature (Ta), the length of the amplicon, the studied region, and the number of CpG sites within it. MS-HRM analyses were performed using a protocol recently developed, validated with pyro-sequencing, and fully described by us [27].

Each sample was run in duplicate. Fully methylated and unmethylated DNA (EpiTect methylated and unmethylated human control DNA, bisulfite converted, Qiagen, Milan, Italy, Catalogue N° 59695) were mixed to obtain the following ratios of methylation: 0%, 12,5%, 25%, 50%, 75%, 100%. Standard curves with known methylation ratios were included in each assay and used to deduce the methylation ratio of each sample (Figure 1). In order to obtain single methylation percentage values from MS-HRM assays, rather than a range, we applied an interpolation method recently developed and described by us [28], that allowed obtaining precise HRM methylation values.

All the employed procedures have been recently fully described and validated by us, and any further technical data on the set-up and validation procedures of the MS-HRM protocol for the analysis of *MTHFR* promoter methylation can be found in our recent methodological paper [27].

2.4. Statistical analysis

Differences in mean methylation levels of the *MTHFR* gene between groups were compared by analysis of variance (ANOVA) correcting for age at sampling and smoking habits. ANOVA was also used to evaluate the contribution of smoking habits to the studied endpoints. Linear regression analysis was performed to search for correlation between age at sampling and *MTHFR* gene methylation levels, between age at sampling and the BNMN frequency, and between *MTHFR* gene methylation levels and the BNMN frequency. Analyses were performed with the STATGRAPHICS 5.1 Plus software package for Windows. The statistical power of the study was evaluated with the online calculator ClinCalc.com (http://clincalc.com/Stats/SampleSize.aspx). A P value < 0.05 was considered as statistically significant.

3. Results

Figure 2 shows the mean *MTHFR* methylation levels obtained in MDS and control mothers. We observed a mean *MTHFR* promoter methylation of 33.3 ± 8.1% in MDS and of 28.3 ± 5.8% in control mothers (Crude p value = 0.002; Adjusted p value = 0.001). The sample size was chosen to have an a-priori power of more than 80% to detect an average 5% DNA methylation difference between groups, which represents the statistical power usually required for genome-wide DNA methylation studies [29]. A post-hoc analysis performed using the observed means and standard deviations revealed that the present study has 89.7% power to detect the observed methylation difference between groups.

Linear regression analysis revealed no correlation between *MTHFR* methylation and age at sampling (r = 0.02; p = 0.84). Data on chromosome damage events were already available from our cohort and, as shown in Table 1, the mean frequency of BNMN lymphocytes in MDS was significantly higher if compared to the control mothers (16.1 ± 8.6‰ vs 10.5 ± 4.3‰; p = 0.0004) (Table 1). Linear regression analysis revealed correlation between age at sampling and the frequency of BNMN lymphocytes (r = 0.25; p = 0.04), furthermore we observed a significant correlation between *MTHFR* promoter methylation and BNMN frequency (r = 0.33; p = 0.006) (Figure 3).

We already observed that smoking habits had no significant effect on the BNMN frequency in our cohort [4]. ANOVA analysis also revealed no significant contribution of smoking habits to *MTHFR* promoter methylation levels (smokers vs. never-smokers: p = 0.20; smokers + ex-smokers vs. never-smokers: p = 0.60). With a total of 64 never-smokers and 20 smokers and ex-smokers, we had 80% power to detect a mean methylation difference of 5% or higher between groups.

4. Discussion

In the present study we investigated *MTHFR* promoter methylation in blood DNA of women who conceived a child with DS in young age and matched control women, observing that MDS have a significantly higher methylation level of the gene under investigation than control mothers. In addition, a significant correlation between *MTHFR* promoter methylation levels and the frequency of BNMN lymphocytes was observed in our cohort.

MTHFR is one of the major enzymes of the folate metabolic pathway, whose genetic polymorphisms have been often associated with chromosome damage and maternal risk of birth of a child with DS [11,30]. DNA methylation in the promoter region of a gene is an epigenetic event resulting in reduced gene expression, and there is accumulating evidence of increased *MTHFR* promoter methylation in several human pathological conditions [17-24]. In the present study we investigated a functional CpG island in the promoter region of the *MTHFR* gene whose methylation levels have previously been linked to gene expression levels [20]. To the best of our knowledge the present is the first study addressing the methylation levels of a gene involved in folate metabolism in MDS, and present results suggest that *MTHFR* promoter methylation is increased in MDS and correlates with markers of genome instabily. Therefore, present results support a link between impaired folate metabolism and chromosome damage in those women.

One of the major limits for the study of the molecular mechanisms leading to chromosome 21 malsegregation is the unavailability of human egg cells from MDS, so that most of the studies aimed at linking folate metabolism to chromosome 21 malsegregation have been performed with surrogate cells and tissues, including peripheral lymphocytes [3-7]. In this regard, *in vitro* studies in human lymphocytes have shown that folate restriction induces chromosome 21 aneuploidy [31,32]. Similarly, the studies performed so far in peripheral lymphocytes from MDS revealed that those cells are characterized by several markers of genome instability and premature ageing, including an increased frequency of micronuclei, short telomeres, and global changes in DNA methylation [3-

10]. Those biomarkers could be indicators of altered pathways, such as impaired folate metabolism, accounting for an increased risk to give birth to a DS child in young age or to develop age-related neurodegenerative diseases later in life [3-10].

In order to minimize the contribution of environmental factors and poly-medication to the observed *MTHFR* methylation levels we adopted very stringent inclusion criteria to exclude from the study alcohol consumers, users of vitamin supplements, or people with a documented occupational or medical exposure to chemical, physical or biological agents known or suspected to interfere with DNA methylation or micronucleus formation. In addition, to minimize the effects of ethnic or geographical factors, case and control mothers were all Caucasians and residents of central Italy (Pisa and neighbouring areas) at recruitment. Furthermore, we restricted our analysis to women who conceived before 35 years of age, because after that age limit the risk for DS is mainly due to advanced maternal age [1]. Unfortunately, in most of the cases we could not measure *MTHFR* methylation soon after pregnancy, but often some to several years later. However, there was no correlation between age at sampling and *MTHFR* promoter methylation in our cohort. Some of the women (20 out of 84) were smokers or ex-smokers, but their exclusion would have drastically reduced the statistical power of the study. Smoking is a known environmental factor able to induce changes in DNA methylation, its contribution to *MTHFR* promoter methylation is controversial [20,22], and no significant effect of smoking to global DNA methylation levels was observed in lymphocytes of MDS [10]. In the present study case and control mothers were matched for smoking habits and we found no significant effect of smoking to *MTHFR* promoter methylation levels. However, due to the relatively small sample-size of our cohort, additional studies are recommended to further address the contribution of smoking to gene promoter methylation in blood DNA of MDS.

Limitations of the present study are the relatively small case-control cohort and the iclusion of only Caucasian women residents of central Italy. Therefore, the present must be considered as a pilot study to evaluate *MTHFR* gene methylation in MDS, and confirmation is required in

subsequent cohorts as well as in other populations than Italians. Indeed, many authors have demonstrated that geographic and ethnic factors can modulate folate metabolism as well as DNA methylation reactions [13-16]. The present is also a retrospective study based on the analysis of DNA samples obtained from women who conceived a DS child when they were young. Prospective studies are therefore required in order to address whether or not increased *MTHFR* promoter methylation at peri-conception is linked to an increased maternal risk for a DS birth.

Overall, the present study pointed out an increased *MTHFR* promoter methylation in lymphocytes from MDS and a correlation between *MTHFR* promoter methylation levels and chromosome instability in those cells. Very interestingly, recent studies have shown that *MTHFR* promoter methylation is also linked to circulating folate, vitamin B12 and Hcy levels in individuals affected by Alzheimer's disease, cardiovascular disease, as well as in healthy aged subjects, suggesting that the *MTHFR* methylation status could be a mediator of impairments of the folate metabolic pathway [22,27,33]. Therefore, we suggest that an increased *MTHFR* promoter methylation in lymphocytes of MDS could contribute to impaired folate metabolism in those cells with similar effects of those exerted by genetic polymorphisms, serving as a potential upstream player in genomic instability related disorders, such as chromosome 21 malsegregation or age-related neurodegeneration [34-36].

5. Conclusions

In summary, in the present pilot study investigating the methylation status of the *MTHFR* gene in peripheral lymphocytes, we observed that women who conceived a child with DS in young age have a statistically significant higher promoter methylation than matched control women. Furthermore, we observed that *MTHFR* promoter methylation correlates with chromosome damage evaluated as the frequency of BNMN lymphocytes. Many authors observed increased *MTHFR* promoter methylation in human diseases characterized by impaired folate metabolism, such as for example cancer, vascular disorders and male infertility, and there is accumulating evidence of correlation between *MTHFR* promoter methylation and impaired folate metabolism [17-26]. Functional *MTHFR* genetic polymorphisms linked to reduced protein activity represent the most frequently studied candidate polymorphisms in genetic association studies for the maternal risk of birth of a child with DS [13-16]. Changes in *MTHFR* promoter methylation are increasingly recognised as a source of inter-individual variability in protein activity whose effects might be similar to those conferred by genetic polymorphisms, but except for the present study no data is yet available concerning the *MTHFR* epigenetic regulation in MDS [13,25]. Therefore, present data are indicative that not only mutations in folate-pathway genes, but also their epimutations are likely to contribute to the increased genomic instability observed in cells from MDS, suggesting that they could play a role in the risk of birth of a child with DS, as well as in the onset of age related diseases in those women.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] J.K. Morris, D.E. Mutton, E. Alberman, Revised estimates of the maternal age specific live birth prevalence of Down's syndrome, J. Med. Screen. 9 (2002) 2-6.

[2] F. Coppedè, The complex relationship between folate/homocysteine metabolism and risk of Down syndrome, Mutat. Res. 682 (2009) 54-70.

[3] L. Migliore, G. Boni, R. Bernardini, F. Trippi, R. Colognato, I. Fontana, et al., Susceptibility to chromosome malsegregation in lymphocytes of women who had a Down syndrome child in young age, Neurobiol. Aging 27 (2006) 710–716.

[4] F. Coppedè, F. Migheli, S. Bargagna, G. Siciliano, I. Antonucci, L. Stuppia, et al., Association of maternal polymorphisms in folate metabolizing genes with chromosome damage and risk of Down syndrome offspring, Neurosci. Lett. 449 (2009) 15-19.

[5] R.L. Silva-Grecco, G.C. Navarro, R.M. Cruz, M.A. Balarin, Micronucleated lymphocytes in parents of Down syndrome children, Braz. J. Med. Biol. Res. 45 (2012) 573-577.

[6] S. Abdel Hady, H.H. Afifi, E.A. Abdel Ghany, M.B. Taher, M.M. Eid, Micronucleus assay as a biomarker for chromosome malsegregation in young mothers with Down syndrome children, Genet. Couns. 26 (2015) 13-19.

[7] I. Albizua, B.L. Rambo-Martin, E.G. Allen, W. He, A.S. Amin, S.L. Sherman, Association between telomere length and chromosome 21 nondisjunction in the oocyte, Hum. Genet. (2015) Sep 25. [Epub ahead of print], doi: 10.1007/s00439-015-1603-0.

[8] N. Schupf, D. Kapell, J.H. Lee, R. Ottman, R. Mayeux, Increased risk of Alzheimer's disease in mothers of adults with Down's syndrome, Lancet 344 (1994) 353-356.

[9] N. Schupf, D. Kapell, B. Nightingale, J.H. Lee, J. Mohlenhoff, S. Bewley, et al., Specificity of the fivefold increase in AD in mothers of adults with Down syndrome, Neurology 57 (2001) 979-984.

[10] I. Babić Božović, A. Stanković, M. Živković, J. Vraneković, M. Kapović M, B. Brajenović-Milić, Altered LINE-1 Methylation in Mothers of Children with Down Syndrome, PLoS One 10 (2015) e0127423.

[11] S.J. James, M. Pogribna, I.P. Pogribny, S. Melnyk, R.J. Hine, J.B. Gibson, et al., Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome, Am. J. Clin. Nutr. 70 (1999;70:495–501.

[12] M.L. Martínez-Frías, The biochemical structure and function of methylenetetrahydrofolate reductase provide the rationale to interpret the epidemiological results on the risk for infants with Down syndrome, Am. J. Med. Genet. A 146A (2008) 1477–1482.

[13] F. Coppedè, The genetics of folate metabolism and maternal risk of birth of a child with Down syndrome and associated congenital heart defects, Front. Genet. 6 (2015) 223.

[14] M.A. Costa-Lima, M.R. Amorim, I.M. Orioli, Association of methylenetetrahydrofolate reductase gene 677C>T polymorphism and Down syndrome, Mol. Biol. Rep. 40 (2013) 2115–2125.

[15] X. Wu, X. Wang, Y. Chan, S. Jia, Y. Luo, W. Tang, Folate metabolism gene polymorphisms MTHFR C677T and A1298C and risk for Down syndrome offspring: a meta-analysis, Eur. J. Obstet. Gynecol. Reprod. Biol. 167 (2013) 154–159.

[16] M. Yang, T. Gong, X. Lin, L. Qi, Y. Guo, Z. Cao, et al., Maternal gene polymorphisms involved in folate metabolism and the risk of having a Down syndrome offspring: a meta-analysis, Mutagenesis 28 (2013) 661–671.

[17] N. Khazamipour, M. Noruzinia, P. Fatehmanesh, M. Keyhanee, P. Pujol, MTHFR promoter hypermethylation in testicular biopsies of patients with non-obstructive azoospermia: the role of epigenetics in male infertility, Hum. Reprod. 24 (2009) 2361-2364.

[18] W. Wu, O. Shen, Y. Qin, X. Niu, C. Lu, Y. Xia, et al., Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR), PLoS One 5 (2010) e13884.

[19] J.C. Rotondo, S. Bosi, E. Bazzan, M. Di Domenico, M. De Mattei, R. Selvatici, et al., Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion, Hum. Reprod. 27 (2012) 3632-3638.

[20] T. Vaissière, R.J. Hung, D. Zaridze, A. Moukeria, C. Cuenin, V. Fasolo, et al., Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors, Cancer Res. 69 (2009) 243-252.

[21] A. Botezatu, D. Socolov, I.V. Iancu, I. Huica, A. Plesa, C. Ungureanu, et al., Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and promoter methylation in cervical oncogenic lesions and cancer, J. Cell Mol. Med. 17 (2013) 543-549.

[22] L.K. Wei, H. Sutherland, A. Au, E. Camilleri, L.M. Haupt, S.H. Gan, et al., A potential epigenetic marker mediating serum folate and vitamin B12 levels contributes to the risk of ischemic stroke, Biomed. Res. Int. 2015 (2015) 167976.

[23] M. Ghattas, F. El-Shaarawy, N. Mesbah, D. Abo-Elmatty, DNA methylation status of the methylenetetrahydrofolate reductase gene promoter in peripheral blood of end-stage renal disease patients, Mol. Biol. Rep. 41 (2014) 683-688.

[24] J. Ge, J. Wang, F. Zhang, B. Diao, Z.F. Song, L.L. Shan, et al., Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical significance, Genet. Mol. Res. 14 (2015) 8021-8028.

[25] M.J. Bonder, S. Kasela, M. Kals, R. Tamm, K. Lokk, I. Barragan, et al. Genetic and epigenetic regulation of gene expression in fetal and adult human livers. BMC Genomics 15 (2014) 860.

[26] F. Coppedè, E. Grossi, F. Migheli, L. Migliore, Polymorphisms in folate-metabolizing genes, chromosome damage, and risk of Down syndrome in Italian women: identification of key factors using artificial neural networks, BMC Med. Genomics. 3 (2010) 42.

[27] P. Tannorella, A. Stoccoro, G. Tognoni, L. Petrozzi, M.G. Salluzzo, A. Ragalmuto, et al. Methylation analysis of multiple genes in blood DNA of Alzheimer's disease and healthy individuals, Neurosci. Lett. 600 (2015) 143-147.

[28] F. Migheli, A. Stoccoro, F. Coppedè, W.A. Wan Omar, A. Failli, R. Consolini, et al., Comparison study of MS-HRM and pyrosequencing techniques for quantification of APC and CDKN2A gene methylation, PLoS One 8 (2013) e52501.

[29] K. Lunnon, R. Smith, E. Hannon, P.L. De Jager, G. Srivastava, M. Volta, et al., Methylomic profiling implicates corticalderegulation of ANK1 in Alzheimer's disease, Nat. Neurosci. 17 (2014) 1164–1170.

[30] F. Coppedè, R. Colognato, A. Bonelli, G. Astrea, S. Bargagna, G. Siciliano, et al., Polymorphisms in folate and homocysteine metabolizing genes and chromosome damage in mothers of Down syndrome children, Am. J. Med. Genet. A 143A (2007) 2006–2015.

[31] X. Wang, P. Thomas, J. Xue, M. Fenech, Folate deficiency induces aneuploidy in human lymphocytes in vitro-evidence using cytokinesis-blocked cells and probes specific for chromosomes 17 and 21, Mutat. Res. 551 (2004) 167-180.

[32] S. Beetstra, P. Thomas, C. Salisbury, J. Turner, M. Fenech, Folic acid deficiency increases chromosomal instability, chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei, Mutat. Res. 578 (2005) 317-326.

[33] L. Wang, J. Zhang, S. Wang, Demethylation in the promoter region of MTHFR gene and its mRNA expression in cultured human vascular smooth muscle cells induced by homocysteine, Wei Sheng Yan Jiu 36 (2007) 291-294.

[34] P. Thomas, M. Fenech, Buccal Cytome Biomarkers and Their Association with Plasma Folate, Vitamin B12 and Homocysteine in Alzheimer's Disease, J. Nutrigenet. Nutrigenomics 8 (2015) 57-69.

[35] F. Coppedè, L. Migliore, DNA damage in neurodegenerative diseases, Mutat. Res. 776 (2015) 84-97.

[36] G.C. Román, MTHFR Gene Mutations: A Potential Marker of Late-Onset Alzheimer's Disease?, J. Alzheimers Dis. 47 (2015) 323-327.

Table 1. Demographic characteristics of mothers of DS children (MDS) and control mothers.

Variable	MDS N° = 40	Control mothers N° = 44	*p*-Value
Age at sampling: years (mean ± SD)	46.1 ± 11.5	47.8 ± 7.1	0.41[a]
never-smokers	31 (77.5%)	33 (75.0%)	0.69 [b]
ex-smokers	4 (10.0%)	3 (7.0%)	
smokers	5 (12.5%)	8 (18.0%)	
BNMN ‰ (mean ± SD)	16.1 ± 8.6	10.5 ± 4.3	0.0004 [c]

[a] Student's T test.
[b] Fisher exact test.
[c] Analysis of variance (ANOVA) with corrections for age at sampling and smoking habits.

Table 2. Sequence of the primers, annealing temperature (T_a), length of the amplicon, studied region and number of CpG sites.

Primer sequences	T_a	Amplicon length	Promoter region	CpG sites
F: 5'-TTTTAATTTTTGTTTGGAGGGTAGT-3' R: 5'- AAAAAAACCACTTATCACCAAATTC-3'	54°C	155 bp	From +30 to +184	7

Figure Legends

Figure 1. Melting curve of the *MTHFR* gene showing the standard samples with known methylation percentage (0%, 12.5%, 25%, 50%, 75%, 100%) and a sample in duplicate (indicated with an arrow).

Figure 2. Mean methylation levels of the *MTHFR* promoter in mothers of Down syndrome individuals (MDS) and control women. We observed a mean *MTHFR* promoter methylation of 33.3% in MDS and of 28.3% in control mothers ($p = 0.001$, ANOVA with corrections for age at sampling and smoking habits). The graph shows means ± standard errors of the means.

Figure 3. Linear regression analysis between *MTHFR* promoter methylation and BNMN frequency: ($r = 0.33$; $p = 0.006$).

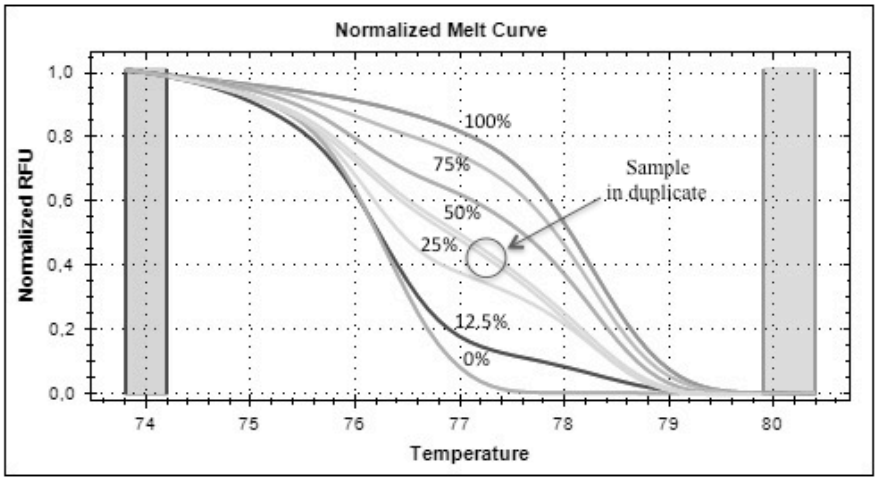
Normalized Melt Curve
Normalized RFU
1,0
0,8
0,6
0,4
0,2
0,0
74
75
76
77
78
79
80
Temperature
100%
75%
50%
25%
12,5%
0%
Sample
in duplicate

Figure 1

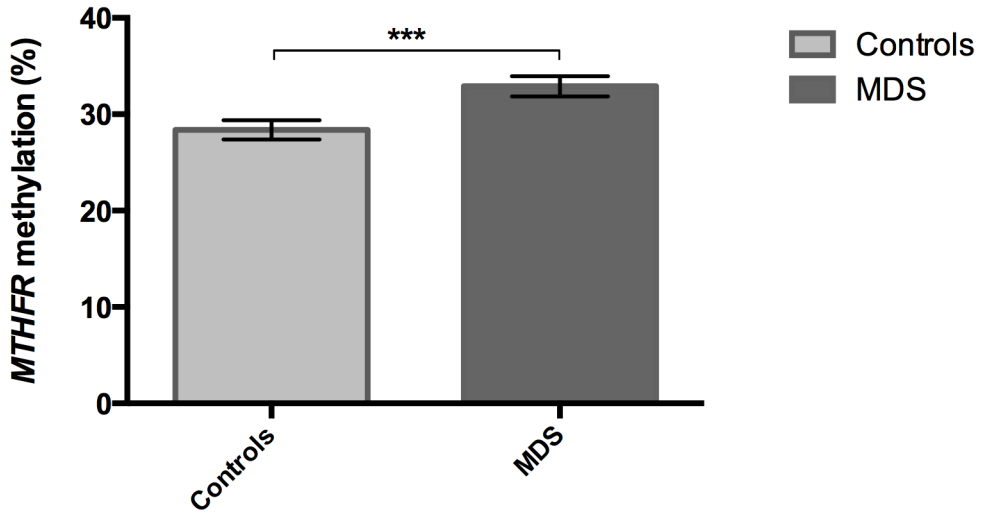

Figure 2

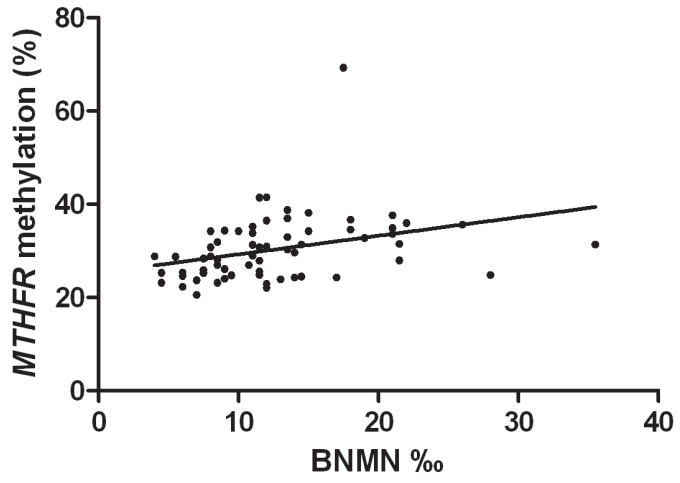

Figure 3