

Prevalence of the Polymorphism *MTHFR* A1298C and not *MTHFR* C677T Is Related to Chromosomal Aneuploidy in Brazilian Turner Syndrome Patients

perspectives

ABSTRACT

KELLY CRISTINA DE OLIVEIRA
BIANCA BIANCO
IEDA T. N. VERRESCHI
ALEXIS DOURADO GUEDES
BIANCA BORSATO GALERA
MARCIAL FRANCIS GALERA
CAIO P. BARBOSA
MONICA VANNUCCI NUNES LIPAY

Endocrinology Division –
Department of Medicine (KCO,
BB, ITNV, ADG, MVNL); Genetics
Division - Department of
Morphology and Genetics –
Federal University of São Paulo –
Unifesp (MVNL), São Paulo, SP,
Brazil; Medical Genetics and
Molecular Biology Unit of the
General Hospital from University
of Cuiabá (UNIC) (BBG, MFG),
Cuiabá, MT, Brazil; Genetics
Division of Medicine College
from ABC (CPB), São Paulo,
SP, Brazil.

Background: Dysfunctions in the folate metabolism can result in DNA hypomethylation and abnormal chromosome segregation. Two common polymorphisms of this enzyme (C677T and A1298C) reduce its activity, but when associated with aneuploidy studies the results are conflicting. The objective of the present study is to analyze the *MTHFR* gene polymorphisms in women with Turner Syndrome and in a control group, correlating the findings to the chromosomal aneuploidy. **Methods:** The study comprised 140 patients with Turner Syndrome, of which 36 with chromosome mosaicism and 104 non-mosaics, and a control group of 209 fertile and healthy women without a history of any offspring with aneuploidy. Polymorphisms C677T and A1298C were studied by RFLP-PCR and the results were statistically analyzed. **Results:** The frequency of genotypes *MTHFR* 677CC, 677CT and 677TT in the patients with Turner Syndrome and chromosome mosaicism was, respectively, 58.3%, 38.9% and 2.8%. Among the patients with non-mosaic Turner Syndrome, 47.1% presented genotype 677CC, 45.2% genotype 677CT, and 7.7% genotype 677TT. Among the 209 individuals of the control group, genotypes 677CC, 677CT and 677TT were found at the following frequencies: 48.3%, 42.1% and 9.6%, respectively. As for polymorphism A1298C, the patients with Turner Syndrome and chromosome mosaicism presented genotypes 1298AA, 1298AC and 1298CC at the following frequencies: 58.3%, 27.8% and 13.9%, respectively. Among the non-mosaic Turner Syndrome patients, genotype 1298AA was found in 36.5%, genotype 1298AC in 39.4%, and genotype 1298CC in 22.1%. In the control group, genotypes 1298AA, 1298AC and 1298CC were present at the following frequencies: 52.6%, 40.7% and 6.7%, respectively. **Conclusion:** No correlation was observed between the *MTHFR* gene polymorphism 677 and chromosomal aneuploidy in the Turner Syndrome patients. However, the *MTHFR* gene polymorphism at position 1298, mainly genotype 1298CC that reduces the enzyme efficiency, was more frequent in the group of Turner Syndrome patients, suggesting its involvement in mechanisms related to chromosomal imbalances. (*Arq Bras Endocrinol Metab* 2008; 52/8:1374-1381)

Keywords: Turner syndrome; *MTHFR* gene; Polymorphism; Aneuploidy; Chromosomal imbalance

RESUMO

A Prevalência do Polimorfismo A1298C e não do C677T do Gene *MTHFR* está Relacionada à Ocorrência de Aneuploidias Cromossômicas em Mulheres Brasileiras Portadoras da Síndrome de Turner.

Introdução: Disfunções no metabolismo dos folatos podem resultar em hipometilação do DNA e na segregação cromossômica anormal. Dois polimorfismos comuns no gene *MTHFR* (C677T e A1298C) reduzem a atividade da enzima e, quando associados a estudos de aneuploidias apresentam resultados conflitantes. O objetivo do presente estudo foi a análise dos polimorfismos do gene *MTHFR* em mulheres portadoras da síndrome de Turner

Received in 25/8/2008
Accepted in 20/9/2008

e em indivíduos de grupo-controle, correlacionando os achados ao mecanismo de formação de aneuploidias cromossômicas. **Métodos:** Foram estudadas 140 portadoras da síndrome de Turner sendo 36 com mosaïcismo cromossômico e 104 não-mosaicos, e um grupo-controle composto por 209 mulheres férteis e saudáveis sem história de prole com aneuploidia. Os polimorfismos *MTHFR* C677T e A1298C foram estudados por RFLP-PCR e os resultados analisados estatisticamente. **Resultados:** A frequência dos genótipos *MTHFR* 677CC, 677CT e 677TT nas pacientes portadoras de síndrome de Turner e mosaïcismo cromossômico foi, respectivamente, 58,3%, 38,9% e 2,8%. Das pacientes portadoras de síndrome de Turner não-mosaico, 47,1% apresentaram o genótipo 677CC, 45,2% o genótipo 677CT e 7,7% apresentaram o genótipo 677TT. Nos 209 indivíduos do grupo-controle, os genótipos 677CC, 677CT e 677TT foram encontrados nas seguintes frequências: 48,3%, 42,1% e 9,6%, respectivamente. Quanto ao polimorfismo A1298C, as portadoras de síndrome de Turner e mosaïcismo cromossômico apresentaram os genótipos 1298AA, 1298AC e 1298CC nas seguintes frequências: 58,3%, 27,8% e 13,9%, respectivamente. Já nas portadoras de Síndrome de Turner não-mosaico, o genótipo 1298AA foi encontrado em 36,5%, o genótipo 1298AC em 39,4% e o genótipo 1298 CC em 22,1%. No grupo-controle, os genótipos 1298AA, 1298AC e 1298CC estavam presentes nas frequências 52,6%, 40,7% e 6,7%. **Conclusão:** Não foi observada correlação entre o polimorfismo C677T do gene *MTHFR* e a aneuploidia cromossômica presente nas portadoras de síndrome de Turner. O polimorfismo A1298C do gene *MTHFR*, principalmente o genótipo 1298CC, foi mais freqüente nas portadoras de síndrome de Turner, sugerindo seu envolvimento no mecanismo de formação de aneuploidias cromossômicas. (Arq Bras Endocrinol Metab 2008; 52/8:1374-1381)

Descritores: Síndrome de Turner; Gene *MTHFR*; Polimorfismo; Aneuploidia

INTRODUCTION

Approximately 15-20% of the clinically recognized pregnancies are spontaneously aborted, most of them during the first trimester (1). Cytogenetic studies of spontaneous abortions have revealed that chromosome abnormalities are the main cause, contributing with about 50 to 60% of the cases (2,3). Sex chromosome monosomy is the most common chromosomal abnormality in humans, occurring in about 1 to 2% of all pregnancies (4). The liveborn 45,X individuals present the clinical characteristics of the Turner Syndrome (TS). Some authors believe that the presence of another sex chromosome is essential at some time of the embryonic life, once 99% of the 45,X conceptuses do not reach term (5). The high frequency of mosaicism makes the Turner Syndrome an anomaly of great interest in the search for clarification of the nondisjunction mechanism.

The molecular mechanisms which determine chromosomal nondisjunction are not well known so far, but dysfunctions in folate metabolism and methylation may result in DNA hypomethylation and in abnormal chro-

mosome segregation. There are data in the literature correlating metabolic folate deficiency with aneuploidy of chromosomes 17 and 21 in human lymphocytes (6). Methylenetetrahydrofolate reductase (*MTHFR*) plays a crucial role in the regulation of DNA methylation.

The *MTHFR* gene, located on the short arm of chromosome 1 (1p36.3), presents two common polymorphisms involving nucleotides C677T and A1298C. The change of C for T at position 677 causes the substitution of alanine for valine in the *MTHFR* protein and the consequent reduction in enzyme activity. The specific activity of the *MTHFR* enzyme is reduced by 35% in the presence of heterozygosis, genotype C/T, compared to the normal genotype C/C, and by 70% in homozygosis, genotype T/T. Polymorphism A1298C brings about the substitution of a glutamate for a valine, causing a reduction in the enzyme activity that is more effective when in homozygosis (7-10).

MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate, the main methyl donor for the remethylation of homocysteine to methionine. Homocysteine is an amino acid formed by the demethylation of methionine, an essential amino acid (Figure 1).

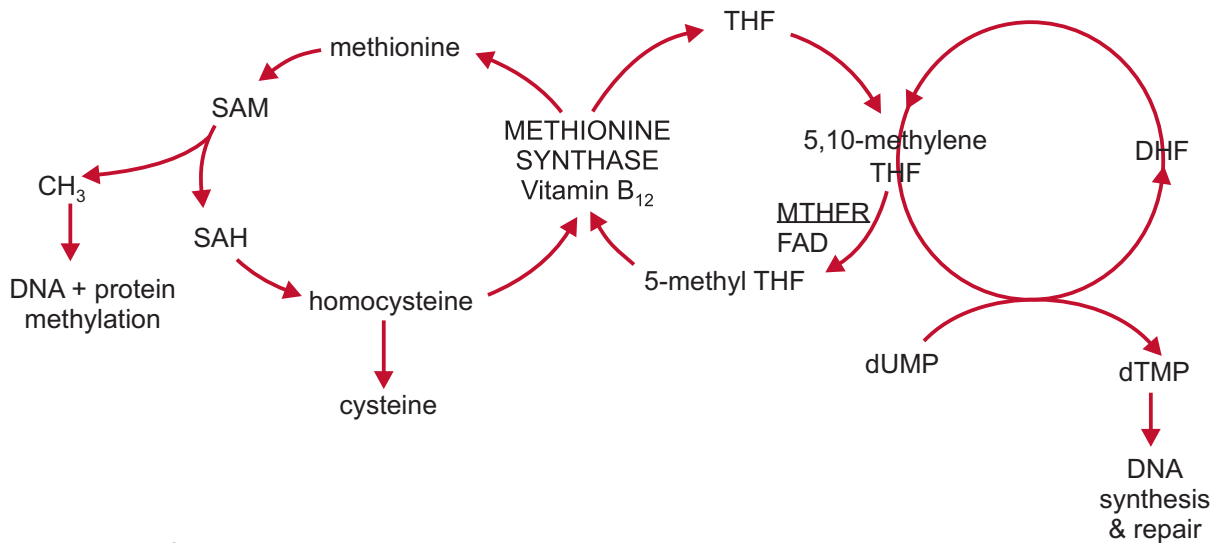


Figure 1. Folate cycle⁸.

Polymorphism C677T is located within the catalytic domain of the protein, whereas A1298C is located within the presumably regulatory domain. Polymorphism A1298C influences the specific activity of the enzyme and also the folate concentration, but with less impact than polymorphism C677T (10).

Folate is essential for the synthesis of nucleotide precursors (used in DNA synthesis), for the methylation reactions (7) and for the conversion of uracil (dUMP) to thymidylate (dTMP) (8). In the eukaryotic cells, about 5% of the cytosine residues are methylated forming 5-methylcytosine that have both a structural and regulatory significance (9).

A reduction in the MTHFR enzyme activity requires an increased folic acid intake to keep the remethylation of homocysteine into methionine normal (9). Consequently, low folate concentrations in individuals with a reduced MTHFR enzyme activity result in an increase in the homocysteine levels and a decrease in plasma methionine. Intracellular homocysteine is associated with DNA-methyltransferase inhibition and DNA hypomethylation (11).

The metabolic pathways of folate can be modified by polymorphisms in relevant genes such as *MTHFR*, or by the action of carcinogenic elements, as for example alcohol or tobacco (12).

According to Santos and cols. (13), Turner syndrome may be an investigation model for *MTHFR* gene polymorphisms for somatic chromosomal non-disjunction, due to the high frequency of chromosome mosaicism in these patients.

These authors studied 49 TS patients and 200 controls and found a high frequency of genotype C677T/C677T in TS patients group. They concluded that, when in homozygosis, this mutation can have a somatic effect on chromosomal non-disjunction through a decrease in *MTHFR* activity in TS patients.

Thus, the objective of the present study is to analyze polymorphisms C677T and A1298C of the *MTHFR* gene in women with Turner Syndrome with and without chromosome mosaicism and in a control group, correlating the findings with the origin of chromosomal aneuploidy.

SUBJECTS AND METHODS

Patients and controls

Screening of the individuals was performed at the Gynads and Development Outpatient Clinic of the Discipline of Endocrinology of Unifesp-EPM and of the Medical Genetics and Molecular Biology Unit of the General Hospital from University of Cuiaba, and a total of 140 women who were under clinical follow-up and had a confirmed cytogenetic diagnosis of Turner Syndrome were included in the study. Of these, 36 had chromosome mosaicism cytogenetically detected and 104 were non-mosaics (Table 1). The control group comprised blood sample of 209 fertile women in good health and without history of children with chromosome aneuploidies from Familiar Planning Outpatient

Table 1. Karyotype and number of patients with Turner syndrome evaluated.

Number of Patients	Karyotype
95	45,X
1	46,X,+mar
18	45,X/46,XX
1	45,X/47,XXX
5	45,X/46,X,+mar
3	45,X/46,X,i(X)(q10)
1	45,X/45,X,add(15)(p11)
2	45,X/46,X,r(X)
7	46,X,i(X)(q10)
1	45,X/46,X,idic(Yp)
1	45,X/46,XX,p+
1	45,X/46,XY
1	46,X,der(X)(p11.2)
1	46,X,der(X)/45,X
1	45,X/46,X,dup(X)(q25-q21)
Total	140

Clinic of the Medicine College from ABC. The study protocol was approved by the local research ethics committee (CEP 1902/06). All TS patients and/or their parents gave informed consent for the study.

Evidencing the current trend towards an earlier diagnosis of TS, the reasons for seeking medical attention went from prenatal diagnosis, short stature, and primary amenorrhea to infertility. A hormone profile consistent with hypergonadotropic hypogonadism or an unexplained short stature in females led to cytogenetic evaluation.

Methods

Karyotypes were determined by standard cytogenetic analysis of peripheral blood lymphocytes, and the number of metaphases analyzed by G banding followed Hook's (1977) (14) criteria for detecting 8% mosaicism in 40 metaphases, with a confidence interval of 95%. For genomic DNA extraction, peripheral blood was collected according to the protocol developed by Lahiri and Numberger (1991) (15), with modifications.

The primers for amplification of regions 677 and 1298 of the *MTHFR* gene were the following: 677F - (5'-TGAAGGAGAAGGTGTCTGCGGGA-3'), and

677R - (5'-AGGACGGTGCGGTGAGAGTG-3') (16); and 1298F - (5'-CAAGGAGGAGCTGCTGAAGA-3') exonic primer and 1298R (5'-CAACTCCAGCATCACT-3') intronic primer (17) (Figure 2).

PCR was performed in a final reaction volume of 50 μ L containing 200 ng of genomic DNA, 10 mM of $MgCl_2$, 10 mM, 2.5 U of Taq DNA polymerase (Invitrogen), 10 mM of Tris-HCl (pH8.4), 50 mM of KCl, 3.0 mM of $MgCl_2$, 0.2 μ M of each primer. Amplification was carried out in a thermal cycler (Perkin Elmer 7500[®]) and consisted of a 5 min denaturing step at 95°C, followed by 35 cycles of 1 min at 95°C (denaturing), 1 min of annealing at 61°C and 1 min at 72°C (extension), followed by a final extension cycle of 7 min at 72°C. The whole reaction product was electrophoresed on 1.0% agarose gel and stained with ethidium bromide to verify the success of the amplification.

For identification of polymorphism C677T we had used the *HinfI* enzyme. Digestion with the restriction enzyme was carried out in a final volume of 50 μ L, using 30 μ L of PCR product, 13.5 μ L of distilled water, 15 units of *HinfI* enzyme, and 5.0 μ L of buffer. The samples were incubated for at least 3h at 37°C. Size analysis of the restriction fragments was visualized after separation of the PCR products digested by electrophoresis gel containing 3% agarose and stained with ethidium bromide.

For identification of polymorphism A1298C we had used the *MboII* enzyme. Digestion with the restriction enzyme was carried out in a final volume of 50 μ L, using 30 μ L of PCR product, 14 μ L of distilled water, 10 units of *HinfI* enzyme, and 5.0 μ L of buffer. The samples were incubated for at least 1h at 37°C. Size analysis of the restriction fragments was visualized after separation of the PCR products digested by electrophoresis gel containing 3% agarose and stained with ethidium bromide.

Polymorphism C677T creates a recognition sequence for the restriction enzyme *HinfI*, and this is detected by digestion of the 198-bp PCR product, generating 23- and 175-bp fragments for the polymorphism in homozygosis (genotype TT), because the 23-bp fragment is not retained in the gel. Genotype CC is characterized by the presence of a 198-bp fragment, and genotype CT is characterized by the presence of two fragments, one with 198 bp and the other with 175 bp.

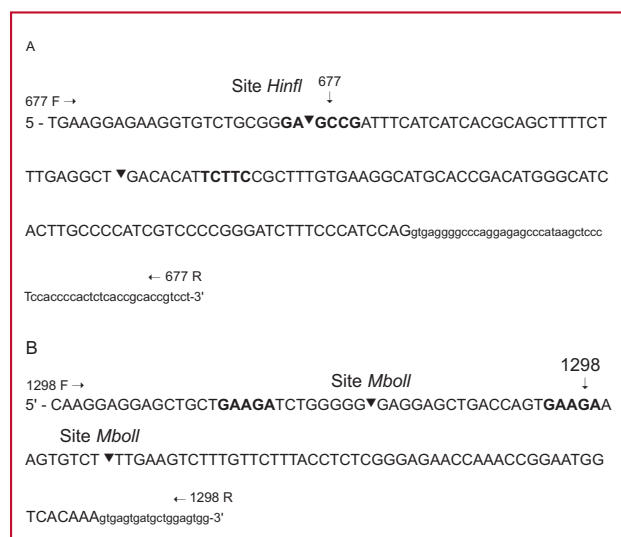


Figure 2. MTHFR gene fragment with primer sequence and the recognition site of the restriction enzymes used in the experiment¹⁷.

There are present two sites of recognition for the restriction enzyme *MboII* in the 128-bp fragment obtained by PCR that contains the 1298 region. After digestion, the most common genotype, 1298AA, has originated three fragments (28, 28 and 72-bp) but is characterized just by the fragment that contains 72-bp, as another two were not retained in the gel. The genotype CC is determined by the 100-bp alone and the AC

genotype is determined by the presence of both fragments of 72 and 100-bp in the gel (Figure 3). The results were statistically analyzed using the chi-square test.

RESULTS

The distribution of genotypes *MTHFR* 677CC, 677CT and 677TT, and of genotypes 1298AA, 1298AC and 1298CC in Turner Syndrome patients with and without chromosomal mosaicism and in the controls is shown in Table 2.

The frequency of genotypes *MTHFR* 677CC, 677CT and 677TT in the patients with Turner Syndrome and chromosomal mosaicism was, respectively, 58.3%, 38.9% and 2.8%. Among the patients with non-mosaic Turner Syndrome, 47.1% presented genotype 677CC, 45.2% genotype 677CT, and 7.7% genotype 677TT. Among the 209 individuals of the control group, genotypes 677CC, 677CT and 677TT were found at the following frequencies: 48.3%, 42.1% and 9.6%, respectively. As for polymorphism A1298C, the patients with Turner Syndrome and chromosomal mosaicism presented genotypes 1298AA, 1298AC and 1298CC at the following frequencies: 58.3%, 27.8% and 13.9%, respectively. Among the non-mosaic Turner Syndrome patients, genotype 1298AA was found in 36.5%, genotype 1298AC in 39.4%, and genotype 1298CC in 22.1%. In the control group, genotypes 1298AA, 1298AC and 1298CC were present at the following frequencies: 52.6%, 40.7% and 6.7%, respectively.

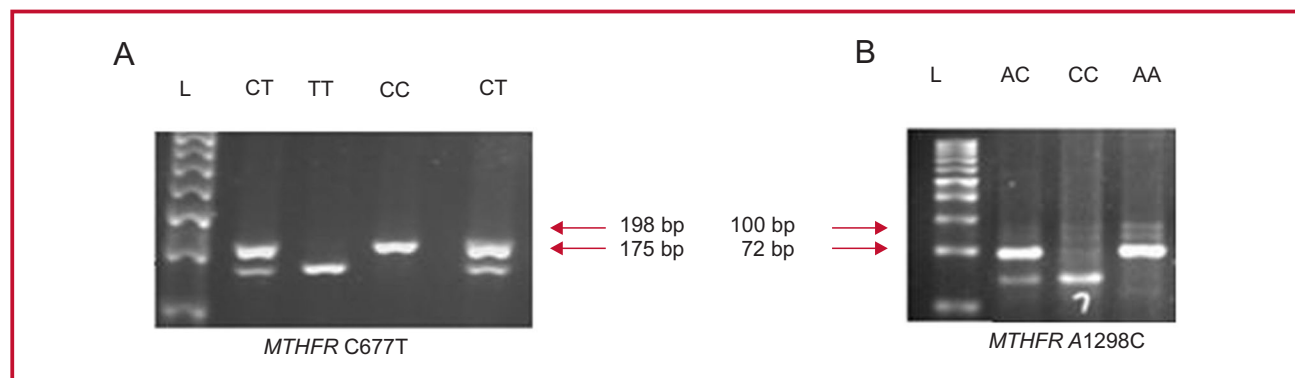


Figure 3. Polymorphism analysis of the methylenetetrahydrofolate reductase gene (*MTHFR*) amplicons by agarose gel electrophoresis after restriction endonuclease digestion (RFLP). A) – 677 position was digested by *Hinfl* revealing the genotypes CC(wild type)(198bp band), CT and TT (175bp band). B) – 1298 position was digested by *MboII* and presented the genotypes AA (wild type, 100bp band), AC and CC (72bp band).

Table 2. Frequency of *MTHFR* gene polymorphisms C677T and A1298C detected in patients presenting Turner Syndrome with and without chromosome mosaicism and in the individuals of the control group.

Gene polymorphism	Gendtype	TS patients with mosaicism		TS patients without mosaicism		Controls		p
		n	%	n	%	n	%	
<i>MTHFR 677</i>	CC	21	58,3	49	47,1	101	48,3	0,557
	CT	14	38,9	47	45,2	88	42,1	0,548
	TT	1	2,8	8	7,7	20	9,6	0,502
Total		36		104		209		
<i>MTHFR 1298</i>	AA	21	58,3	38	36,5	110	52,6	0,46
	AC	10	27,8	41	39,4	85	40,7	0,490
	CC	5	13,9	23	22,1	14	6,7	<0,001
Total		36		104		209		

Statistical significance was determined at $p < 0,05$.

There were no significant differences in the genotype distributions considering three groups (TS patients, TS with somatic mosaicism and control group) for the 677TT polymorphism. However, when compared the genotype frequency between the control group and TS patients was observed a statistical significant difference for 1298CC genotype ($p = 0.001$), that was associated with an increased risk of aneuploidy. Considering the presence of both simultaneously 677TT and 1298CC, no statistically difference was also found.

DISCUSSION

Turner Syndrome (TS) is one of the most common chromosome anomalies in humans, present in 1:2500 of female liveborns. It is characterized by short stature, gonadal dysgenesis, primary amenorrhea, sexual infantilism, and multiple congenital anomalies (5).

A 45,X karyotype is found in 40-60% of TS patients (5), the others being mosaics with a 45,X cell line accompanied by others, with two or more X chromosomes. Approximately 30% of all cases present structural alterations of the X chromosome whether in homogeneous or in mosaic karyotypes including a 45,X cell line, and finally about 5% are accounted for by patients with structural alterations of the Y chromosome as well as mosaics, with a cell line accompanied by others which include at least one Y chromosome, complete or not.

It is estimated that 15-20% of the human conceptions carry chromosomal anomalies due to errors in meiosis which are most of the time lethal to the fetus

(18). The mechanism most frequently involved in the generation of aneuploidies is chromosome nondisjunction, when the homologous chromosomes or the sister chromatids fail to separate. Chromosomal nondisjunction is a rare adverse event that occurs preferentially during oocyte maturation (meiosis I), originating gametes which are nullisomic or disomic for a given chromosome (19) or at the time of conception (meiosis II) (20,21). To date, advanced age is the only well-established risk factor.

Besides nondisjunction, other two mechanisms can generate aneuploidies: anaphase delay, a delay in the separation of homologues or sister chromatids during anaphase (16); and a defect in the reactions mediated by methionine synthase, caused by genetic and/or diet-related factors, leading to abnormal segregation of the chromosomes by an indirect effect of the DNA methylation patterns in the oocytes (22).

Some studies had revealed that chromosomal mal-segregation effects could occur due to nondisjunction, also in somatic cells in young women that beared a child with Down syndrome, suggesting the existence of genetic or environmental factors responsible for the formation of monosomic/disomic gametes (23).

The study by James and cols. (24) was the first to suggest an association between abnormal folate metabolism and the occurrence of trisomy 21-affected pregnancies in a sample of young mothers. Folate and homocysteine metabolism can be influenced by genetic variants not only in *MTHFR*, but in other genes related to folate metabolism. However, some studies consider

that nutritional deficiencies in folic acid or vitamin B12 are often necessary to detect phenotypic expression of these gene variants (9,25), as can be associated with elevated levels of plasma homocysteine. Chango and cols (25), studying *MTHFR* polymorphism on A677T in a French population are in accordance with some previous studies that could not establish a correlation between TT and homocysteine, suggesting that folate intake in France may nullify any effect of this deleterious genotype in the carrier. Similar results have also been found in an Italian study conducted by Bosco and cols. (26). Both studies suggested that the Mediterranean diet high in folate could nullify a possible effect of *MTHFR* polymorphism on risk of chromosomal aneuploidy development. These results are also consistent with the low frequency of neural-tube defects in both countries (27).

Chronic folate deficiency, and consequently of the methyl radical, has been associated with abnormal DNA methylation (28), non-disjunction by hypomethylation (7,24), DNA strand breaks, alterations in DNA recombination, aberrant chromosomal segregations, and excessive incorporation of uracil in the DNA (8). There are studies showing that DNA hypomethylation, caused by a decrease in the folate concentration, can induce chromosome losses, probably resulting from the low condensation of the pericentromeric region (28).

Hobbs and cols. (7) reported that pericentromeric DNA methylation is of extreme importance to chromosome stability and normal segregation. Stern and cols. (29) investigated whether the *MTHFR* gene mutation C677T affected the genomic DNA methylation and found that individuals with the mutated genotype have a greater capacity to receive the methyl group than normal individuals, indicating hypomethylation in genotype 677TT. DNA methylation can be used as a functional indicator of the folate levels, once folate plays a critical role in the prevention of chromosome breaks and DNA hypomethylation (28).

Several studies have been published that aim to evaluate the role of the *MTHFR* polymorphisms in the risk of chromosomal aneuploidies, specially Down syndrome and the results are often conflicting, specially due to the small size of the samples. However, these studies are useful, as they can both underline particular associations in a defined population or be indicative of a more general potential association so that, when evaluating combined results produced to date, some general information can be drawn (6).

The interaction between different polymorphisms may totally modify their individual effect and that some of those effects are different in carriers that had child with chromosomal aneuploidy and normal ones (30).

The present observations are in conflict with previous results of the literature, studying less than 50 TS patients with multiple karyotype presentation (13). Some considerations may be done related to the small sample of TS patients and the heterogeneity of the control group. We found no correlation between chromosomal nondisjunction in Turner Syndrome patients and the C677T polymorphism of the *MTHFR* gene. Polymorphism *MTHFR* A1298C was more frequent among the Turner Syndrome patients in the present study and, although it has less impact on the enzyme activity than polymorphism *MTHFR* C677T, data suggests that this polymorphism contributes to the formation of aneuploidies.

No correlation was observed between polymorphism C677T of the *MTHFR* gene and the chromosome nondisjunction of the Turner Syndrome patients. Polymorphism A1298C of the *MTHFR* gene, mainly genotype 1298CC, was more frequent in the patients with Turner Syndrome, suggesting its involvement in the origin of chromosomal imbalances.

Acknowledgments: Financial support Fapesp (2007/03295-0). No potential conflict of interest relevant to this article was reported.

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Correspondence to:

Monica Vannucci Nunes Lipay
 Disciplina de Endocrinologia
 Rua Pedro de Toledo, 781, 13º. Andar
 04039-032 São Paulo, SP
 E-mail: malipay@uol.com.br

This article has received corrections in agreement with the ERRATUM published in Volume 53 Number 1.