Maternal risk for Down syndrome is modulated by genes involved in folate metabolism

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Abstract. Studies have shown that the maternal risk for Down syndrome (DS) may be modulated by alterations in folate metabolism. The aim of this study was to evaluate the influence of 12 genetic polymorphisms involved in folate metabolism on maternal risk for DS. In addition, we evaluated the impact of these polymorphisms on serum folate and plasma methylmalonic acid (MMA, an indicator of vitamin B₁₂ status) concentrations. The polymorphisms transcobalamin II (TCN2) c.776C>G, betaine-homocysteine S-methyltransferase (BHMT) c.742A>G, methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) c.677 C>T and the MTHFR 677C-1298A-1317T haplotype modulate DS risk. The polymorphisms MTHFR c.677C>T and solute carrier family 19 (folate transporter), member 1 (SLC19A1) c.80 A>G modulate folate concentrations, whereas the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) c.66A>G polymorphism affects the MMA concentration. These results are consistent with the modulation of the maternal risk for DS by these polymorphisms.

Keywords: Down syndrome, genetic polymorphism, folate metabolism

1. Introduction

Down syndrome (DS), or trisomy 21 (MIM 190685), is the most common genetic disorder with a prevalence of 1 in 660 live births [27]. The only well-established risk factor for DS is advanced maternal age [7]. However, many DS children are born to mothers younger than 35 years, suggesting that other factors can also influence DS etiology. James et al. [48] hypothesized that pericentromeric hypomethylation, resulting from impaired folate metabolism secondary to a polymorphism on methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) gene, could impair chromosomal segregation and increase the risk for chromosome 21 nondisjunction in young mothers. Since then, several studies have revealed that polymorphisms in genes involved in the folate pathway modulate the maternal risk for DS [6,17,35,39,49] and the concentrations of metabolites involved in the folate pathway [14,30,43].

Folate metabolism vitally participates in the biosynthesis of nucleotides and S-adenosyl-methionine (SAM), the major methyl donor for DNA methylation reactions (Fig. 1). A folate deficiency has been associated with DNA hypomethylation, DNA damage, chromosomal instability, abnormal chromosome segregation and aneuploidy of chromosome 21 [45,47].
This study aimed to evaluate associations between 12 genetic polymorphisms, MTHFR c.677C>T, MTHFR c.1298A>C, MTHFR c.1317T>C, 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) c.2756 A>G, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) c.66A>G, cystathionine-beta-synthase (CBS) c.844ins68, CBS c.833T>C, solute carrier family 19 (folute transporter), member 1 (SLC19A1), also known as reduced folate carrier – RFC1 c.80A>G, transcobalamin II (TCN2) c.776C>G, TCN2 c.67A>G, methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methylenetetrahydrofolate cyclohydrolase, formylmethylenetetrahydrofolate synthetase (MTHFD1) c.1958G>A and betaine-homocysteine S-methyltransferase (BHMT) c.742G>A, and the maternal risk for DS. In addition, we evaluated the impact of the polymorphisms on serum folate and plasma methylmalonic acid (MMA, an indicator of the vitamin B12 status) concentrations.

2. Material and methods

The study protocol was approved by the Research Ethics Committee of São José do Rio Preto Medical School (CEP-FAMEERP), State of São Paulo, and by the National Research Commission (CONEP), Brazil. Fasting peripheral blood samples were obtained from 105 women (case mothers) with karyotypically confirmed full trisomy 21 (translocation or mosaicism were not included) liveborn offspring and from 185 mothers with at least one healthy offspring and no history of miscarriage (control mothers). Case mothers were from the same region of São Paulo (in the northwest). Thus, they likely share similar socio-demographic characteristics and racial backgrounds.

Genomic DNA was isolated from leucocytes in peripheral blood according to Miller et al. [46] or using the GFX™ Genomic Blood DNA Purification Kit (GE Healthcare, USA). The polymorphisms MTHFR c.677C>T, MTHFR c.2756A>G, SLC19A1 c.80A>G, TCN2 c.776C>G, CBS c.833T>C and MTHFD1 c.1958G>A were determined as previously described [11,17,23,33,41]. The variant TCN2 c.776C>G was analyzed via the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using primers from Pietrzyk & Bik-Multanowski (2003) [20], and the PCR products were digested with the SceI enzyme. The polymorphisms MTRR c.66A>G, TCN2 c.67A>G and BHMT c.742G>A were analyzed by real time PCR allelic discrimination (Taqman SNP Genotyping Assays, C_3068176_10, C_25967461_10 and C_11646606_20 respectively, Applied Biosystems, Foster City, CA, USA). Automated sequencing was used to investigate the MTHFR c.1298A>C and c.1317T>C polymorphisms as previously described [37]. The analysis, except for the purification procedure, was performed using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas Life Sciences, Brazil) according to the manufacturer’s instructions. Plasma MMA concentrations were determined by liquid chromatography-tandem mass spectrometry as previously described [51], and folate concentrations were determined by chemiluminescence (Immulite Kit, DPC Medlab, Brazil).

2.1. Statistical analysis

The Hardy-Weinberg (HW) equilibrium was assessed via a chi-square test using the BioEstat program, and the genotype frequencies were compared between case and control mothers by the likelihood ratio test. The relationship between the number of deleterious alleles for the 12 loci tested and the maternal risk for DS was assessed by logistic regression analysis. For this analysis, the sample was divided in two subsets (0–7 and 8–14 alleles for the total group and for the subgroup of women with maternal age ≤ 35 years old) according to the median value of deleterious alleles (median = 7). Previously published data [2,9,16] and the results in...
Table 1

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele deleterious</th>
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<tbody>
<tr>
<td>MTHFR c.677C&gt;T</td>
<td>T</td>
</tr>
<tr>
<td>MTHFR c.1298A&gt;C</td>
<td>C</td>
</tr>
<tr>
<td>MTHFR c.1317T&gt;C</td>
<td>C</td>
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<tr>
<td>MTR c.2756A&gt;G</td>
<td>G</td>
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<td>MTRR c.66A&gt;G</td>
<td>G</td>
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<td>SLC19A1 c.80A&gt;G</td>
<td>G</td>
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<tr>
<td>TNC2 c.67A&gt;G</td>
<td>G</td>
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<tr>
<td>TNC2 c.776C&gt;G</td>
<td>G</td>
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<tr>
<td>CBS c.844ins68*</td>
<td>I</td>
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<tr>
<td>CBS c.833T&gt;C</td>
<td>C</td>
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<tr>
<td>MTHFD1 c.1958G&gt;A</td>
<td>A</td>
</tr>
<tr>
<td>BHMT c.742G&gt;A</td>
<td>G</td>
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</table>

* The results of the CBS c. 844ins68 genotypes were defined as I for the allele with the 68bp insertion and W for the wild – type allele.

The results of the CBS c. 844ins68 genotypes were defined as W for the wild-type allele and I for the allele with the 68bp insertion.

Multiple logistic regression analyses, with forward stepwise selection by the likelihood ratio, were performed using SPSS19 software. Genotype data for the 12 polymorphisms, maternal age and folate and MMA concentrations were included in the original models to verify the influence of each of these factors on the maternal risk for DS. Only the women that provided all the data were included in this analysis. The genotype data used in the logistic regression model was analyzed in two different ways: considering either (1) the dominant model (heterozygous + mutant homozygous versus wild-type homozygous) or (2) the recessive model (wild-type homozygous + heterozygous versus mutant homozygous). Once advanced maternal age is shown to be an important risk factor for DS, the analyses were also performed in a sub-group formed only by women ≤ 35 years old (case: 54; control:173). These tests were applied to generate an odds ratio (OR) and 95% confidence intervals (CI).

Stepwise forward multivariate logistic regression analyses were also performed for the total group (case and control together) to verify the factors that influence biochemical parameters. For MMA analysis, the concentrations were categorized considering the values above (case mothers = 21; control mothers = 44) or equal to/above (case mothers = 66; control mothers = 137) the 75th percentile, and the genotype data for the 12 polymorphisms (recessive and dominant models) as well as the folate concentrations were used as predictors.

According to the likelihood ratio test, the genotype
gorized considering values below (case mothers = 21; control mothers = 44) or equal to/above (case mothers = 66; control mothers = 137) the 25th percentile, and the genotype data for the 12 polymorphisms (recessive and dominant models) as well as the MMA concentrations were used as predictors.

The computer-assisted statistical analyses were carried out using Minitab for Windows program (Release 14) and SPSS19 software. Values of P ≤ 0.05 were considered significant.

3. Results
and control groups

Haplotype frequencies of the morphisms HW equilibrium in both groups, except for the polymorphisms (Table 2). The genotype frequencies were in frequencies were not different between DS and control groups. The genotype frequencies were in frequencies were not different between DS and control groups. The genotype frequencies were in

frequency in the control group (0.14 ± 0.05–0.81).

The haplotype frequencies of the MTHFR, TCN2 and CBS genes are presented in Table 3. The MTHFR gene exhibited linkage disequilibrium (LD) between the polymorphisms c.677 C>T and c.1298A>C (LOD = 11.05; D’ = 1.0), c.677C>T and c.1317T>C (LOD = 3.23; D’ = 1.0) and c.1298A>C and c.1317T>C (LOD = 3.83; D’ = 1.0). A significantly higher frequency of the C-A-T haplotype (wild-type alleles) was observed in the control group compared to the case group (P = 0.01). The TCN2 polymorphisms c.67A>G and c.776C>G are weakly linked (LOD = 2.46; D’ = 0.63), whereas the CBS variants at positions 833 and 844 are strongly linked (LOD = 74.17; D’ = 1.0). There was no difference in the haplotype frequencies for the TCN2 and CBS genes between the groups. The CBS haplotypes 833T/844I and 833C/844W were not present in either group.

When considering the dominant model using logistic stepwise regression analysis, maternal age (OR, 1.12; 95% CI, 1.075–1.174; P < 0.0005) and the MTHFR c.677 CT or TT genotypes (OR, 1.76; 95% CI, 1.011–3.073; P = 0.04) significantly contributed independently to DS risk. Maternal age (OR, 1.13; 95% CI, 1.080–1.183; P < 0.0005) and the TCN2 c.776G>G genotype (OR, 2.45; 95% CI, 1.038–5.788; P = 0.04) and BHMT c.742G>A genotype (OR, 0.26; 95% CI, 0.078–0.843; P = 0.02) were significant modifiers of DS risk under the recessive model (Table 4).

With respect to the factors that exert influence on biochemical parameters, folate concentrations below the 25th percentile were associated with the presence of MTHFR c.677 CT or TT (OR, 2.19; 95% CI, 1.223–3.920; P = 0.01), whereas MMA concentrations above the 75th percentile were associated with the MTRR c.66 AG or GG genotypes (OR, 1.98; 95% CI, 1.122–3.495; P = 0.02), both in the dominant model (Table 5).

When we analyzed only women ≤ 35 years old at conception, the most predictive independent risk factors for DS were maternal age (OR, 1.21; 95% CI, 1.098–1.321; P < 0.0005) and the MTHFR c.677 CT or TT genotypes (OR, 2.30; 95% CI, 1.135–4.661; P =
The presence of MMA concentrations above the 75th percentile was associated with the presence of BHMT c.742G>A polymorphisms in mothers of DS children in a Brazilian population. In addition, the influence of the polymorphisms BHMT c.742G>A and TCN2 c.67A>G on DS risk have never been studied until now.

The polymorphism TCN2 c.776 GG, which has been previously investigated for DS risk by our group in a smaller sample [21] and by Fintelman-Rodrigues et al. [38], with negative results, was associated with increased DS risk in this study. Moreover, we observed that the TCN2 c.776 GG genotype influenced the risk for DS in both the total group and in the group with the conception age of women ≤ 35 years. The presence of the TCN2 c.776 GG genotype has been shown to negatively affect the serum concentration of the TCN2 protein-vitamin B12 complex [28] and to be associated with low concentrations of SAM in childbearing-age women [43]. Considering that SAM is the major methyl donor for DNA methylation reactions, the variant TCN2 c.776G>G may influence maternal risk for DS by modifying the DNA methylation pattern. We are the first group to study the influence of the TCN2 c.67A>G polymorphism on maternal risk for DS, where no association was observed with DS risk. The LD between the variants TCN2 c.67A>G and c.776C>G observed in this study is consistent with a previous study [26].

Our group is the first to evaluate the role of the BHMT c.742G>A polymorphism on the risk of bearing a DS child, where an association between the BHMT c.742 AA genotype and decreased maternal risk for DS was observed. The BHMT protein catalyzes an alternative route of homocysteine (Hcy) remethylation (Fig. 1). The polymorphism produces two distinct alloenzymes, which exhibit significant differences in Km values for Hcy and betaine [10]. The Km values are lower for the variant alloenzyme compared to the wild-type. The low Km of the alloenzyme may be responsible for the increased efficiency of Hcy remethylation using betaine as a methyl group donor [42]. The decreased effect of the BHMT c.742A allele on DS risk could be expected when the maternal AA genotype

### Table 5

| Variables associated with folate and methylmalonic acid (MMA) concentration according to the multiple logistic regression analyses with forward stepwise selection |

<table>
<thead>
<tr>
<th>OR</th>
<th>95% CI</th>
<th>P value*</th>
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<tbody>
<tr>
<td><strong>Total Group</strong></td>
<td></td>
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<tr>
<td><strong>Dominant Model</strong></td>
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<tr>
<td>MTHFR c.677 C&gt;T</td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>CT or TT</td>
<td>2.19</td>
<td>1.223–3.920</td>
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<tr>
<td><strong>MMA</strong></td>
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<td>MTMR c.66A&gt;G alelo</td>
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<tr>
<td>GG</td>
<td>1.00 (reference)</td>
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</tr>
<tr>
<td>AA or AG</td>
<td>1.98</td>
<td>1.122–3.495</td>
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<tr>
<td><strong>Recessive Model</strong></td>
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<tr>
<td>SLC19A1 c.80 A&gt;G</td>
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<tr>
<td>AA or AG</td>
<td>1.98</td>
<td>1.122–3.495</td>
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<td><strong>Dominant Model</strong></td>
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<td>CC</td>
<td>1.00 (reference)</td>
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<tr>
<td>CT or TT</td>
<td>2.01</td>
<td>1.052–3.831</td>
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<tr>
<td><strong>MMA</strong></td>
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<tr>
<td>MTMR c.66A&gt;G alelo</td>
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<tr>
<td>GG</td>
<td>2.20</td>
<td>1.110–4.351</td>
</tr>
<tr>
<td>AA or AG</td>
<td>1.88</td>
<td>1.028–3.440</td>
</tr>
</tbody>
</table>

0.02) in the dominant model and the TCN2 c.776 GG (OR, 3.47; 95% CI, 1.353–8.917; \( P = 0.01 \)) and BHMT c.742 AA genotypes (OR, 0.12; 95% CI, 0.015–0.974; \( P = 0.05 \)) in the recessive model (Table 4).

In women ≤ 35 years old, folate concentrations below the 25th percentile were associated with the presence of MTHFR c.677 CT or TT (OR, 2.01; 95% CI, 1.052–3.831; \( P = 0.03 \)) in the dominant model and with the presence of SLC19A1 c.80 GG (OR, 2.20; 95% CI, 1.110–4.351; \( P = 0.02 \)) in the recessive model. The presence of MTMR c.66 AG or GG were associated with MMA concentrations above the 75th percentile (OR, 1.88; 95% CI, 1.028–3.440; \( P = 0.04 \)) in the dominant model (Table 5).

The median number of deleterious alleles did not differ between the groups, both in the total group and in the group of women ≤ 35 years old.

### 4. Discussion

Although advanced maternal age at conception represents an important and well-established risk factor for DS [7], as confirmed in this study, the occurrence of DS births by young mothers suggests that other risk factors are also involved in the etiology of this syndrome. Abnormal folate metabolism has been identified as a maternal risk factor for DS in several populations [9]. This study reveals that polymorphisms in folate metabolism genes modulate the maternal risk for bearing a child with DS. This is the first study evaluating the role of MTHFR c.1317T>C, CBS c.833T>C, TCN2 c.67A>G, MTHFD1 c.1958G>A and BHMT c.742G>A polymorphisms in mothers of DS children in a Brazilian population. In addition, the influence of the polymorphisms BHMT c.742G>A and TCN2 c.67A>G on DS risk have never been studied until now.

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was protective against neural tube defects (NTD) in the offspring [2,16]. Moreover, NTDs and DS are influenced by the same genetic factors involved in folate metabolism [13].

Polymorphisms in the MTHFR gene have been extensively analyzed for influences on folate and methyl metabolisms in maternal risk for DS. The MTHFR enzyme plays an important role in regulating DNA methylation reactions through the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methylTHF (Fig. 1). A common polymorphism in the MTHFR gene, c.677 C>T, is known to decrease the affinity of the enzyme for its flavin adenine dinucleotide (FAD) cofactor, thereby decreasing the enzyme activity [4,25]. The heterozygous MTHFR c.677 CT genotype reduces the enzyme activity by approximately 35%, and the homozygous TT genotype reduces activity by 70% [41]. In this study, the presence of the MTHFR c.677 CT or TT genotypes was associated with increased maternal risk for DS, which corroborates previous associations between the MTHFR c.677 C>T polymorphism and the modulation of the maternal risk for DS [32,39,49]. Coppedè et al. [8] have observed an association between the MTHFR c.677 T allele and the occurrence of chromosome damage and missegregation events in mothers of DS individuals, supporting the role of this allele in the etiology of trisomy 21. Previously, these authors observed a significant increase in the rate of aneuploidy of chromosome 21 in these mothers [31].

The LD between the MTHFR polymorphisms c.677C>T, c.1298A>C and c.1317T>C observed in this study is consistent with the literature, which has illustrated LD between the MTHFR c.677 C>T and c.1298A>C [9,34]. Moreover, the silent polymorphism at position 1317 is near the one at 1298 position. The higher frequency of the MTHFR 677C-1298A-1317T haplotype in the control group confirms the protective maternal effect of these alleles against DS, which is indicated by the rare alleles 677T and 1298C that have been associated with increased maternal risk for DS in several studies [22,32,39,49]. In addition, this result corroborates the association between the 677T-1298C haplotype and the maternal risk for DS observed by Scala et al. [17].

MTRR, an enzyme codified by the MTRR gene, is responsible for the maintenance of the activated form of the MTR enzyme [29]. Several studies have observed an association between the MTRR c.66A>G polymorphism, alone and combined with other genetic variants, and DS risk and an elevated Hcy concentration [3, 5,6,36,40,44,49]. Additionally, a steady state kinetic analysis revealed a significant decrease in the affinity for MTRR accompanying a c.66A>G substitution, revealing a significant difference in the relative efficacies of the common MTRR polymorphism c.66A>G [15]. These findings further validate the association between the MTRR c.66 AG and GG genotypes and higher concentrations of MMA, likely a consequence of the variant enzyme activity that results in a higher Hcy concentration and consequently higher MMA concentrations.

The SLC19A1 gene encodes an enzyme that participates in folic acid absorption, transporting for 5-methylTHF, an important determinant of folate concentration, to the interior of a variety of cells [50,52]. The SLC19A1 gene is polymorphic at nucleotide 80 (A>G), and an assessment of the impact of this polymorphism on protein function has demonstrated a difference in its affinity for substrates and/or its efficiency in transport compared to the wild type enzyme [24]. Chang et al. [1] observed that SLC19A1 c.80AA/ MTHFR c.677CT individuals exhibited higher plasma folate levels than SLC19A1 c.80GG/ MTHFR c.677CT individuals, which corroborates our observations of lower folate concentrations in the presence of the SLC19A1 c.80 GG genotype.

Because most polymorphisms, with the exception of CBS c.844ins68 and c.833 T>C, did not deviate from the HW equilibrium, our sample set was appropriately ascertained [18,19]. Departure from the HW equilibrium may have resulted from random selection or a small sample size.

A major strength of our study was the number of polymorphisms in folate metabolism genes investigated. Out of 12 polymorphisms, five have never been analyzed in a Brazilian population until now, including the BHMT c.742G>A and TCN2 c.67A>G polymorphisms that influence the maternal risk for DS. A potential limitation of our study is that folate and MMA concentrations were not measured at the child’s delivery in both case and control mothers. Although the measurement of the concentrations at the time of conception would have been more relevant, the current quantification is a likely reflection of adult dietary patterns, once an adult’s dietary tends to have a similar pattern over the time. Also, the influence of the polymorphisms on MMA and folate concentrations was analyzed in the total group, including DS and control mothers, to investigate the polymorphism influence on the concentrations regardless of the presence of a DS child. The small size of the case group, which could reduce the power of the statistical analysis and complicate an investigation
of possible genotype combinations that may influence the maternal risk for DS, was an additional limitation of this study. However, studies that have investigated an association between folate gene polymorphisms and the risk of DS offspring have been conducted with relatively small sample sizes [9], primarily due to difficulties in the recruitment of these mothers, with significant results.

In conclusion, the results of this study indicate that the TCN2 c.776C>G, BHMT c.742A>G, and MTHFR c.677 C>T polymorphisms and the MTHFR 677C-1298A-1317T haplotype modulate the risk for DS. The polymorphisms MTHFR c.677C>T and SLC19A1 c.80 A>G modulate folate concentrations, whereas the MTRR c.66A>G polymorphism affects MMA concentrations. These findings contribute to future research aimed at identifying metabolic interventions that will aid in preventing nondisjunction of the 21 chromosome. Future studies may benefit from sample sizes that are large enough to identify specific gene-gene interactions.

Acknowledgments

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