Daniella Balduino Victorino

Revisão Sistemática e Metanálise da Associação entre Polimorfismos Genéticos Maternos Envolvidos no Metabolismo do Folato e o Nascimento de Indivíduos com Síndrome de Down

São José do Rio Preto

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Orientadora: Prof.ª Dr.ª Érika Cristina Pavarino

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Victorino, Daniella Balduino
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SUMÁRIO

Dedicatória

Aos meus pais, Regina Maura e José Rodolfo

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Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível (Charles Chaplin).

Lista de Figuras

Artigo 1

Artigo 2

Artigo 3

Lista de Tabelas

Artigo 1

Artigo 2

Artigo 3

Lista de Abreviaturas e Símbolos

10-formil-THF	10-formiltetrahidrofolato
5-MTHF	5-metiltetrahidrofolato / 5-methyltetrahydrofolate
5,10-metenil-THF	5,10-meteniltetrahidrofolato
5,10-metileno-THF	5,10-metilenotetrahidrofolato
Cbl	Cobalamina / cobalamin
CI	Confidence interval
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CβS	Cistationina β-sintase / Cystathionine β-synthase
DHF	Dihidrofolato
DNA	Ácido desoxirribonucleico
DS	Down syndrome
DSM	Case mothers
FAMERP	Faculdade de Medicina de São José do Rio Preto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FE	Fixed-effects
Hcy	Homocisteína / homocysteine
HWE	Hardy-Weinberg equilibrium
IC	Intervalo de confiança
LILACS	Literatura Latino-Americana e do Caribe em Ciências da Saúde
MeSH	Medical subject heading
M-H	Mantel-Haenszel method
MTHFD1	Metilenotetrahidrofolato desidrogenase 1 / Methylenetetrahydrofolate dehydrogenase 1

MTHFR	Metilenotetrahidrofolato redutase / Methylenetetrahydrofolate reductase
MTR	Metionina sintase / Methionine synthase
MTRR	Metionina sintase redutase / Methionine synthase reductase
NUTECC	Núcleo Transdisciplinar para Estudo do Caos e da Complexidade
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction - restriction fragment length polymorphism
PUBMED	Public/Publisher Medline
RE	Random-effects
RFC1	Carreador de folato reduzido 1 / Reduced folate carrier 1
RNA	Ácido ribonucléico
SAH	S-adenosylhomocysteine
SAM	S-adenosilmetionina / S-adenosyl-methionine
SD	Síndrome de Down
SLC19A1	Solute carrier family 19 (folate transporter), member 1
TC2	Transcobalamina 2 / Transcobalamin 2
THF	Tetrahidrofolato
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular

Resumo

Introdução Síndrome de Down (SD) é atribuída à presença de três cópias do cromossomo 21, decorrente da não-disjunção cromossômica meiótica materna em 95% dos casos. Polimorfismos genéticos maternos envolvidos no metabolismo do folato foram associados ao nascimento de indivíduos com a SD, porém os resultados dos estudos são contraditórios. **Objetivos** Avaliar, por meio de revisão sistemática e metanálise, a associação entre os polimorfismos genéticos maternos *Metilenotetrahidrofolato redutase* (*MTHFR*) C677T e A1298C, *Metionina sintase redutase* (*MTRR*) A66G, *Metionina sintase* (*MTR*) A2756G, *Carreador de folato reduzido 1* (*RFC1*) A80G, *Cistationina β-sintase* (*CβS*) 844ins68, *Metilenotetrahidrofolato desidrogenase 1* (*MTHFD1*) G1958A e *Transcobalamina 2* (*TC2*) C776G e o nascimento de indivíduos com a SD. **Métodos** As buscas bibliográficas foram realizadas anteriormente a maio de 2014 e os bancos de dados utilizados foram: PUBMED, EMBASE, LILACS, lista de referências bibliográficas dos artigos selecionados, busca manual em anais de congressos e comunicação pessoal. Foram incluídos estudos caso-controle que avaliaram a presença dos polimorfismos genéticos em mães de crianças com SD por trissomia livre do cromossomo 21 (mães-caso) e em mães de crianças sem histórico de anormalidades cromossômicas, síndromes ou malformações (mães-controle). Os critérios de exclusão consistiram em estudos que incluíram mães de crianças com SD por translocação ou mosaicismo, relatos de caso, editoriais e artigos de revisão. A extração dos dados e a avaliação da qualidade dos estudos foram feitas por dois investigadores. A metanálise avaliou a associação entre cada polimorfismo e o risco materno para a SD por meio dos modelos genéticos

dominante, recessivo, codominante e alélico. Medidas de desfecho dicotômicas foram sumarizadas utilizando-se modelos de efeito fixo e randômico e os resultados foram expressos em *odds ratio* (OR) com intervalo de confiança de 95% (IC 95%). A heterogeneidade entre estudos foi calculada pelo teste *Q* e pela estatística I^2 e suas potenciais fontes foram investigadas pelas análises de sensibilidade e subgrupo. O viés de publicação foi estimado pelos *funnel plot* e teste de regressão linear. **Resultados** Coletivamente, 30 estudos caso-controle preencheram os critérios de elegibilidade, o que totalizou 3.101 mães-caso e 3.967 mães-controle. Foi verificada associação significativa entre os polimorfismos *MTHFR* C677T e *MTRR* A66G e o risco materno para SD. As análises de subgrupo de acordo com a etnia revelaram associações significativas para o polimorfismo *MTHFR* C677T e o risco materno para a SD em caucasianos, brasileiros e asiáticos e para o polimorfismo *MTRR* A66G em caucasianos. Adicionalmente, foi encontrada associação significativa para o polimorfismo *RFC1* A80G e o risco materno para a SD e também nas análises de subgrupo de asiáticos e de mães com idade materna inferior a 35 anos no momento da concepção. Finalmente, o genótipo *MTHFD1* 1958GA revelou-se fator de risco materno para o nascimento de indivíduos com SD quando a análise foi restringida aos estudos cujo grupo controle estava em equilíbrio de Hardy-Weinberg. Nenhuma associação foi verificada para os polimorfismos *MTHFR* A1298C, *MTR* A2756G, *CβS* 844ins68 e *TC2* C776G. **Conclusões** Os polimorfismos *MTHFR* C677T, *MTRR* A66G, *RFC1* A80G e *MTHFD1* G1958A são fatores de risco materno para a SD.

Abstract

Introduction Down syndrome (DS) is caused by the presence of three copies of chromosome 21 in consequence to chromosome nondisjunction in maternal meiosis observed in about 95% of cases. Genetic polymorphisms involved in folate metabolism were associated with the maternal risk for DS. However, the results are contradictories. **Objectives** To perform a systematic review and meta-analysis in order to evaluate the association between *Methylenetetrahydrofolate reductase* (*MTHFR*) C677T and A1298C, *Methionine synthase reductase* (*MTRR*) A66G, *Methionine synthase* (*MTR*) A2756G, *Reduced folate carrier 1* (*RFC1*) A80G, *Cystathionine β-synthase* (*CβS*) 844ins68, *Methylenetetrahydrofolate dehydrogenase 1* (*MTHFD1*) G1958A and *Transcobalamin 2* (*TC2*) C776G genetic polymorphisms and the maternal risk for DS. **Methods** Studies were searched up to May 2014 on MEDLINE, EMBASE, LILACS, hand searched reference lists of published articles and conference meetings and personal communication. Case-control studies that evaluated the association between genetic polymorphisms in case mothers (DSM) and controls mothers (CM) were included. DSM are considered mothers that have gave birth to children with free trisomy of 21 chromosome and CM are considered mothers that have gave birth to children without chromosomal abnormality, syndrome or malformation. Studies with mothers of DS individuals with translocation or mosaicism, case reports, editorials and review articles were excluded. Data extraction and quality assessment were performed independently by two investigators. Meta-analysis assesses the associations between each genetic polymorphism and maternal risk for DS by dominant, recessive, codominant and allelic genetic models. Dichotomous outcome measures were pooled using fixed and random

effects models and the results were expressed by odds ratio (OR) with 95% confidence intervals (95% CI). Heterogeneity between studies was evaluated using Q test and the I^2 and subgroup and sensitivity analyses were performed in order to investigate the potential sources of heterogeneity. Publication bias was estimated using funnel plot and linear regression test. **Results** Collectively, 30 case-control studies including 3,101 DSM and 3,967 CM were included. Significant association between *MTHFR* C677T and *MTRR* A66G polymorphisms and maternal risk for DS was found when all population is considered. Subgroup and sensitivity analyses according ethnicity showed significant associations for the *MTHFR* C677T polymorphism in Caucasians, Brazilians and Asians and for the *MTRR* A66G polymorphism in Caucasians. Additionally, the results of the *RFC1* A80G polymorphism demonstrated significant association, it was also found in Asians and maternal age less than 35 years at conception subgroups analyses. Finally, *MTHFD1* 1958GA genotype was revealed as maternal risk factor for DS when only studies with control group in Hardy-Weinberg equilibrium were considered. No association among *MTHFR* A1298C, *MTR* A2756G, *CβS* 844ins68 and *TC2* C776G polymorphisms and maternal risk for DS was found. **Conclusions** *MTHFR* C677T, *MTRR* A66G, *RFC1* A80G and *MTHFD1* 1958GA polymorphisms are associated with maternal risk for DS.

INTRODUÇÃO

1. INTRODUÇÃO

A síndrome de Down (SD) ou trissomia do 21 (OMIM 190685) é a causa mais comum de deficiência cognitiva de etiologia genética,[1] presente em aproximadamente 1 a cada 660 nascidos vivos.[2] Apesar de existirem características clínicas marcantes na SD, a presença delas é bastante variável entre os indivíduos, tanto em gravidade quanto em ocorrência.[3]

Cerca de 90% dos casos de SD resultam da não-disjunção cromossômica durante a meiose materna, principalmente na meiose I.[4,5] Está bem estabelecido que a idade materna avançada é fator de risco para a não-disjunção cromossômica.[5-9] Entretanto, o nascimento de indivíduos com SD de mães jovens sugere o envolvimento de outros fatores etiológicos.[10-12] Em 1999, numa tentativa de elucidar os mecanismos moleculares envolvidos neste fenômeno, James e colaboradores propuseram que a ocorrência da SD independente da idade materna está relacionada à hipometilação do DNA como consequência do metabolismo anormal do folato.[13]

A metilação do DNA possui vários papéis funcionais, incluindo controle da expressão gênica[14,15] e manutenção da integridade e estabilidade genômica.[16,17] De fato, mostrou-se que a hipometilação prejudica a formação da heterocromatina e o estabelecimento do cinetocoro, complexo DNA-proteína que garante a divisão precisa dos cromossomos entre as células filhas por meio da ligação do centrômero aos microtúbulos do fuso mitótico. Assim, a hipometilação está associada à instabilidade do DNA e, consequentemente, à segregação anormal dos cromossomos e à ocorrência de aneuploidias.[12,17-21]

O metabolismo do folato (Figura 1) é constituído de dois ciclos principais: um envolvendo a síntese de purinas e pirimidinas, essencial para síntese e reparo de DNA, e

outro de metilação celular, essencial para o fornecimento de grupos metil para metiltransferases celulares, que atuam no controle associado à expressão gênica e na manutenção da estabilidade genômica.[22] Após a ingestão dietética, o folato é rapidamente reduzido a dihidrofolato (DHF) e, em seguida, à sua forma ativa, tetrahidrofolato (THF). Posteriormente, THF é sequencialmente convertido em seus derivados 10-formiltetrahidrofolato (10-formil-THF), 5,10-meteniltetrahidrofolato (5,10-metenil-THF) e 5,10-metilenotetrahidrofolato (5,10-metileno-THF) pela ação da enzima trifuncional metilenotetrahidrofolato desidrogenase 1 (MTHFD1). A atividade da enzima MTHFD1 é essencial para a síntese de DNA, já que providencia 10-formil-THF e 5,10-metileno-THF para a síntese *de novo* de purinas e pirimidinas.[23]

A enzima metilenotetrahidrofolato redutase (MTHFR) catalisa a conversão de 5,10-metileno-THF para 5-metiltetrahidrofolato (5-MTHF), a principal forma circulante de folato, necessário para a doação de grupos metil para a remetilação de Homocisteína (Hcy) à metionina.[24] Esta reação de remetilação é catalisada pela enzima metionina sintase (MTR), que requer vitamina B12 ou cobalamina (Cbl) como cofator,[25,26] e resulta na formação de S-adenosilmetionina (SAM), o maior doador intracelular de grupos metil para reações de metilação do DNA.[27] Adicionalmente, devido à oxidação da Cbl, a enzima MTR se torna inativa e sua regeneração funcional requer a ação da enzima metionina sintase redutase (MTRR).[28]

A enzima cistationina β-sintase (CβS), na presença de vitamina B6, catalisa a reação de transulfuração em que Hcy e serina são condensadas em cistationina, etapa intermediária na formação de cisteína.[29] Sob condições fisiológicas normais, toda Hcy é remetilada a metionina ou catalisada para cistationina. Sendo assim, o aumento na

concentração de Hcy representa anormalidades no metabolismo do folato e, por conseguinte, nas reações de metilação.[22]

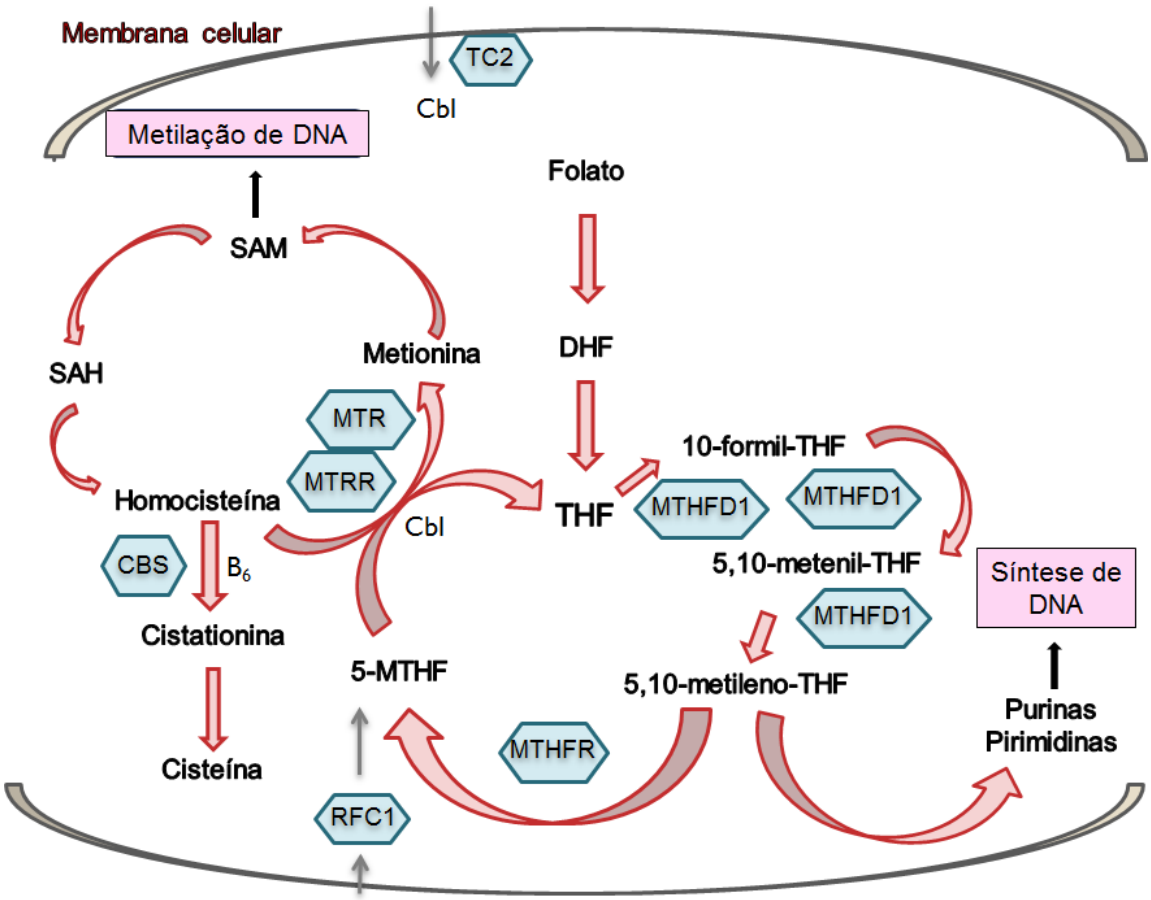

Figura 1. Metabolismo do folato com as principais enzimas envolvidas. B_6 = Vitamina B_6, CβS = cistationina β-sintase, Cbl = Cobalamina, DHF = Dihidrofolato, MTHFD1 = Metilenotetrahidrofolato desidrogenase 1, MTHFR = Metilenotetrahidrofolato redutase, MTR = Metionina sintase, MTRR = Metionina sintase redutase, 5,10-metileno-THF = 5,10-metilenotetrahidrofolato, 5-MTHF = 5-metiltetrahidrofolato, 5,10-metenil-THF = 5,10-meteniltetrahidrofolato, 10-formil-THF = 10-formiltetrahidrofolato, RFC1 = Carreador de folato reduzido 1, SAH = S-adenosilhomocisteína, SAM = S-adenosilmetionina TC2 = Transcobalamina 2, THF = Tetrahidrofolato.

Proteínas transportadoras de folato reduzido, como a enzima carreadora de folato reduzido 1 (RFC1)[30], e de Cbl, como a enzima transcobalamina 2 (TC2), também são importantes para o metabolismo do folato. A proteína RFC1 localiza-se na membrana das células da mucosa intestinal e desempenha um papel essencial na absorção do folato, através do transporte de 5-MTHF para o interior de uma variedade de células[30], constituindo um importante determinante das concentrações de folato disponíveis no meio intracelular.[30,31] Por sua vez, a proteína TC2 é sintetizada no endotélio vascular da vilosidade intestinal e liga-se à Cbl no fluido intersticial, formando o complexo TC2-Cbl que passa, então, a microcirculação da vilosidade intestinal e finalmente atinge a circulação sistêmica por meio da veia porta.[32]

Polimorfismos em genes que codificam enzimas envolvidas no metabolismo do folato têm sido apontados como fatores de risco materno para a SD,[13,33-39] visto que podem interferir nas concentrações de Hcy e SAM.

O gene *MTHFR* apresenta dois polimorfismos associados à redução da atividade enzimática, C677T e A1298C.[40,41] Diversos estudos evidenciaram que o polimorfismo *MTHFR* C677T contribui para o aumento da concentração de Hcy plasmática,[35,40,42,43] assim como influencia no risco materno para a SD.[13,35,38,44-47] Adicionalmente, também mostraram associação significativa os estudos que investigaram o papel do polimorfismo *MTHFR* A1298C para o risco materno para a SD e o aumento da concentração de Hcy plasmática.[38,39,41,42,48,49] No entanto, outros estudos não verificaram associação entre o polimorfismo *MTHFR* C677T e o risco materno para a SD,[25,26,37,50,51] bem como para o polimorfismo *MTHFR* A1298C.[25,26,46,52,53]

O gene *MTRR* se apresenta polimórfico no nucleotídeo 66, em que a substituição de adenina por guanina (A66G) ocasiona a substituição de isoleucina por metionina na

proteína.[54] Estudos sustentam um papel independente do polimorfismo *MTRR* A66G no risco materno para a SD na presença do genótipo homozigoto *MTRR* 66GG,[35,47,50] bem como quando combinado com outros polimorfismos como *MTHFR* C677T[33,47,48,51] e *MTR* A2756G.[55] Entretanto, estudos adicionais não encontraram associação entre este polimorfismo e o risco materno para a SD, seja isolado ou combinado com outros polimorfismos.[26,34,39]

O polimorfismo de uma substituição de adenina por guanina na posição 2756 (A2756G) no gene *MTR* também está relacionado com alterações na via metabólica do folato e, consequentemente, associado ao risco materno para a SD, na presença dos genótipos AG ou GG,[55] e em combinação com outros polimorfismos desta via.[33,34,36,46,55,56] Em adição, o alelo *MTR* 2756G foi encontrado em maior frequência, tanto em homozigose quanto em heterozigose, em mães de indivíduos com SD quando comparadas às mães de indivíduos sem a síndrome.[50] Entretanto, alguns estudos não confirmaram estes achados.[26,33,39,46]

O polimorfismo *RFC1* A80G é responsável pela substituição de adenina por guanina na posição 80 do gene. Este polimorfismo foi associado à redução nos níveis de expressão da proteína RFC1, com consequente comprometimento na eficiência do transporte[57] e redução dos níveis plasmáticos de folato.[58-61] Alguns estudos caso-controle não observaram contribuição deste polimorfismo como fator de risco materno independente para a SD,[26,56,62] enquanto outros mostraram uma associação significativa.[39,63] Adicionalmente, estudos sugerem um papel para este polimorfismo no risco materno para a SD quando combinado com o polimorfismo *MTHFR* C677T (*MTHFR* 677TT/*RFC1* 80GG)[64] ou conjuntamente com outros polimorfismos como *MTHFR* A1298C, *MTRR* A66G, *MTR* A2756G e *CβS* 844ins68.[33,36]

O gene *CβS* se apresenta polimórfico no nucleotídeo 844, com inserção de 68 pb (*CβS* 844ins68). Estudos mostram associação entre este polimorfismo e a redução nas concentrações de Hcy[65-67] e o aumento da atividade da enzima CβS[66-68] na presença do fragmento inserido. É possível que a melhora da atividade enzimática e, consequentemente, diminuição das concentrações de Hcy, comprometa a via de remetilação da Hcy para metionina, o que reduz a síntese de SAM e as reações de metilação celulares.[65] O polimorfismo 844ins68 foi investigado como fator de risco materno independente para a SD e nenhuma associação foi encontrada,[26,33,39,46,56] porém há evidência de associação entre este polimorfismo e o risco materno para a SD na presença de outros polimorfismos como *MTHFR* C677T, *MTHFR* A1298C, *MTRR* A66G, *MTR* A2756G e *RFC1* A80G.[33,46]

O polimorfismo *MTHFD1* G1958A, em que guanina é substituída por adenina na posição 1958 do gene, encontrado no domínio 10-formiltetrahidrofolato sintetase, foi relacionado com alteração funcional da enzima,[69] uma vez que essa substituição de aminoácidos foi associada a alterações nas concentrações de folato e Hcy.[70,71] O estudo conduzido por Scala *et al.* mostrou uma associação entre o genótipo *MTHFD1* 1958AA e o risco materno para a SD quando combinado com o genótipo *RFC1* 80GG.[39] Entretanto, estudos posteriores não verificaram associação entre este polimorfismo e o risco materno para a SD.[62,72]

O gene *TC2* é polimórfico no nucleotídeo 776 (C→G). Estudos indicam que o polimorfismo *TC2* C776G ocasiona perda da afinidade da enzima TC2 com a vitamina B12 devido a alterações no sítio de ligação ou a modificações na estrutura secundária da enzima,[73] o que pode influenciar negativamente na quantidade de vitamina B12 disponível no organismo[74] e ocasionar alterações no nível de transcrição do gene

TC2.[75] Em estudo de Barbosa *et al.*, o polimorfismo *TC2* C776G foi associado a variações nas concentrações de SAM em mulheres em idade reprodutiva, uma vez que mulheres com os genótipos *TC2* 776CG ou 776GG apresentaram concentrações mais baixas do que mulheres com os genótipos 776CC.[76] Considerando que SAM é o principal doador de grupos metil para as reações de metilação do DNA,[27] é possível que o polimorfismo *TC2* C776G exerça influência no risco materno para a SD. Por outro lado, no estudo conduzido por Biselli *et al.*, nenhuma associação foi verificada entre o polimorfismo *TC2* C776G e risco materno para a SD.[77] Ainda, Fintelman-Rodrigues *et al.* encontraram maior número de genótipos combinados *TC2* 776CC/*MTHFR* 677TT e *TC2* 776CC/*MTR* 2756AG em mães de indivíduos sem SD.[56]

Os estudos que avaliaram a associação entre os polimorfismos genéticos envolvidos no metabolismo do folato e o risco materno para a SD são inconclusivos ou contraditórios. Tal divergência pode decorrer, principalmente, devido ao tamanho amostral, variações geográficas nas frequências dos alelos de um determinado gene entre diferentes populações e fatores ambientais de cada região, como a quantidade de folato ingerido.[48,78,79]

Visto que resultados discordantes constantemente emergem de estudos diferentes que abordaram a mesma questão e estudos individuais esporadicamente possuem poder estatístico suficiente para proporcionar respostas definitivas, as revisões sistemáticas com metanálise possuem grande relevância como ferramenta de investigação científica, com custos mais baixos do que os exigidos para a realização de estudos de larga escala.[80] A revisão sistemática é um tipo de pesquisa minuciosamente planejada, com o objetivo de sumarizar estudos primários da literatura e que responde a uma questão clínica específica. Para tal, adota métodos apropriados para identificar, selecionar e

avaliar criticamente os estudos, e também para analisar os dados dos estudos incluídos na revisão.[81,82] Estes dados podem ser quantitativamente agrupados por métodos estatísticos denominados metanálise[83,84] que pode ser descrita como a análise estatística de uma coletânea de estudos individuais, que contrasta e combina os achados dos diferentes estudos, com o objetivo de identificar padrões consistentes e fontes de discordância entre seus resultados.[85] Com o uso desta análise pode-se estudar associações entre exposições e desfechos para as quais existe um número grande de estudos não conclusivos ou com resultados contraditórios.

Assim, o uso da metanálise pode contribuir para esclarecer o papel dos polimorfismos genéticos envolvidos no metabolismo do folato em relação ao risco da não-disjunção do cromossomo 21. Além disso, pode permitir a compreensão das inconsistências observadas nos achados dos diferentes estudos, com vistas a, eventualmente, estabelecer fatores de risco para a ocorrência da SD.

1.1 OBJETIVOS

O objetivo deste estudo foi determinar, por meio de revisão sistemática e metanálise, se o nascimento de indivíduos com síndrome de Down está associado à presença de polimorfismos genéticos maternos envolvidos no metabolismo do folato (*MTHFR* C677T, *MTHFR* A1298C, *MTRR* A66G, *MTR* A2756G, *RFC-1* A80G, *CBS* 844ins68, *TC2* C776G, *MTHFD* G1958A).

ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

Os resultados referentes aos objetivos propostos para esta dissertação estão apresentados em formato de artigos científicos.

Artigo 1

Título: Meta-Analysis of *Methylenetetrahydrofolate reductase* maternal gene in Down syndrome: increased susceptibility in women carriers of the *MTHFR* 677T allele

Autores: Daniella Balduino Victorino, Moacir Fernandes de Godoy, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino

Periódico: *Molecular Biology Reports*, artigo publicado.

Artigo 2

Título: Positive association between the *Reduced folate carrier 1* A80G Polymorphism (*RFC1* A80G) and Maternal Risk for Down Syndrome

Autores: Daniella Balduino Victorino, Moacir Fernandes de Godoy, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino

Periódico: *American Journal of Medical Genetics A*, artigo submetido para publicação.

Artigo 3

Título: Genetic polymorphisms involved in folate metabolism and maternal risk for Down syndrome: a meta-analysis

Autores: Daniella Balduino Victorino, Moacir Fernandes de Godoy, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino

Periódico: *Disease Markers*, artigo aceito para publicação.

Title: Meta-Analysis of *Methylenetetrahydrofolate reductase* maternal gene in Down syndrome: increased susceptibility in women carriers of the *MTHFR* 677T allele

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ABSTRACT

Because a number of data studies include some controversial results about *MTHFR* (Methylenetetrahydrofolate reductase) polymorphisms and Down syndrome (DS), we performed a meta-analysis to determine a more precise estimation of this association. Studies were searched on PUBMED, EMBASE and LILACS, up to April 2013, and they were eligible if they included case mothers (DSM) that have gave birth to children with DS, and controls mothers (CM) that have gave birth to healthy children without chromosomal abnormality, syndrome or malformation. The combined odds ratio with 95% confidence intervals was calculated by fixed or random effects models to assess the strength of associations. Potential sources of heterogeneity between studies were evaluated using Q test and the I^2. Publication bias was estimated using Begg's test and Egger's linear regression test. Sensitivity analyses were performed by using allelic, dominant, recessive and codominant genetic models, Hardy-Weinberg equilibrium and ethnicity. Twenty two studies with 2,223 DSM and 2,807 CM were included for *MTHFR* C677T and 15 studies with 1,601 DSM and 1,849 CM were included for *MTHFR* A1298C. Overall analysis suggests an association of the *MTHFR* C677T polymorphism with maternal risk for DS. Moreover, no association between the *MTHFR* A1298C polymorphism and maternal risk for DS was found. There is also evidence of higher heterogeneity, with I^2 test values ranging from 8% to 89%. No evidence of publication bias was found. Taken together, our meta-analysis implied that the T allele carriers might carry an increased maternal risk for DS.

Keywords Down syndrome; meta-analysis; *MTHFR* C677T; *MTHFR* A1298C; folate pathway.

INTRODUCTION

First described in 1866, Down syndrome (DS) is one of the most commonly identified genetic forms of intellectual disability, which affect about 1 in 660 live births [1]. The disorder is caused by a complete or partial (translocations or mosaicism) triplication of chromosome 21 resulting in multiple congenital abnormalities of variable severity [2,3]. In the majority of DS cases (90%), the nondisjunction event is of maternal origin, occurring primarily during meiosis I in the maturing oocyte [3].

Advanced maternal age at conception is the only well known risk factor for the great majority of DS pregnancies, as chromosome trisomies are more prevalent in children born to mothers aged 35 years and older [4,5]. However, several children with DS are born to women younger than 35 years at conception, indicating a predisposition to chromosome nondisjunction in these women [6]. Chromosomal nondisjunction and folate metabolism have received great attention. James et al. [7] were the first to present evidence that the occurrence of chromosome 21 nondisjunction is associated with DNA hypomethylation due to abnormal folate metabolism.

Folic acid is essential for normal DNA synthesis and normal cellular methylation reactions. The 5,10-Methylenetetrahydrofolate reductase (MTHFR) enzyme catalyzes the synthesis of 5-methylenetetrahydrofolate, the methyl donor for the B12-dependent remethylation of homocysteine (Hcy) to methionine. Methionine is the precursor for S-adenosyl-methionine (SAM), the major cellular methyl donor for DNA, RNA, proteins, and phospholipids methylation [6]. Hence, all these pathways might be affected by the *MTHFR* C677T or A1298C functional polymorphism, which could both reduce the enzyme activity [8-11].

In reference to this association, many studies have been carried out to determine the relationship among several polymorphisms of genes involved in this metabolic pathway and maternal risk for DS [7, 12-34]. However, the data from these studies have shown conflicting results for their small sample size and unified ethnicity, and the question is still unsolved.

Therefore, in order to evaluate these contradictory results and further explore the association between the *MTHFR* C677T and A1298C polymorphisms and the maternal risk for DS, we conducted a systematical review and a meta-analysis. Additionally, the heterogeneity among studies and the existence of potential bias were explored.

METHODS

Search strategy: Identification of eligible studies

PUBMED, EMBASE and LILACS-SCIELO electronic database were retrieved in order to find studies focusing on the association between *MTHFR* C677T or *MTHFR* A1298C polymorphisms and the birth of children with DS (last search update, April 2013). The medical subject heading (MeSH) terms and keywords used in the search strategy are shown in Figure 1. Two distinct authors read the retrieved studies independently in their entirety to assess their appropriateness for the inclusion in this meta-analysis. The relevant articles and publications on the same topic in reference lists of the reviewed articles were also retrieved according to the inclusion criteria. Only the articles published in English, Spanish and Portuguese were included.

Criteria of inclusion and exclusion

Any human association study based on validated genotyping methods, regardless of sample size and published up to April 2013 was eligible (1) if it pertained to the association between *MTHFR* C677T or A1298C polymorphisms and the maternal risk for DS; (2) case–control studies that determined the distributions of the *MTHFR* C677T or *MTHFR* A1298C genotypes in case mothers and in a control group of mothers; (3) case mothers (DSM) are considered mothers that gave birth to at least one child with DS with free trisomy 21, and the controls mothers (CM) are considered mothers that have gave birth to healthy children, without chromosomal abnormality, syndrome or malformation; (4) it presented sufficient data to calculate an odds ratio (OR) with 95% confidence interval (CI); (5) for the articles with overlap data of the same population resource, only the latest or largest report was included. Mothers of DS individuals with translocation or mosaicism, review articles, case reports, abstracts, letters, comments, editorials and animal studies were excluded.

Data extraction and quality assessment

The following information was extracted from each eligible study: first author's name, the journal, the year of publication, the ethnicity of subjects, the country, the study design, demography characteristics of cases and controls, genotyping method, genotype frequency and the number of cases and controls for the *MTHFR* C677T and A1298C genotypes. The allele frequencies were calculated from the corresponding genotype distributions. Baseline information and data were extracted by two reviewers independently using the same standard. Another reviewer adjudicated the differences between them.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) among the controls was estimated by chi-square test to compare the observed genotype frequencies with the expected ones and violations of HWE were defined as $P \leq 0.05$. Sensitivity analysis was conducted by limiting the meta-analysis to studies conformed to HWE.

We evaluated the maternal risk for (1) allelic model (minor allele versus major allele); (2) codominant model (heterozygous versus common homozygous carriers and rare homozygous versus common homozygous carriers); (3) dominant model (minor allele carriers versus common homozygous carriers); (4) recessive model (minor homozygous carriers versus common allele carriers). The associations between the *MTHFR* C677T and *MTHFR* A1298C polymorphisms and the risk of birth of a child with DS were further analyzed by categorizing into different ethnic populations. They were indicated as a pooled odds ratio (OR) and 95% confidence intervals (CI).

The pooled OR was estimated using fixed-effects (FE) [35] and random-effects (RE) [36] models. The RE model assumes different studies show substantial diversity and assesses both within-study sampling error and between-study variation [36]. The FE model assumes that all of the studies are estimating the same underlying effect and considers only within-study variance [35]. The associations with $P \leq 0.05$ were considered as being statistically significant.

Heterogeneity among included studies was assessed by Chi-square based Q-test [37].We also quantified the effect of heterogeneity using $I^2 = 100\% \times (Q-df)/Q$ [38], which ranges from 0 (minimum heterogeneity) and 100% (maximum heterogeneity), and measures the degree of inconsistency in the studies by calculating what proportion of the total variation across studies should be attributed to heterogeneity [38]. The

overall estimate of risk was obtained by Mantel-Haenszel method in a FE model or DerSimonian and Laird method in a RE model in the absence ($P > 0.05$) or in the presence ($P \leq 0.05$) of heterogeneity, respectively [35,36].

Publication bias was examined visually by a Begg's test (funnel plot method) [39], in which the standard error of log (OR) of each study was plotted against its log (OR). If there was publication bias, the funnel plot would be asymmetric. Funnel plot asymmetry was further assessed by Egger's linear regression test [40], which measures funnel plot asymmetry on the natural logarithm scale of the OR and rank correlation. $P \leq 0.05$ was considered statistically significant. Software Review Manager 5.2, BioEstat 5.3 and StatsDirect 1.9.15 was used for all analyses.

RESULTS

A flow chart summarizing the retrieved studies and the studies excluded, with specified reasons, is shown in Figure 2. Finally, 22 studies published between 2000 and 2012 were included in this meta-analysis. Studies were conducted in different ethnic populations: twelve involved Caucasian [12-16,20,23,26,28,30-32], five mixed Brazilian [17,18,25,27,33] and five Asian [19,21,24,29,34].

Some of the articles reported that CM was composed by women who had no experience with miscarriages [12,15,17,18,21,23,25,28-33], while others articles did not bring any information about miscarriages [13,14,19,20,24,26,34]. On the other hand, two studies did report CM who had previously experiences with miscarriages [16,27].

From the twelve studies included in this meta-analysis, only three studies report of the parental origin of the extra chromosome 21 [19,25,30]. Moreover, only a subgroup of mothers along with father and DS child was selected for the parental origin analysis.

All eligible studies used validated genotyping methods to determine the genetic polymorphisms, as polymerase chain reaction (PCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [9,41,42].

A list of the main characteristics extracted from the studies included in the meta-analysis is summarized in Table 1.

The distribution of genotypes in the controls of all eligible studies was consistent with HWE, except for six studies (Acacio et al. [18] for *MTHFR* C677T and Boduroglu et al. [15], Meguid et al. [23], Santos-Rebouças et al. [25], Cyril et al. [29] and Sadiq et al. [32] for *MTHFR* A1298C) (Table 1).

MTHFR C677T polymorphism and maternal risk for DS

The association between *MTHFR* C677T polymorphism and DS was investigated in 22 studies including 2,223 DSM and 2,807 CM. We found statistically significant differences in polymorphic allele frequencies between cases and controls in Brazilian and Asian populations, as well as when all the populations were considered (Table 2). Overall, an association between maternal risk for DS and the polymorphic genotypes was observed in different genetic models when all studies were pooled into the meta-analysis (Figure 3 and Table 3).

We further examined the association between *MTHFR* C677T polymorphism and DS according to distribution of genotypes in CM conforming to HWE and ethnicity because there was significant heterogeneity between studies (Table 3). In one study [18], the distribution of genotypes in CM deviated from HWE (Table 1). A sensitivity analysis (exclusion of this study) was carried out. Results showed that there still was large heterogeneity among studies; however, an association between maternal risk for

DS and the polymorphic genotypes was observed in all genetic models. We performed a sensitivity analysis to investigate the effect of each individual study, since the exclusion of a given article may isolate the remaining subgroup from the article's particular effect. After eliminating the results of Wang et al. [21] and Sadiq et al. [32], the heterogeneity decreased, which indicated that these studies may be the main origin of the heterogeneity observed. However, despite eliminating the data of these studies, our results did not change (data not show).

Subgroup analysis by the ethnicity revealed significant associations in TT vs CC codominant and allelic genetic models on Caucasians and in dominant, CT vs CC codominant and allelic genetic models on Brazilians (Table 3). Additionally, a statistically significant association was found for the comparison of CT vs CC in the Asian population. There was moderate heterogeneity between the studies performed in Caucasian and Asian populations but not in Brazilian population. Sensitivity analysis was also performed by omitting one study each time to assess the effect of individual study. After eliminating the results of Sadiq et al. [32] on the Caucasian subgroup, and Kohli et al. [24] on the Asian subgroup, heterogeneity decreased, which indicated that these studies may be the main origin of the heterogeneity on these subgroups. No individual study affected pooled results significantly in Caucasian analysis subgroup. However, Kohli et al. [24] affected the association between *MTHFR* C677T and maternal risk for DS. Thus we dropped this study, the results showed a significant association with the overall ORs and no statistical heterogeneity on Asian subgroup analysis. The result of *MTHFR* C677T in fixed-effects model was OR 2.26 (95% CI 1.54 to 3.31) in dominant model, OR 5.78 (95% CI 2.43 to 13.76) in recessive model,

OR 8.63 (95% CI 3.42 to 21.79) in TT vs CC and OR 2.32 (95% CI 1.69 to 3.16) in T vs C. In the discussion section, this subject will be more deeply analyzed.

MTHFR A1298C polymorphism and maternal risk for DS

There were 15 studies with 1,601 DSM and 1,849 CM examining the association of *MTHFR* A1298C polymorphism and maternal risk for DS. There were no statistically significant differences of polymorphic allele frequencies between cases and controls in Caucasian, Brazilian and Asian populations, as well as when all the populations were considered (Table 2).

The meta-analysis failed to reveal an association between the *MTHFR* A1298C polymorphism and maternal risk for DS when all studies were pooled into it (Table 3). Similar results were observed in the subgroup analyses by ethnicity and no significant association was observed between the *MTHFR* A1298C polymorphism and maternal risk for DS in Caucasian, Brazilian or Asian populations. There was significant heterogeneity for the comparison of CC vs AC + AA, CC vs AA and C vs A in all populations. However, the significant heterogeneity disappeared when we excluded studies [15,23,25,29,32] which all showed deviations from HWE, or when we stratified by Brazilian ethnicity. Sensitivity analysis was also performed by omitting one study each time to assess the effect of individual study. After eliminating the results of Meguid et al. [23], heterogeneity decreased, which indicated that this study contribute to the heterogeneity in Caucasians. However, despite eliminating the data of these studies, our results did not change (data not show). Additionally, in the Asia subgroup, this analysis was not possible because only two studies were included.

Publication bias

Begg's test (funnel plot method) [39] and Egger's linear regression test [40] were used to assess the publication bias of the currently available literature. The shape of the funnel plot does not revealed any evidence of asymmetry for the *MTHFR* C677T and A1298C polymorphisms in all genetic models analyses (Table 3). Moreover, the Egger's test also showed no evidence of publication bias for both polymorphisms (Table 3).

DISCUSSION

Our meta-analysis showed a significant association between *MTHFR* C677T polymorphism and maternal risk for DS in an overall analysis, and regarding to all genetic models. Such association was well supported by Caucasian and Brazilian subgroups. After eliminating the study which was considered the main origin of heterogeneity, the meta-analysis also showed a significant association in Asian populations. Moreover, the subsequently made sensitivity analysis supported such an association. However, no significant evidence of association between *MTHFR* A1298C and maternal risk for DS was observed in our study.

Folate is an important key factor involved in complex metabolic pathways including synthesis and repair of DNA and DNA methylation [6]. Functional maternal polymorphisms at genes encoding key enzymes in folate pathway, as *MTHFR* C677T and A1298C, are known to reduce the enzyme activity [8-11]. Therefore, changes in DNA methylation or DNA stability and integrity may be induced by folate deficiency and predispose it to the development of DS [6-11].

The genetic mechanisms of DS have been substantially explored. In 1999, James et al. [7] suggested that DNA hypomethylation and abnormal chromosomal segregation were derived from an abnormal folate pathway and since then, several studies have demonstrated a relationship between the *MTHFR* C677T and A1298C polymorphisms and maternal risk for DS. However, most of the studies have shown conflicting results and the question is still unsolved. Because the inconsistent results from relatively small studies are underpowered for detecting polymorphism effects, the meta-analysis has become a very powerful tool for combining results of various studies, enabling summarization of the main conclusions, and providing high statistical power for testing research hypotheses. This meta-analysis of 22 published articles for *MTHFR* C677T polymorphism and 15 published articles for *MTHFR* A1298C polymorphism from different ethnicities afforded a greater possibility to reach reasonably strong conclusions, in order to give an explanation for the genetic association between *MTHFR* C677T and A1298C polymorphisms and maternal risk for DS.

When all the eligible studies were pooled into the meta-analysis, our results support a genetic association between the *MTHFR* C677T polymorphism and maternal risk for DS, which corroborates some of the previous case-control studies [20-23,32,43]. Additionally, the meta-analysis of *MTHFR* C677T have shown stable and robust results, while the sensitivity analyses demonstrated that the results would not be reversed by any of the Caucasians and Brazilians studies considered in our work.

Although the exact functions of the *MTHFR* C677T polymorphism in DS maternal risk is not yet clear, our current analysis would have sufficient statistical power to detect this association. It may seem plausible to consider that the reduction of the enzyme activity can be explained by the C to T substitution at the 677 nucleotide of the

MTHFR gene [7,9,10], and it is connected with an increased requirement for folic acid, elevated levels of plasma Hcy and a decrease in SAM levels, which is the major methyl group donor for methylation reactions as DNA, proteins and lipids methylation [7]. Some researchers showed that the plasma Hcy concentrations was significantly higher in DS mothers when compared to control mothers in studies that investigated the effect of plasma Hcy concentrations on maternal risk for DS [7,13,17,22].

Folate deficiency is associated with DNA hypomethylation [44,45] and aneuploidy of chromosome 21 [46,47]. In the absence of sufficient folic acid, chronic elevation in intracellular Hcy can lead to a decrease in the ratio of SAM to S-adenosylhomocysteine (SAH) which is associated with inhibition of the DNA methyltransferase and DNA hypomethylation. The consequences of such events are: pericentromeric hypomethylation, impaired chromosome segregation and increasing the risk of chromosome 21 nondisjunction [7,46,48-50]. One study reported a decrease in the DS offspring of mothers who were supplemented with high doses of folic acid (~6 mg/d) during the first gestational month [51]. Such study supports the hypothesis of an involvement of folate in the etiology of DS. Furthermore, the *MTHFR* C677T polymorphism is associated with DNA hypomethylation [52-54] in the presence of the polymorphic T allele. These findings support the hypothesis of a relationship between the increased frequency of maternal *MTHFR* C677T polymorphism (observed in this study) and the etiology of DS.

As showed by our meta-analysis results for the *MTHFR* A1298C polymorphism, *MTHFR* A1298C polymorphism was not associated with independent maternal risk factor for DS. However, *MTHFR* C677T and *MTHFR* A1298C are both in the *MTHFR* gene and so their interaction is possible to occur [8]. Furthermore, dietary intake,

especially for folate, is different for different ethnic populations [14,53,55]; this variation can impact on the prevalence of DS, and so it must not be excluded. Finally, the between-study heterogeneity can also affect the results.

One of the most essential purposes of meta-analysis is to find the sources of the between-study heterogeneity, since heterogeneity can lead to problems in the results interpretation of a meta-analysis [56]. In our meta-analysis, heterogeneity evaluation was always conducted. Thus, subgroup analysis was performed. In the stratified analysis by ethnicity, significant associations were found in Caucasian and Brazilian populations for the *MTHFR* C677T polymorphism in most genetic models. However, there was moderate heterogeneity among the studies performed in Caucasian but not in Brazilian population. Sensitivity analysis showed that the Sadiq's [32] study on the Caucasian subgroup was the main source of the heterogeneity in this subgroup analysis. However, the OR was not significantly changed by omitting this study, indicating that our results were robust and reliable.

Clinical heterogeneity like maternal age and dietary intake may also explain the between-study heterogeneity since different populations may show differences in dietary intake of folate and vitamin B12 [14,53,55]. Another explanation may be that different genetic backgrounds may have caused the heterogeneity since the T allele frequencies of the *MTHFR* C677T polymorphism in DS mothers varies on different populations [57,58]. The Brazilian population is one of the most mixed and heterogeneous populations. Such heterogeneity is a consequence of inter-ethnic crosses between Europeans, Africans and Amerindians [59]. Predisposition to diseases and allele frequencies is substantially variable around populations as a result of genetic drift and adaptation to local selective factors such as climate and available nutrients [60]. It is

possible that risk markers observed in genetically homogeneous populations, such as the Caucasian group, do not apply to mixed ones [61]. However, our findings have shown that inspite of the Brazilian population's genetic diversity, there is a significant association between the *MTHFR* C677T polymorphism and the maternal risk for DS in such a population.

The sensitivity analysis of *MTHFR* C677T in Asian population showed that the study of Kohli et al. [24] affected the results. Under the review of this paper, Kohli et al. [24] showed contradictory results when compared to results from other included studies, since it showed that the frequency of *MTHFR* C677T polymorphism in north Indian mothers of babies with DS was 28%, compared to 35% in controls mothers (677CT and 677TT). Additionally, after removing such study, the relationship between *MTHFR* C677T polymorphism and the maternal risk for DS showed a significant association on the overall ORs, while the heterogeneity was sharply reduced, showing that this particular study is the main responsible for the heterogeneity in the Asian subgroup. Since the sample size was too limited (301 DSM and 347 CM), after removal of the Kohli's study, we must be cautious about the observed association and more studies are required to improve the precision of the result.

Recently, to the best of our knowledge, three meta-analyses papers reported the association of *MTHFR* C677T and A1298C polymorphisms with maternal risk for DS [62-64]. The studies included in these meta-analyses and the sample number differed from each other and reported different results. A previous meta-analysis, conducted in 2012 by Wu et al. [62], evaluated the relationship between *MTHFR* C677T and A1298C polymorphisms and the maternal risk for DS based on 28 publications including 2,806 cases and 4,597 controls for *MTHFR* C677T and 18 studies including 1,854 cases and

2,364 controls for *MTHFR* A1298C polymorphism. There are some discrepancies between the Wu's study [62] and our study. Moreover, we carried out some independent and original subgroup analyses. Subgroup analysis of Brazilian population was not performed in the Wu's study [62], but it was included in this meta-analysis. We also conducted stratified analyses by codominant models, and almost all results revealed significant association between *MTHFR*C677T polymorphism and maternal risk for DS. In the controls of the studies conducted by Acacio et al. [18] for *MTHFR* C677T and by Boduroglu et al. [15], Meguid et al. [23], Santos-Rebouças et al. [25], Cyril et al. [29] and Sadiq et al. [32] for *MTHFR* A1298C, the genotype distribution deviated from HWE, and so that is the reason why they were excluded from our sensitivity analysis. On the other hand, they were included in the Wu et al. [62] article. Furthermore, the inclusion and exclusion criteria's were not strict and so may lead to poor quality of the studies included in their meta-analysis.

Another meta-analysis including 20 studies, performed almost at the same time, was conducted by Costa-Lima et al. [63]. The literature on *MTHFR* A1298C polymorphism was not included in the Costa-Lima's study [63], but it was included in our study. In the analysis result, nineteen articles were shared between the Costa-Lima's study [63] and our study. However, our study included three additional articles (including different and newly published literature) about *MTHFR* C677T [29,33,34], as well as fifteen additional articles about *MTHFR* A1298C polymorphism [15-20,23,25-29,31-33]. We also conducted stratified analyses by dominant and recessive model, which was not made in the Costa-Lima et al. [63] article. Finally, the third similar meta-analysis conducted by Yang et al. [64] did not include genetic models analyses, but it was included in this meta-analysis. The distinct results may, generally, be due to the

differences in the studies included in the meta-analysis. These factors lead to different conclusions. For these reasons and compared with previous meta-analyses, we demonstrated more exact and stronger evidence to clarify the association between *MTHFR* C677T and A1298C polymorphisms and maternal risk for DS.

Although we have increased the statistical power to detect slight associations by combining the data of individual studies, there are still some limitations we must describe. First of all, only studies written in English, Spanish and Portuguese were included. This selection pattern may generate biases on the results. Second, although we have collected all the eligible studies, the results of the subgroup stratification analysis must be carefully interpreted due to the limited number of published studies. Third, analyses of multiples interaction between gene-gene and gene-environment were not performed. It is reasonable to consider that specific environmental and lifestyle factors may alter these associations between genetic polymorphisms and maternal risk for DS. Furthermore, although previous reports have suggested that *MTHFR* C677T and A1298C polymorphisms are in linkage disequilibrium [65,66] and that they have a synergistic effect on enzyme activity [8], we could not investigate the association between the haplotypes and combined genotypes of the *MTHFR* C677T and A1298C polymorphisms and maternal risk for DS due to the lack of detailed original data in the included studies in this meta-analysis. So, further detailed studies are required to perform a haplotypes and combined genotypes analysis to explore the associations between these two polymorphisms and maternal risk for DS. Finally, almost all Caucasians in the study are European descent and statistical power for analyses in other ethnicities is limited. The main conclusions from this paper are based on analyses between Caucasian and Brazilian women, since Asian studies have shown restricted

sample sizes. Future studies with larger samples are also necessary to clarify current findings across studies of these distinct ethnic groups.

Nevertheless, our meta-analysis has shown several advantages. First, a meta-analysis of the association between *MTHFR* C677T and A1298C polymorphisms and maternal risk for DS is statistically more powerful than any single study. Second, our strict inclusion criteria are satisfactorily assured by the great quality of the studies included in our meta-analysis. Third, all controls in the included studies were mothers that had gave birth to healthy children, without reported abnormalities. The distribution of genotype in CM followed the HWE in all studies except for one study [18] in the meta-analysis for the *MTHFR* C677T, and for five [15,23,25,29,32] in the meta-analysis for the *MTHFR* A1298C. When excluding these studies, the pooled OR was not significantly changed indicating that the control group could represent the base population.

Taken together, our meta-analysis implied that the T allele carriers might carry an increased maternal risk for DS. However, it is conceivable that the *MTHFR* C677T polymorphism has a heterogeneous effect on the maternal risk for DS across different ethnicities. Surely, DS is the consequence of the interaction among several factors such as genetic, epigenetic, environmental and ethnic origin. In addition, our meta-analysis also highlights the need to consider potential gene-gene and gene-environment interactions, when one tries to comprehend and combine observed data. Future studies should lead to better and comprehensive understanding of the association between the *MTHRF* C677T polymorphism and maternal risk for DS.

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Table 1 Information of the Included Studies that Investigated the Relationship Between *Methylenetetrahydrofolate Reductase* C677T and A1298C polymorphisms and Down Syndrome

First Author (Year)[a]	Ethnicity (Country)	DSM	CM	*MTHFR* C677T											*MTHFR* A1298C										
				Distribution of genotypes						Frequency of alleles				P^{HWE}	Distribution of genotypes						Frequency of alleles				P^{HWE}
				TT		CT		CC		T		C			CC		AC		AA		C		A		
				DSM	CM	DSM	CM	DSM	CM	DSM	CM	DSM	CM		DSM	CM	DSM	CM	DSM	CM	DSM	CM	DSM	CM	
Hobbs et al. (2000) [12]	Caucasian (USA and Canada)	157	140	22	14	84	59	51	67	128	87	186	193	0.84	-	-	-	-	-	-	-	-	-	-	-
O'Leary et al. (2002) [13]	Caucasian (Ireland)	41	192	2	18	21	84	18	90	25	120	57	264	0.80	-	-	-	-	-	-	-	-	-	-	-
Stuppia et al. (2002) [14]	Caucasian (Italy)	64	112	12	23	32	62	20	27	56	108	72	116	0.25	-	-	-	-	-	-	-	-	-	-	-
Boduroglu et al. (2004) [15]	Caucasian (Turkey)	152	91	11	3	55	30	86	58	77	36	227	146	0.71	11	10	97	60	44	21	119	80	185	102	0.001
Chango et al. (2005) [16]	Caucasian (France)	119	119	12	12	64	58	43	49	88	82	150	156	0.38	11	12	49	56	59	52	71	79	167	159	0.58
da Silva et al. (2005) [17]	Brazilian (Brazil)	154	158	15	7	72	67	67	84	102	81	206	235	0.15	3	6	37	32	30	50	42	44	98	132	0.79
Acacio et al. (2005) [18]	Brazilian (Brazil)	70	88	5	9	30	25	35	54	40	42	100	134	0.03	6	7	49	50	99	101	62	63	246	253	0.77
Rai et al. (2006) [19]	Asian (India)	149	165	16	18	43	54	20	39	64	43	234	287	0.58	22	5	39	37	28	28	83	47	95	93	0.12
Scala et al. (2006) [20]	Caucasian (Italy)	94	256	12	2	40	39	97	124	87	239	101	273	0.75	17	25	39	108	38	128	73	158	115	364	0.74
Wang et al. (2008) [21]	Asian (China)	64	70	18	5	32	29	14	36	68	39	60	101	0.79	-	-	-	-	-	-	-	-	-	-	-
Meguid et al. (2008) [23]	Caucasian (Egypt)	42	48	5	3	17	12	20	33	27	18	57	78	0.21	14	1	20	29	8	18	48	31	36	65	0.008
Kohli et al. (2008) [24]	Asian (India)	104	109	0	6	29	32	75	71	30	44	178	174	0.35	-	-	-	-	-	-	-	-	-	-	-
Santos-Rebouças et al. (2008) [25]	Brazilian (Brazil)	103	108	9	12	43	47	51	49	60	69	146	147	0.88	5	2	40	49	58	57	49	52	157	164	0.01
Martínez-Frías et al. (2008) [26]	Caucasian (Spain)	146	190	24	27	61	85	61	78	109	139	183	241	0.62	13	19	57	78	76	91	82	116	210	260	0.70
Brandalize et al. (2009) [27]	Brazilian (Brazil)	239	197	32	18	113	93	94	86	177	130	301	264	0.31	12	8	84	76	143	113	105	98	373	296	0.27
Coppede et al. (2009) [28]	Caucasian (Italy)	94	113	17	18	52	55	25	40	86	91	102	135	0.90	6	7	38	53	44	47	50	67	126	147	0.11
Cyril et al. (2009) [29]	Asian (India)	36	60	0	0	3	0	33	60	3	0	69	120	1.00	3	13	19	21	14	26	25	47	47	73	0.03
Pozzi et al. (2009) [30]	Caucasian (Italy)	74	184	16	29	30	93	28	62	62	151	86	217	0.54	-	-	-	-	-	-	-	-	-	-	-
Vranekovic et al. (2010) [31]	Caucasian (Croatia)	111	141	13	11	49	64	49	66	75	86	147	196	0.40	7	10	56	68	48	63	70	88	152	194	0.14
Sadiq et al. (2011) [32]	Caucasian (Jordan)	53	29	3	1	27	5	23	23	33	7	73	51	0.31	0	1	29	18	24	10	29	20	77	38	0.04
Zampieri et al. (2012) [33]	Brazilian (Brazil)	105	185	10	18	55	73	40	94	75	109	135	261	0.49	6	9	48	73	51	101	60	91	150	279	0.36
Mohanty et al. (2012) [34]	Asian (India)	52	52	0	0	8	3	44	49	8	3	96	101	0.83	-	-	-	-	-	-	-	-	-	-	-

Table 2 Comparisons of variant allele frequencies between cases and controls in three ethnic populations

Polymorphism	Allele	Population	NO. Data (case/control)	Allele frequency (mean±SD)		P-value[a]
				Case Mothers	Control Mothers	
*MTHFR*C677T	T	Caucasian	12 (2,294/3,230)	0.368±0.066	0.323±0.111	0.407
		Brazilian	5 (1,342/1,472)	0.325±0.039	0.286±0.040	0.009
		Asian	5 (810/912)	0.200±0.195	0.127±0.115	< 0.0001
		Total	22 (4,446/5,614)	0.320±0.121	0.270±0.126	0.006
*MTHFR*A1298C	C	Caucasian	8 (1,610/1,968)	0.350±0.100	0.334±0.044	0.449
		Brazilian	5 (1,342/1,470)	0.249±0.042	0.237±0.021	0.973
		Asian	2 (250/260)	0.408±0.082	0.362±0.038	0.104
		Total	15 (3,202/3,698)	0.324±0.097	0.305±0.062	0.357

[a]Test of difference between cases and controls.

P-values are based on chi-square test.

NO. Data, number of included studies and total number of case/controls mothers.

The results are shown in absolute numbers.

Table 3 Main Results of Pooled Odds Ratios, 95% Confidence Intervals and Heterogeneity for the Association of *Methylenetetrahydrofolate Reductase* C677T and A1298C Polymorphisms and Down Syndrome

				Test of Association		Test of Heterogeneity			Tests of Publication Bias (P-value)	
Polymorphism	Comparison	Population	Study (n)	OR (95% CI)	P-value (Z test)	χ^2	I^2	P-value	Rank test (Begg & Mazumdar)	Linear regression (Egger et al)
MTHFR C677T	TT / CT vs CC (dominant)	All	22	**1.32 (1.11, 1.58)[a]**	0.002	42.32	50%	0.004	0.45	0.09
		All in HWE	21	**1.32 (1.09, 1.58)[a]**	0.004	41.87	52%	0.003	0.67	0.11
		Caucasian	12	1.24 (0.98, 1.56)[a]	0.07	21.27	48%	0.03	0.38	0.14
		Brazilian	5	**1.31 (1.05, 1.64)[b]**	0.02	4.33	8%	0.36	0.81	0.89
		Asian	5	1.95 (0.93, 4.07)[a]	0.08	14.74	73%	0.005	0.75	0.57
	TT vs CT / CC (recessive)	All	22	**1.32 (1.10, 1.59)[b]**	0.003	24.41	22%	0.18	0.62	0.33
		All in HWE	21	**1.35 (1.12, 1.63)[b]**	0.002	23.05	22%	0.19	0.36	0.20
		Caucasian	12	1.24 (0.98, 1.56)[b]	0.07	4.52	0	0.95	0.94	0.75
		Brazilian	5	1.23 (0.86, 1.77)[b]	0.26	4.74	16%	0.31	0.48	0.42
		Asian	5	2.13 (0.28, 16.25)[a]	0.47	8.90	78%	0.01	-	-
	TT vs CC (codominant)	All	22	**1.46 (1.10, 1.94)[a]**	0.009	31.77	40%	0.03	0.26	0.15
		All in HWE	21	**1.50 (1.12, 2.01)[a]**	0.007	31.04	42%	0.03	0.17	0.11
		Caucasian	12	**1.28 (1.00, 1.65)[b]**	0.05	7.87	0	0.72	0.54	0.38
		Brazilian	5	1.38 (0.95, 2.02)[b]	0.09	4.58	13%	0.33	0.48	0.52
		Asian	5	2.49 (0.24, 25.85)[a]	0.44	11.18	82%	0.004	-	-
	CT vs CC (codominant)	All	22	**1.27 (1.07, 1.52)[a]**	0.007	37.04	43%	0.02	0.78	0.40
		All in HWE	21	**1.25 (1.05, 1.50)[a]**	0.01	35.66	44%	0.02	0.54	0.07
		Caucasian	12	1.20 (0.93, 1.54)[a]	0.16	21.99	50%	0.02	0.38	0.14
		Brazilian	5	**1.30 (1.04, 1.62)[b]**	0.02	4.87	18%	0.30	0.81	0.56
		Asian	5	**1.46 (1.05, 2.03)[b]**	0.02	8.88	55%	0.06	0.75	0.36

MTHFR A1298C	T vs C (allelic)	All	22	**1.25 (1.09, 1.44)[a]**	0.001	43.99	52%	0.002	0.35	0.12
		All in HWE	21	**1.26 (1.09, 1.45)[a]**	0.002	43.97	55%	0.002	0.42	0.13
		Caucasian	12	**1.17 (1.04, 1.31)[b]**	0.009	15.63	30%	0.16	0.15	0.06
		Brazilian	5	**1.22 (1.04, 1.43)[b]**	0.02	3.59	0	0.47	0.48	0.76
		Asian	5	1.95 (0.93, 4.07)[a]	0.08	14.74	73%	0.005	0.75	0.80
	CC / AC vs AA (dominant)	All	15	1.03 (0.89, 1.18)[b]	0.70	15.72	11%	0.33	0.55	0.30
		All in HWE	10	1.05 (0.90, 1.22)[b]	0.56	9.57	6%	0.39	0.48	0.15
		Caucasian	8	0.96 (0.79, 1.17)[b]	0.69	9.50	26%	0.22	0.54	0.73
		Brazilian	5	1.06 (0.86, 1.32)[b]	0.58	4.44	10%	0.35	0.23	0.22
		Asian	2	1.35 (0.81, 2.27)[b]	0.25	0.12	0	0.73	-	-
	CC vs AC / AA (recessive)	All	15	1.17 (0.79, 1.74)[a]	0.42	27.06	48%	0.02	0.59	0.70
		All in HWE	10	1.27 (0.95, 1.70)[b]	0.10	11.09	19%	0.27	0.72	0.43
		Caucasian	8	1.15 (0.67, 1.99)[a]	0.61	15.48	55%	0.03	0.38	0.47
		Brazilian	5	1.12 (0.68, 1.87)[b]	0.65	2.02	0	0.73	0.48	0.81
		Asian	2	1.23 (0.10, 15.15)[a]	0.87	8.92	89%	0.003	-	-
	AC vs AA (codominant)	All	15	1.00 (0.86, 1.15)[b]	0.95	12.08	0	0.60	0.69	0.31
		All in HWE	10	1.02 (0.86, 1.19)[b]	0.85	8.06	0	0.53	0.60	0.24
		Caucasian	8	0.92 (0.75, 1.13)[b]	0.42	4.12	0	0.77	0.71	0.94
		Brazilian	5	1.07 (0.82, 1.40)[b]	0.62	5.80	31%	0.21	0.23	0.23
		Asian	2	1.25 (0.73, 2.16)[b]	0.42	0.65	0	0.42	-	-
	CC vs AA (codominant)	All	15	1.18 (0.79, 1.78)[a]	0.42	26.83	48%	0.02	0.27	0.53
		All in HWE	10	1.26 (0.93, 1.70)[b]	0.14	11.15	19%	0.27	0.60	0.64
		Caucasian	8	1.11 (0.60, 2.08)[a]	0.73	18.01	61%	0.01	0.38	0.51
		Brazilian	5	1.17 (0.70, 1.97)[b]	0.54	1.26	0	0.87	0.81	0.60
		Asian	2	1.43 (0.15, 14.04)[a]	0.76	6.48	85%	0.01	-	-
	C vs A (allelic)	All	15	1.06 (0.91, 1.24)[a]	0.45	27.52	49%	0.02	0.23	0.35
		All in HWE	10	1.06 (0.94, 1.19)[b]	0.38	14.00	36%	0.12	0.21	0.22
		Caucasian	8	1.04 (0.81, 1.33)[a]	0.76	19.11	63%	0.008	0.90	0.58

Brazilian	5	1.02 (0.86, 1.22)[b]	0.79	3.04	0	0.55	0.81	0.18
Asian	2	1.32 (0.92, 1.90)[b]	0.13	3.61	72%	0.06	-	-

Abbreviations: OR, Odds Ratio; CI, Confidence Interval.

[a]Random-effect model.

[b]Fixed-effect model.

- insufficient strat.

Bold values indicate significant associations.

PUBMED search strategy:
1 Down syndrome
2 Down's syndrome
3 Downs Syndrome
4 Syndrome, Down
5 Syndrome, Down's
6 Trisomy 21
7 Trisomy 21, Meiotic Nondisjunction
8 Trisomy 21, Mitotic Nondisjunction
9 #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8
#10 Methylenetetrahydrofolate Reductase
#11 Methylene-THF Reductase
#12 Methylenetetrahydrofolate Reductase
#13 5,10-Methylenetetrahydrofolate Reductase
#14 Methylene Tetrahydrofolate Reductase
#15 Tetrahydrofolate Reductase, Methylene
#16 MTHFR
#17 #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16
#18 Polymorphisms, Genetic
#19 Genetic Polymorphism
#20 Polymorphism (Genetics)
#21 Genetic Polymorphisms
#22 C677T
#23 A1298C
#24 #18 OR #19 OR #20 OR #21 OR #22 OR #23
#25 #9 AND #17 AND #24

Figure 1 PUBMED search strategy.

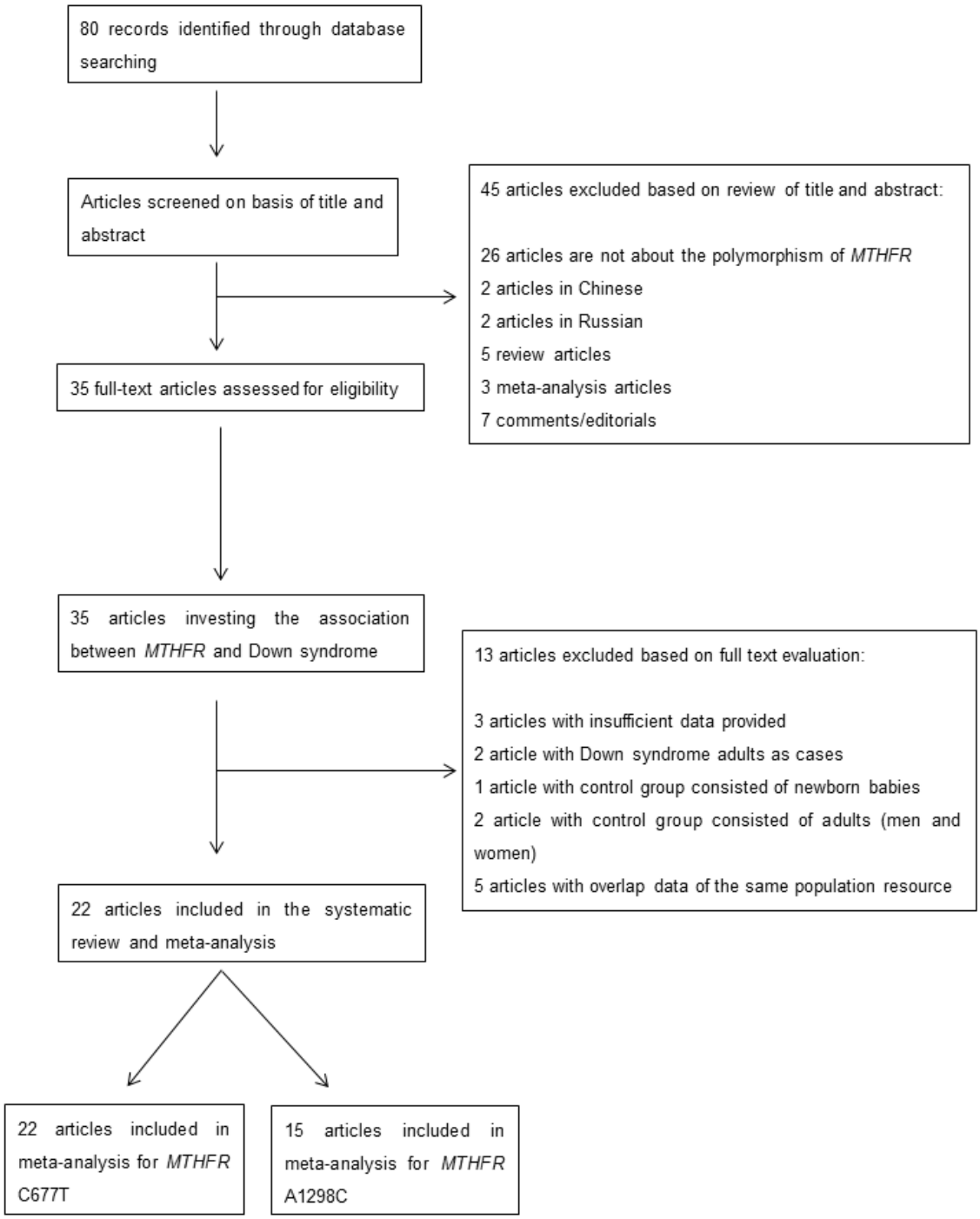

Figure 2 Flow chart of the strategy for study selection concerning the association of maternal *MTHFR* C677T and A1298C polymorphisms and maternal risk for Down syndrome.

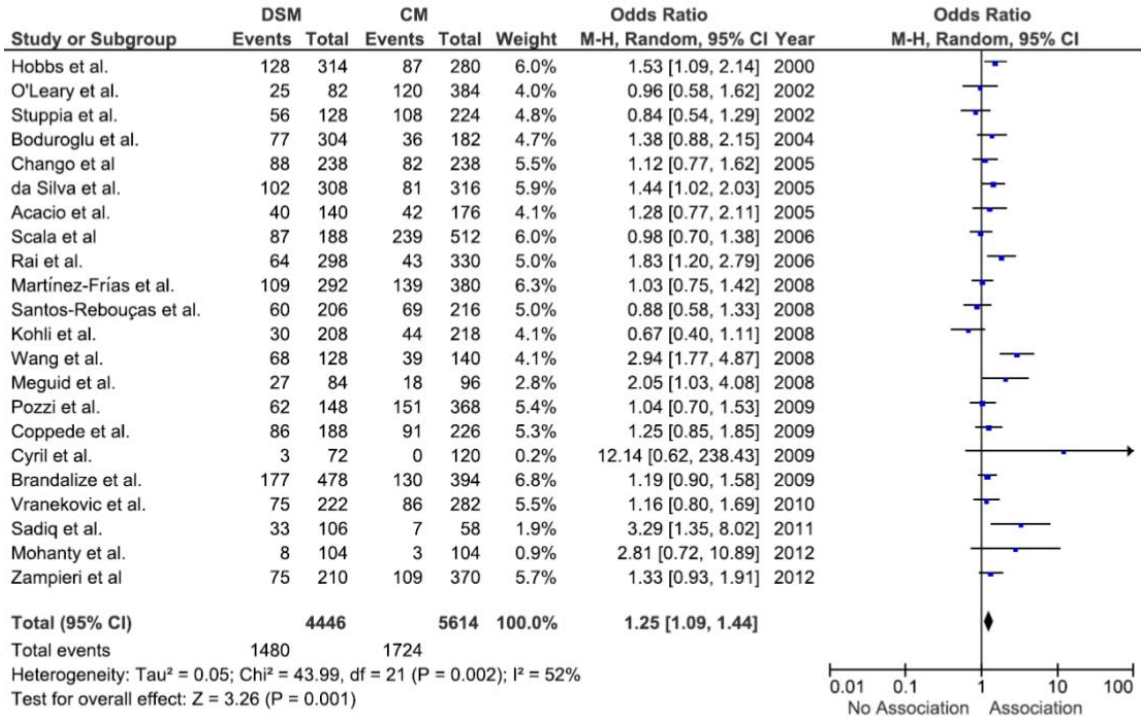

Figure 3 Random-effects (DerSimonian–Laird) meta-analysis of 22 previously published studies, assessing the association between the minor (T) allele of *MTHFR* C677T and DS in 2,223 DSM and 2,807 CM. Overall OR: 1.25 (95% CI 1.09 to 1.44), test for heterogeneity $\chi^2 = 43.99$ (P = 0.002). Each study is shown by the point estimate of the OR and 95% CI. OR = odds ratio; CI = confidence interval. Total: numbers of the allele frequencies of *MTHFR* C677T polymorphism in DSM and CM, respectively. Total events: numbers of the minor (T) allele of *MTHFR* C677T polymorphism in DSM and CM, respectively.

Title: Positive Association between the *Reduced Folate Carrier 1* A80G Polymorphism (*RFC1* A80G) and Maternal Risk for Down Syndrome

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ABSTRACT

Several case-controls studies suggested that the *RFC1* A80G polymorphism may be associated with maternal risk for Down syndrome (DS). However, the results remain inconclusive. We searched electronic databases through January, 2014 for eligible studies. Pooled odds ratios (OR) with 95% confidence intervals (CI) were estimated by fixed or random effects models. Heterogeneity between studies was evaluated using Q test and I2. Publication bias was estimated using Begg's and Egger's tests. A total of 9 case-control studies which comprised 1,070 case mothers and 1,512 controls mothers were included. Q-test results showed homogeneity. The GG vs AA model (OR 1.31, 95% CI 1.04-1.65), the GG/AG vs AA dominant model (OR 1.20, 95% CI 1.00 - 1.44) and the polymorphic G allele (OR 1.13, 95% CI 1.01 – 1.26) were associated with significant maternal risk for DS. Additionally, increased maternal risk for DS was found in the Asians (GG vs AG/AA: OR 2.11, 95% CI 1.13 - 3.94; GG vs AA: OR 2.78, 95% CI 1.30 - 5.95; G vs A: OR 1.50, 95% CI 1.08 - 2.07). Stratified by maternal age less than 35 years at conception, significantly increased maternal risk for DS was found (GG vs AA: OR 1.57, 95% CI 1.05 - 2.33; G vs A: OR 1.22, 95% CI 1.01 - 1.48). No publication bias was found. Our meta-analysis suggested that the *RFC1* A80G polymorphism was associated with maternal risk for DS, even after adjusting the analysis for the maternal age less than 35.

Keywords Down Syndrome; meta-analysis; *SLC19A1* A80G; *RFC1* A80G; folate.

INTRODUCTION

Down Syndrome (DS) is one of the most common chromosomal conditions, characterized by intellectual disability and cognitive delays [Jones, 2007; Contestabile et al., 2010]. This genetic condition results from full or partial (translocation or mosaic) extra copy of chromosome 21 [Ahmed et al., 2005] and it occurs in about one to 890 live births [Loane et al., 2013]. The majority of DS cases is caused by the failure of chromosome segregation during meiosis, and about 90 percent of cases are derived from maternal meiotic errors [Sherman et al., 2005; Freeman et al., 2007; Allen et al., 2009], especially in meiosis I [Allen et al., 2009].

Actually, the causes of chromosome 21 nondisjunction are unknown. Although researchers have shown that the chance of having a baby with DS increases with advanced maternal age [Irving et al., 2008; Melve et al., 2008; Allen et al., 2009, Cocchi et al., 2010], the birth rate of these babies is also high in younger mothers [Eskes et al., 2006]. In 1999, in an attempt to understand the molecular mechanisms underlying this phenomenon, James et al. suggested that the abnormal folate metabolism is associated with DNA hypomethylation, probably at centromeric or peri-centromeric, and with chromosome 21 nondisjunction, consequently [James et al., 2009].

Folate is an essential vitamin required for several metabolic functions such as the synthesis of nucleotide precursors of DNA and RNA, repair of DNA and methylation reactions [Eskes et al., 2006]. The folate metabolism is responsible for synthesis S-adenosyl-methionine (SAM), the main cellular donor of methyl groups for methylation reactions [Finkelstein and Martin, 2000; Eskes et al., 2006]. Once human cells cannot synthesize this factor, folate must be actively transported into cells, and the main responsible for the majority of folate's transportation across intestinal cell membranes is

the solute carrier family 19 (folate transporter), member 1 (SLC19A1, also known as reduced folate carrier 1 - RFC1) [Whetstine et al., 2001; Matherly et al., 2007; Zhao et al., 2011; Zhao and Goldman, 2013]. An important single nucleotide polymorphism at position 80 (A→G) in exon 2 of RFC1 [Whetstine et al., 2001] was related to low levels of RFC1 protein, which is associated to reduced affinity and efficiency in folate transportation [Whetstine et al., 2001] and, consequently, reduced folate plasma levels [Chango et al., 2000; Winkelmayer et al., 2003; Veselá et al., 2005; Devlin et al., 2006].

Several case-controls studies have evaluated the association between *RFC1* A80G polymorphism and maternal risk for DS [Chango et al., 2005; Scala et al., 2006; Coppedè et al., 2006; Ribeiro, 2008; Fintelman-Rodrigues et al., 2009; Brandalize et al., 2010; Neagos et al., 2010; Zampieri et al., 2012; Wang et al., 2013]. Some of these studies showed an association between this polymorphism and maternal risk for DS [Scala et al., 2006; Wang et al., 2013]. However, other studies have found no association [Chango et al., 2005; Fintelman-Rodrigues et al., 2009; Neagos et al., 2010]. Additionally, Coppedè et al. [2006] showed a significant association when this polymorphism is combined with other polymorphisms also involved in folate metabolism such as Brandalize et al. [2010].

These studies have provided initial evidence of the association between *RFC1* A80G polymorphism and maternal risk for DS across different populations. However, the data from these studies have shown conflicting results and the question is still unanswered. Therefore, in the present study, we aggregated published case-controls studies that focused on such an association. We expect to improve the power to detect a precise estimative of association between *RFC1* A80G polymorphism and maternal risk for DS through trans-ethnic systematical review and meta-analysis.

METHODS

Identification and eligibility of relevant studies

To identify all the case-control studies that examined the association between the *RFC1* A80G polymorphism and maternal risk for DS, we conducted a literature research of PUBMED, EMBASE and LILACS (up to January2014) using the MeSH terms and keywords: 'Down syndrome', 'trisomy 21', 'Reduced folate carrier', 'Solute carrier family 19 (folate transporter), member 1','RFC','RFC1','RFC-1','SLC19A1'. Such research was limited to English, Spanish and Portuguese idiom journals. Additional studies were also identified by the references cited in the original studies and review articles. The following criteria were used to select the relevant studies further included in the meta-analysis: (1) if it pertained to the relationship between the polymorphism of *RFC1* A80G and the maternal risk for DS; (2) case–control studies that determined the distributions of the *RFC1* A80G genotypes in case mothers and in a control group of mothers; (3) case mothers (DSM) are considered mothers that gave birth to at least one child with free trisomy 21, and the controls (CM) are mothers that have given birth to children without reported abnormalities; (4) it presented sufficient data to calculate an odds ratio (OR) with 95% confidence interval (CI); (5) for the articles with overlap data for the same population resource, only the latest or largest report was included. In addition, the study authors were contacted directly via email to request any missing data. Family-based association studies were not considered because of different design considerations. Mothers of DS individuals with translocation or mosaicism, case reports, abstracts, letters, comments, editorials, animal studies and review articles were excluded.

Data extraction and quality assessment

All the relevant information was extracted and tabulated by two investigators independently using the same standard. Another reviewer adjudicated the differences between them. The following data retrieved from each study was: the first author's name, the journal, the year of publication, the ethnicity of subjects, the study design, demography characteristics of cases and controls, genotyping method, allele frequencies and genotype distributions in cases and controls and the number of cases and controls.

Statistical analysis

Heterogeneity among included studies was assessed by Chi-square based Q-test [Cochran, 1954]. We also quantified the effect of heterogeneity using I2 = 100%×(Q-df)/Q [Higgins and Thompson, 2002], which ranges from 0 (minimum heterogeneity) and 100% (maximum heterogeneity), and measures the degree of inconsistency in the studies by calculating what proportion of the total variation across studies should be attributed to heterogeneity [Higgins and Thompson, 2002]. The strength of the association between the *RFC1* A80G polymorphism and maternal risk for DS was measured by odds ratios (ORs) with 95% confidence intervals (CIs). We evaluated the association between allele G and maternal risk for DS (G vs A), and made comparisons with the codominant genetic models (AG vs AA and GG vs AA), the dominant genetic model (GG/AG vs AA), and the recessive genetic model (GG vs AG/AA).

The pooled OR was estimated by Mantel-Haenszel method in a fixed-effects model (FE) [Mantel and Haenszel, 1959] or by DerSimonian and Laird method in a random-effects model (RE) [DerSimonian and Laird, 1986] in the absence or in the presence of heterogeneity, respectively. The RE model assumes that different studies

should show substantial diversity and assesses both within-study sampling error and between-study variation [DerSimonian and Laird, 1986]. The FE model assumes that all of the studies are estimating the same underlying effect and considers only within-study variance [Mantel and Haenszel, 1959]. The associations with P-value ≤ 0.05 were considered as being statistically significant. Subgroup analyses were performed by ethnicity and maternal age less than 35 years at conception for DSM and CM. Sensitivity analysis was performed by limiting the meta-analysis to studies conformed to Hardy-Weinberg equilibrium (HWE). The HWE among the controls was estimated by chi-square test to compare the observed genotype frequencies with the expected ones and violations of HWE were defined as P-value ≤ 0.05.

Publication bias was examined visually by a Begg's test (funnel plot method) [Begg and Mazumdar, 1994], in which the standard error of log (OR) of each study was plotted against its log (OR). If there was publication bias, the funnel plot would be asymmetric. Funnel plot asymmetry was further assessed by Egger's linear regression test [Egger et al., 1997], which measures funnel plot asymmetry on the natural logarithm scale of the OR and rank correlation. P-value ≤ 0.05 was considered statistically significant. Software Review Manager 5.2, BioEstat 5.3 and StatsDirect 1.9.15 was used for all analyses.

RESULTS

Study characteristics

A total of 27 potential studies were identified. Of these, 12 studies were excluded after screening the titles and abstracts. The full-text studies were retrieved for a detailed assessment. Six were excluded for specific reasons (4 studies with overlap data of the same population resource and 2 studies with Down syndrome individuals as cases). Finally, 9 case-control studies [Chango et al., 2005; Scala et al., 2006; Coppedè et al., 2006; Ribeiro, 2008; Fintelman-Rodrigues et al., 2009; Brandalize et al., 2010; Neagos et al., 2010; Zampieri et al., 2012; Wang et al., 2013], with a total number of 1,070 DSM and 1,512 CM, were included in the *RFC1* A80G meta-analysis and are illustrated in Figure 1. As presented in Table I, 3 studies were conducted in Europe, 4 studies in South America and 2 studies in Asia. In addition, in 4 of the studies the following condition was observed: both case mothers' age (at conception of the DS child) and control mothers' age (at conception of their last child) were lower than 35 years [Coppedè et al., 2006; Fintelman-Rodrigues et al., 2009; Zampieri et al., 2012; Wang et al., 2013].

Most of the studies reported that CM was composed by women who had no experience with miscarriages [Scala et al., 2006; Coppedè et al., 2006; Ribeiro, 2008; Fintelman-Rodrigues et al., 2009; Neagos et al., 2010; Zampieri et al., 2012; Wang et al., 2013]. On the other hand, two studies did report CM who had previously experienced miscarriages [Chango et al., 2005; Brandalize et al., 2010]. From the nine studies included in this meta-analysis, only one study reports the parental origin of the extra chromosome 21 [Chango et al., 2005].

All included studies used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping method to determine the genetic polymorphism [Chango et al., 2000; Winkelmayer et al., 2003; Födinger et al., 2003; Ananth et al., 2008].

The results of HWE test for the distribution of the genotypes in the control group are presented in Table I. All the included studies were consistent with HWE, except for the controls in Neagos's study [Neagos et al., 2010]. A list of the main characteristics extracted from the included studies in the meta-analysis is summarized in Table I, including the distributions of genotypes and alleles in the DSM and CM.

Evidence synthesis

Since the Q-test of heterogeneity was always not significant, we conducted the meta-analysis using FE model for the comparisons. As shown in Table II and Figure 2A, increased maternal risk for DS was also observed when we compared the GG/AG vs AA dominant model (OR 1.20, 95% CI 1.00 - 1.44). Additionally, the polymorphic homozygote genotype GG was associated with significantly maternal risk for DS (OR 1.31, 95% CI 1.04 - 1.65), compared with the wild-type homozygote genotype AA (Table II and Figure 2B). Moreover, the polymorphic G allele of *RFC1* A80G was associated with significant maternal risk for DS (OR 1.13, 95% CI 1.01 – 1.26), when compared with the wild-type A allele (Table II and Figure 2C). When we stratified by ethnicity, increased maternal risk for DS was found in the Asian populations (GG vs AG/AA: OR 2.11, 95% CI 1.13 - 3.94; GG vs AA: OR 2.78, 95% CI 1.30 - 5.95; G vs A: OR 1.50, 95% CI 1.08 - 2.07) (Table II).

We also performed the analysis stratified by maternal age less than 35 years at conception. There were significant associations for the GG vs AA codominant (OR 1.57, 95% CI 1.05 - 2.33) (Table III and Figure 3A) and allelic (OR 1.22, 95% CI 1.01 - 1.48) (Table III and Figure 3B) comparisons when we limited the DSM and CM by maternal age.

Sensitivity analysis

A sensitivity analysis was performed. It consisted of excluding the study which showed genotypes distribution in CM not conformed to HWE. After excluding this study, the results for overall analyses did not changed substantially and statistically significant associations between maternal risk for DS and *RFC1* A80G polymorphism were observed for these analyses, except for the GG/AG vs AA dominant model (OR 1.19, 95% CI 0.99 - 1.43) (Table II).

Publication bias

Begg's test (funnel plot method) [Begg and Mazumdar, 1994] and Egger's linear regression test [Egger et al., 1997] were performed to assess the publication bias of the included studies. The shapes of the funnel plots do not revealed any visual evidence of asymmetry in all comparisons. In addition, the Egger's test also provided no statistical evidence of funnel plot symmetry and, consequently, no publication bias was found in the included studies (Table II).

DISCUSSION

In order to estimate the association between *RFC1* A80G polymorphism and maternal risk for DS in different ethnic populations, we performed a meta-analysis of 9 eligible studies including 1,070 DSM and 1,512 CM. Overall, our results demonstrated significant associations between *RFC1* A80G polymorphism and maternal risk for DS in several genetic models when all the populations were analyzed. When we stratified by ethnicity, increased maternal risk for DS was observed in the Asians, as well as when we limited the DSM and CM by considering maternal age lower than 35 years at conception. In addition, the association was well supported by the sensitivity analysis and no evidence of publication bias was observed in our study.

Even small reductions in folate levels, according to some studies, may well impact on a variety of diseases such as neural tube defects, cancer or cardiovascular disease [Pietrzik and Bronstrup, 1997; Molloy and Scott, 2001]. However, it has not yet been cleared how reduced folate status may influence these conditions. Folate is one of the members of the B vitamins family and it is essential for the synthesis of SAM, the major cellular methyl donor for methylation of DNA [Finkelstein and Martin, 2000; Eskes et al., 2006].

DNA methylation is a key event in various cellular processes including gene expression, gene integrity [Issa, 1999], conformational configuration and structural stability of DNA [Lewis and Bird, 1991; Linhart et al., 2009; Williams and Jacobson, 2010], binding of transcription factors and other proteins, mutagenesis and imprinting [Hoffmann and Schulz, 2005]. Researchers attested that global DNA hypomethylation caused by folate deficiency [Jacob et al., 1998; Rampersaud et al., 2000; Pufulete et al., 2005; Wasson et al., 2006], may generate strand breaks, mutagenesis through alterations

in chromatin conformation, thus promoting genomic instability [Antequera et al., 1989; Martienssen and Richards, 1995; Wang et al., 2004; Beetstra et al., 2005]. Such DNA instability may predispose to abnormal chromosome segregation [Blount et al., 1997; Hobbs et al., 2002; Fenech et al., 2011], and consequently to chromosome 21 aneuploidy [Wang et al., 2004; Beetstra et al., 2005; Eskes et al., 2006; Linhart et al., 2009; Fenech et al., 2011; Chang et al., 2011].

So, based on the above, James et al. [James et al., 1999] suggested that the occurrence of chromosome 21 nondisjunction in young mothers is associated with centromeric and peri-centromeric hypomethylation due to abnormal folate metabolism, and it is secondary to polymorphism of genes in folate metabolism. In order to be metabolized into an active form, as known 5-methyltetrahydrofolate (5-MTHF), folate needs to be transported into the cells by the RFC1 [Whetstine et al., 2001; Matherly et al., 2007; Zhao et al., 2011; Zhao and Goldman, 2013]. There it sustains key metabolic reactions [Zhao et al., 2011; Zhao and Goldman, 2013]. *RFC1* A80G polymorphism leads to the substitution of a histidine by an arginine [Chango et al., 2000; Whetstine et al., 2001]. Researchers demonstrated that such substitution has impacted in low levels of RFC1 expression, which is associated with a reduction in substrate affinities and/or transport efficiencies [Whetstine et al., 2001]. Additionally, such a deficiency may contribute to disease states associated with decreasing levels of folate [Whetstine et al., 2001] as well as with physiological and developmental problems related to folate deficiency [Hou and Matherly, 2009], such as fetal abnormalities [Butterworth and Bendich, 1996] and chromosome 21 aneuploidy [Wang et al., 2004; Beetstra et al., 2005]. Then, the *RFC1* A80G polymorphism could lead to downstream methylation reactions conducting to DNA hypomethylation and consequently to the reduction of

methyl groups. The final impact may be abnormal chromosome segregation [Blount et al., 1997; Hobbs et al., 2002; Fenech et al., 2011], and occurrence of trisomy of 21.

Consistent with the observations made in the above-mentioned functional studies, our results indicated that polymorphic genotypes (GG/AG and GG) and polymorphic G allele were associated with a significantly increased maternal risk for DS in several genetic models. In the subgroup analysis by ethnicity, we found significant association between *RFC1* polymorphism and maternal risk for DS in Asians but not in Europeans and South Americans. Some factors as ethnic and environmental differences may partly explain this phenomenon. Moreover, differences in diet, such as folate intake, may be also an important cause of this result. Since the sample size was too limited and studies with small sample size may have insufficient statistical power to detect a slight effect [Wacholder et al., 2004], we must considerate the observed association very carefully and other studies are required in order to improve the precision of the results.

Although researchers have shown that the chance of having a baby with DS increases with advanced maternal age (35 years or older) [Irving et al., 2008; Melve et al., 2008; Allen et al., 2009, Cocchi et al., 2010], the birth rate of these babies is high in younger mothers. In the subgroup analysis that considered maternal age at conception lower than 35 years, significantly increased maternal risk for DS was found among the studies for the *RFC1* A80G polymorphism. Then, our findings suggest a possible role of *RFC1* A80G polymorphism in the maternal risk for DS pregnancies in young mothers. It is essential to warn that our results derived from relatively small sample size populations. So they must not be considered as a conclusive analysis, but only as an indicative of possible associations that require further confirmation through larger and more adequately powered designed studies.

There are two similar meta-analyses about the association between the *RFC1* A80G polymorphism and maternal risk for DS. These meta-analyses were based on different groups of studies, as well as on distinct sample sizes. There are some differences between the Yang's study [Yang et al., 2013] and our study. Moreover, we carried out some independent and original analyses, such as comparisons by genetic models. We demonstrated important results in these analyses, since the dominant (GG/AG vs AA), codominant (GG vs AA) and allelic (G vs A) comparisons showed significant associations between the *RFC1* A80G polymorphism and maternal risk for DS, while they only observed a significant association between G allele and maternal risk for DS.

Another meta-analysis, performed almost at the same time, was conducted by Coppedè et al. [2013]. In their study, the subgroup analysis by ethnicity was not performed, but it was included in our meta-analysis and showed a significant association between the *RFC1* A80G polymorphism and maternal risk for DS in Asians. We also conducted stratified analyses by codominant models, and important and significant association results were observed between polymorphic homozygote genotype GG and maternal risk for DS. However, such an analysis was not made in the Coppedè et al. article [2013]. In addition, our meta-analysis included all the published studies and one additional study, which included 200 DSM and 340 CM and accounted for 21% of the total sample size. Finally, our meta-analysis performed a subgroup analysis considering mothers who were younger than 35 years old at conception. Since the only well-established maternal risk for DS is advanced maternal age at conception (35 years or older) [Irving et al., 2008; Melve et al., 2008; Allen et al., 2009, Cocchi et al., 2010], our attempt to investigate this dynamics disconnecting it from the advanced

maternal age at conception premise grows in importance. Thus, compared with previous meta-analyses, we generate more precise and conclusive results of the association between the *RFC1* polymorphism and maternal risk for DS. Thus our results were more precise and persuasive.

Although we have increased the statistical power to detect the associations by combining the data of individual studies, there are still some limitations we must describe. First of all, due to difficulties related to accessing the full texts of studies published in other languages, we decided to include only studies published in English, Spanish and Portuguese. Second, although we have collected all the eligible studies, the results of the subgroup stratification analysis must be carefully interpreted due to the limited number of published studies. Actually as suggested by Hannah and colleagues [2005], the study power is low if the number of studies included in a meta-analysis is 10 or less [Hannah et al., 2005]. Third, analyses of multiples interaction between gene-gene and gene-environment were not performed due to the lack of detailed original data in the included studies in this meta-analysis. It is reasonable to consider that specific environmental and lifestyle factors may alter these associations between genetic polymorphisms and maternal risk for DS.

Nevertheless, our meta-analysis has shown several advantages. First, a meta-analysis of the association between *RFC1* A80G polymorphism and maternal risk for DS is statistically more powerful than any single study. Second, our strict inclusion criteria are satisfactorily assured by the great quality of the studies included in our meta-analysis. Third, the distribution of genotype in CM followed the HWE in all studies except for one study [Neagos et al., 2010]. When excluding this study, the pooled OR was not significantly changed indicating that the control group could represent the base

population. Finally, our results were based on a more precise analysis because we evaluated single-factor estimations with adjustment the data for other risk factors such as advanced maternal age.

In conclusion, based on evidence that abnormal folate metabolism can lead to abnormal chromosomal segregation, this meta-analysis indicates that individuals with polymorphic allele and genotypes of the *RFC1* A80G polymorphism have an associated increased maternal risk for DS. This result comes along even after adjusting the analysis for the maternal age less than 35. Further studies with larger sample sizes and well-matched controls are required to validate this conclusion. Moreover, future studies that consider potential gene-gene and gene-environment interactions may lead to a deeper knowledge about the *RFC1* A80G polymorphism in maternal risk for DS.

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Table I. Information of the Included Studies that Investigated the Relationship Between *Reduced Folate Carrier 1* A80G polymorphism and Down Syndrome

First Author	Year	Country	Geographical Location	DSM[a]	CM[b]	GG		AG		AA		G		A		P_{HWE}[c]
						DSM	CM	DSM	CM	DSM	CM	DSM	CM	DSM	CM	
Chango et al.	2005	France	Europe	119	94	29	26	66	52	24	16	124	105	114	83	0.24
Scala et al.	2006	Italy	Europe	94	263	26	48	41	113	27	102	93	209	95	317	0.09
Coppede et al.	2006	Italy	Europe	69	93	27	31	29	42	13	20	83	104	55	82	0.41
Ribeiro et al.	2008	Brazil	South America	200	340	53	101	106	163	41	76	212	367	188	313	0.50
Fintelman-Rodrigues et al.	2009	Brazil	South America	114	110	25	26	64	55	25	29	114	112	114	108	0.99
Brandalize et al.	2010	Brazil	South America	239	197	65	42	101	91	73	64	229	173	249	221	0.36
Neagos et al.	2010	Romania	Asia	26	46	9	11	16	30	1	5	34	52	18	40	0.02
Zampieri et al.	2012	Brazil	South America	105	185	28	44	48	88	29	53	104	176	106	194	0.52
Wang et al.	2013	China	Asia	104	184	16	13	41	71	47	100	73	97	135	271	0.93

[a]DSM, number of case mothers

[b]CM, number of control mothers

[c]HWE, Hardy-Weinberg equilibrium

Table II. Pooled estimates and stratified analysis for the associations between *Reduced Folate Carrier 1* A80G polymorphism and Down syndrome

Polymorphism	Comparison	Population	Study (n)	Test of Association		Test of Heterogeneity			Tests of Publication Bias (P-value)	
				OR (95%CI)	P-value (Z test)	χ^2	I^2	P-value	Rank test (Begg & Mazumdar)	Linear regression (Egger et al)
RFC1 A80G	GG / AG vs AA	All	9	**1.20 (1.00 – 1.44)** [a]	0.05	4.10	0%	0.85	0.47	0.71
		All in HWE	8	1.19 (0.99 – 1.43) [a]	0.06	3.40	0%	0.85	0.17	0.21
		Europe	3	1.24 (0.86 – 1.77) [a]	0.25	2.25	11%	0.32	-	-
		South America	4	1.12 (0.88 – 1.42) [a]	0.36	0.24	0%	0.97	0.75	0.61
		Asia	2	1.51 (0.94 – 2.41) [a]	0.09	0.42	0%	0.52	-	-
	GG vs AG / AA	All	9	1.17 (0.97 – 1.42) [a]	0.09	10.4	23%	0.24	0.35	0.37
		All in HWE	8	1.16 (0.96 – 1.40) [a]	0.12	9.95	30%	0.19	0.54	0.50
		Europe	3	1.25 (0.88 – 1.77) [a]	0.21	2.84	30%	0.24	-	-
		South America	4	1.05 (0.83 – 1.33) [a]	0.69	2.87	0%	0.41	0.33	0.66
		Asia	2	**2.11 (1.13 – 3.94)** [a]	0.02	0.28	0%	0.60	-	-
	AG vs AA	All	9	1.14 (0.93 – 1.38) [a]	0.20	2.76	0%	0.95	0.61	0.53
		All in HWE	8	1.13 (0.92 – 1.37) [a]	0.24	2.19	0%	0.95	0.90	0.82
		Europe	3	1.13 (0.77 – 1.67) [a]	0.54	1.09	0%	0.58	-	-
		South America	4	1.12 (0.90 – 1.41) [a]	0.31	1.10	0%	0.89	0.33	0.57
		Asia	2	1.29 (0.78 – 2.13) [a]	0.32	0.44	0%	0.51	-	-
	GG vs AA	All	9	**1.31 (1.04 – 1.65)** [a]	0.02	9.07	12%	0.34	0.76	0.42
		All in HWE	8	**1.29 (1.02 – 1.62)** [a]	0.03	8.14	14%	0.32	0.90	0.68
		Europe	3	1.37 (0.89 – 2.12) [a]	0.15	3.63	45%	0.16	-	-

	South America	4	1.24 (0.87 – 1.78) [a]	0.23	0.23	0%	0.89	075	0.99
	Asia	2	**2.78 (1.30 – 5.95) [a]**	0.009	0.13	0%	0.72	-	-
G vs A	All	9	**1.13 (1.01 – 1.26) [a]**	0.04	9.92	19%	0.27	0.91	0.45
	All in HWE	8	**1.12 (1.00 – 1.26) [a]**	0.05	9.41	26%	0.22	0.54	0.65
	Europe	3	1.18 (0.94 – 1.46) [a]	0.15	4.42	55%	0.11	-	-
	South America	4	1.04 (0.90 – 1.21) [a]	0.56	1.39	0%	0.71	0.75	0.94
	Asia	2	**1.50 (1.08 – 2.07) [a]**	0.02	0.01	0%	0.92	-	-

Abbreviations: OR, Odds Ratio; CI, Confidence Interval.

[a]Fixed-effect model.

- insufficient strata.

Bold values indicate significant associations.

Table III. Pooled estimates and stratified analysis by maternal age less than 35 years at conception for the associations between *Reduced Folate Carrier 1* A80G polymorphism and Down syndrome

SubgroupVariable	Comparison	Test of Association		Test of Heterogeneity		
		OR (95%CI)	P-value (Z test)	χ^2	I^2	P-value
Maternal age less than 35 years	GG / AG vs AA	1.32 (0.98 – 1.78)[a]	0.07	0.23	0%	0.97
	GG vs AG / AA	1.35 (0.97 – 1.87)[a]	0.07	3.72	19%	0.29
	AG vs AA	1.21 (0.88 – 1.67)[a]	0.24	0.23	0%	0.97
	GG vs AA	**1.57 (1.05 – 2.33)[a]**	0.03	2.43	0%	0.49
	G vs A	**1.22 (1.01 – 1.48)[a]**	0.04	2.93	0%	0.40

Abbreviations: OR, Odds Ratio; CI, Confidence Interval.

[a]Fixed-effect model.

Bold values indicate significant associations.

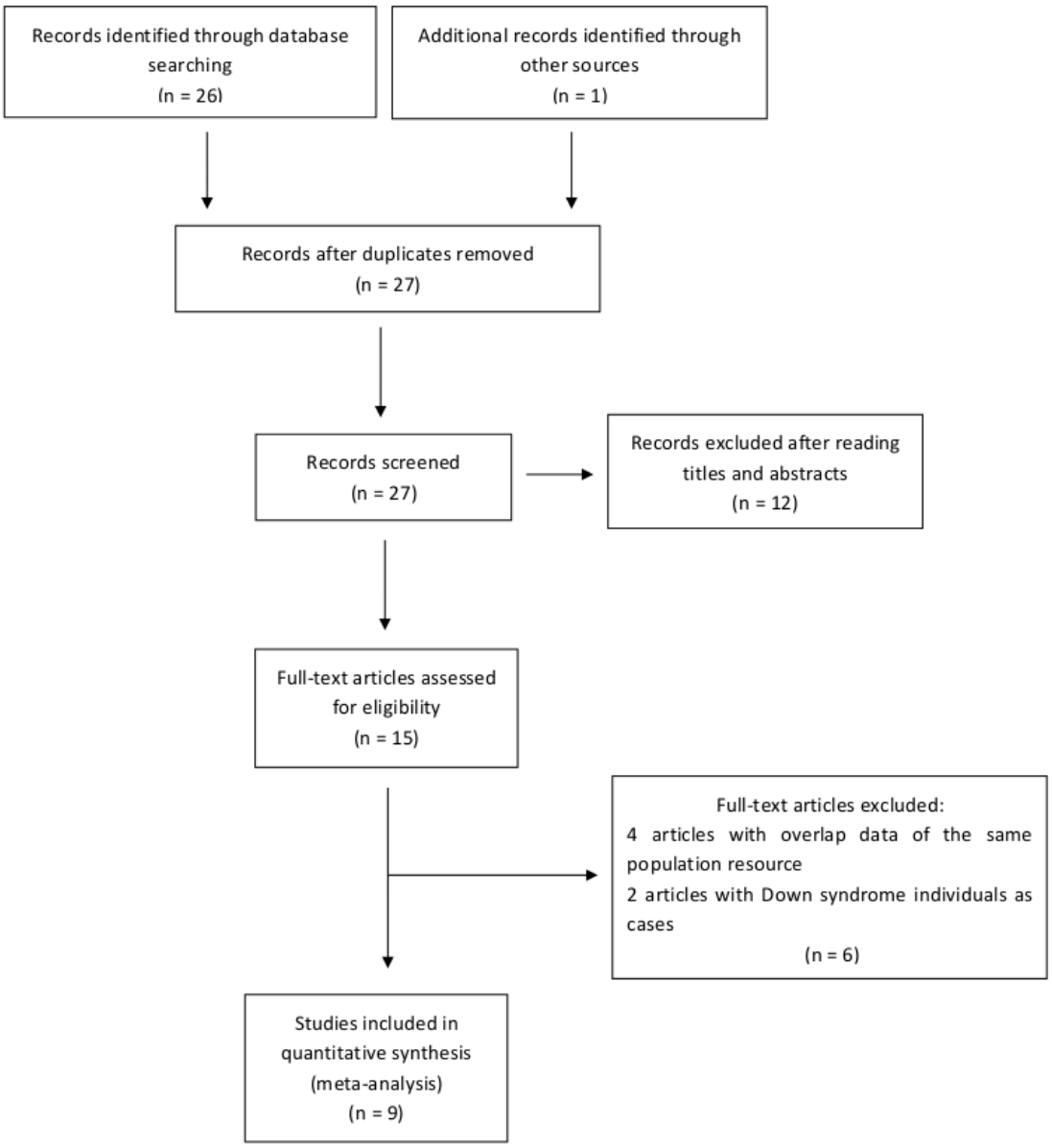

Figure 1. Flow chart of eligible study selection process and studies excluded, with specification of reasons.

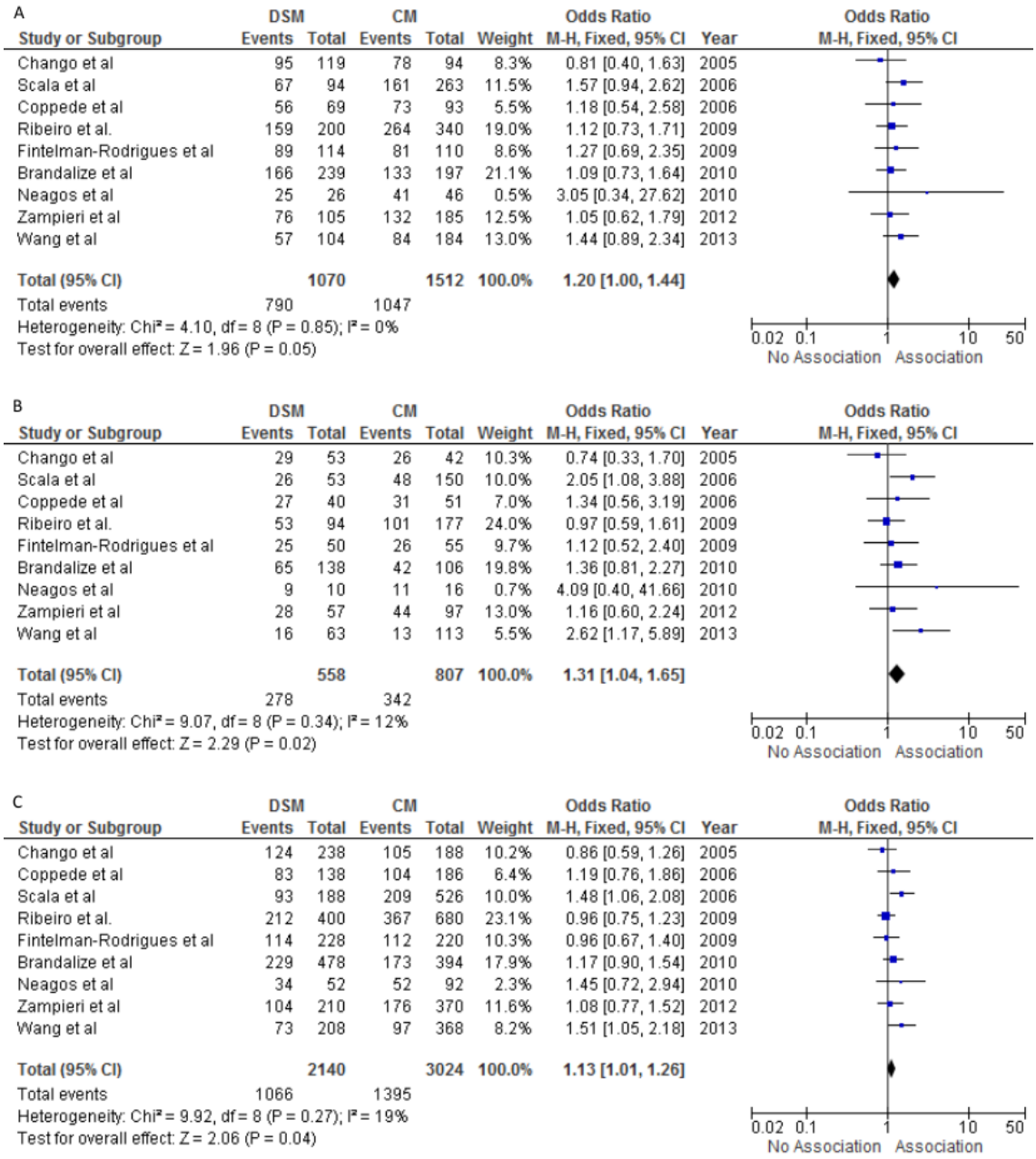

Figure 2. Forest plots of the association between *RFC1* A80G polymorphism and DS under the dominant (A), codominant (B) and allelic (C) genetic models in overall population using a fixed-effects model. Abbreviation: DSM, Down syndrome mothers; CM, control mothers; M-H, Mantel-Haenszel method.

Title: Genetic polymorphisms involved in folate metabolism and maternal risk for Down syndrome: a meta-analysis

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ABSTRACT

Inconclusive results of the association between genetic polymorphisms involved in folate metabolism and maternal risk for Down syndrome (DS) have been reported. Therefore, this meta-analysis was conducted. We searched electronic databases through May, 2014 for eligible studies. Pooled odds ratios with 95% confidence intervals were used to assess the strength of the association, which was estimated by fixed or random effects models. Heterogeneity among studies was evaluated using Q-test and I^2 statistic. Subgroup and sensitivity analyses were also conducted. Publication bias was estimated using Begg's and Egger's tests. A total of 17 case-controls studies were included. There was evidence for an association between the *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS. In the subgroup analysis, increased maternal risk for DS was found in Caucasians. Additionally, the polymorphic heterozygote *MTHFD1* 1958GA genotype was associated with significantly maternal risk for DS, when we limit the analysis by studies conformed to Hardy-Weinberg equilibrium. Finally, considering *MTR* c.2756A>G (rs1805087), *TC2* c.776G>C (rs1801198) and *CBS* c.844ins68, no significant associations have been found, neither in the overall analyses nor in the stratified analyses by ethnicity. In conclusion, our meta-analysis suggested that the *MTRR* c.66A>G (rs1801394) polymorphism and *MTHFD1* c.1958G>A (rs2236225) were associated with increased maternal risk for DS.

Keywords Down Syndrome; meta-analysis; *MTR* c.2756A>G (rs1805087); *MTRR* c.66A>G (rs1801394); *TC2* c.776G>C (rs1801198); *CBS* c.844ins68; *MTHFD1* c.1958G>A (rs2236225); folate metabolism.

INTRODUCTION

Down Syndrome (DS) is the phenotypic manifestation of trisomy of human chromosome 21 and is the most common genetic disorder of intellectual disability, characterized by dysmorphic features that are usual to almost all affected individuals, including craniofacial abnormalities and hypotonia [1, 2]. As reported, the average prevalence is 1 in 660 [3] and in the majority of DS cases (90%), the chromosomal nondisjunction event is of maternal origin, occurring mainly during meiosis I in the maturing oocyte [4].

In a several studies, advanced maternal age at conception (35 years or older) has been associated with increased risk of DS births in various parts of the world [5, 6, 7]. However, several women younger than 35 years at conception have had DS children are also found to be predisposed to early chromosomal nondisjunction [8, 9, 10]. In 1999, James et al [11] were the first to suggest a role for the abnormal folate metabolism in chromosome 21 nondisjunction as elevated maternal risk for DS, independent of maternal age.

Methionine synthase (MTR), methionine synthase reductase (MTRR), transcobalamin 2 (TC2), cystathionine beta synthase (CBS) and methylenetetrahydrofolate dehydrogenase (MTHFD1) are very important enzymes involved in folate/homocysteine (Hcy) metabolism and play essential roles in synthesis and repair of DNA and methylation reactions [12]. The methylation of Hcy to methionine is catalyzed by MTR using cobalamin (vitamin B12) as a cofactor, in which the MTR may become inactivated due to the oxidation of cobalamin cofactor [10, 13, 14]. The transmembrane transport of cobalamin is mediated by cobalamin-transporting proteins, such as transcobalamin 2 (TC2) [15]. Regeneration of inactive form of MTR

into its active form requires reductive methylation of vitamin B12 via a reaction catalyzed by MTRR in which S-adenosylmethionine (SAM) is used as a methyl donor [10, 13, 14].

Cystathionine β-synthase (CBS), an enzyme involved in the transsulfuration cycle, is responsible to metabolize Hcy into cystathionine, a middle step in the synthesis of cysteine [16]. Additionally, methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*), a trifunctional nicotinamide adenine dinucleotide phosphate-dependent cytoplasmic enzyme, catalyzes the sequential interconversion of tetrahydrofolate (THF) into the corresponding 10-formyl-THF, 5,10-methenyl-THF and 5,10-methylene-THF [17], which play a important role in *de novo* purine and pyrimidine biosynthesis and, thus, the synthesis of DNA [18].

Genetic polymorphisms in key enzymes of folate metabolism have been identified in the alteration of the levels of folate and Hcy [19], in the enzyme activity decrease and also in the Hcy remethylation rate [14, 20]. Therefore, changes in folate levels may influence the DNA stability and integrity [21, 22] or affect the methylation patterns and, thus, predispose it to the development of DS [10, 22-24].

Considering the functional effects of the *MTR* c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CBS* c.844ins68 and *MTHFD1* c.1958G>A (rs2236225) polymorphisms, it is expected that these polymorphisms may be associated with the maternal DS risk and several studies have been carried out to determine this association. However, the results remain inconclusive. To explain these issues, we conducted a systematical review and a meta-analysis from all eligible studies, in order to provide more exact estimate of the association among *MTR* c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CBS*

c.844ins68 and *MTHFD1* c.1958G>A (rs2236225) polymorphisms and the maternal risk for DS.

METHODS

Search strategy

A systematic review of literature was performed in PubMed, EMBASE and Lilacs-Scielo databases (last search update, May 2014). The keywords and subject terms used were as follows: (Down syndrome or trisomy 21) and (methionine synthase or methione synthase reductase or transcobalamin or cystathionine beta synthase or methylenetetrahydrofolate dehydrogenase or MTR or MTRR or CBS or TC2 or TCII or MTHFD1 or MTHFD-1 or A2756G or A66G or C776G or 844ins68 or G1958A). The reference lists of the retrieved studies were also screened in order to identify extra articles on this same topic. This research only included papers published in English, Spanish or Portuguese.

Inclusion and exclusion criteria

The following inclusion criteria were used: (a) case-control studies design; (b) association studies that evaluated the association between *MTR* c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CBS* c.844ins68 or *MTHFD1* c.1958G>A (rs2236225) polymorphisms and the maternal risk for DS in case mothers (DSM) and in a control group of mothers (CM); (c) DSM are considered mothers that gave birth to at least one child with DS, and the CM are mothers that have given birth to children without reported abnormalities; (d) studies with detailed genotype and allele frequencies of the DSM and CM or with sufficient

data to calculate them; (e) for articles published by the same population resource or by the same research group, only the article with the largest sample size or most recent study was included in this meta-analysis. Studies with insufficient data, review articles, abstracts, editorials, comments, letters, case reports and animal studies were excluded.

Data extraction and quality assessment

The data were extracted by two reviewers independently. Other reviewer was required in order to resolve the differences between them. The information extracted from each study includes the following: the first author's name, the publication's year, country, ethnicity, demography characteristics, genotyping method, genotype and allele frequencies and the number of DSM and CM.

Statistical analysis

A chi-square test was used to estimate the Hardy-Weinberg equilibrium (HWE) among the control subjects. The maternal risk was evaluated through the following comparisons: (1) allelic model (polymorphic allele versus wild-type allele); (2) codominant models (heterozygous versus wild-type homozygous and polymorphic homozygous versus wild-type homozygous); (3) dominant model (heterozygotes and homozygotes for the polymorphic allele versus wild-type homozygous); (4) recessive model (polymorphic homozygous versus heterozygotes and homozygotes for the wild-type allele). Subgroup analyses based on different ethnic populations (Caucasian, Brazilian and Asian) were also performed. Additionally, sensitivity analysis was used in order to examine the results stability by omitting one study at a time.

The pooled OR was estimated using fixed-effects (FE) [25] and random-effects (RE) [26] models according to heterogeneity. Heterogeneity among studies was calculated using the Chi-square based Q-test [27]. The effect of heterogeneity was also quantified using I^2 statistic [28], which ranges between 0 and 100%. When an absence of heterogeneity between studies was detected, the Mantel-Haenszel method in a FE model was used. In contrast, when heterogeneity between studies was present, the DerSimonian and Laird method in a RE model was adopted. The associations were indicated as a pooled odds ratio (OR) and 95% confidence intervals (CI).

Publication bias was examined by funnel plot method, in which the standard error of log (OR) of each study was plotted against its log (OR). The asymmetry in funnel plot is detected when publication bias is present. Funnel plot asymmetry was also determined by Begg's test [29] and Egger's linear regression test [30]. $P \leq 0.05$ was considered statistically significant. Data analyses were performed using the Cochrane systematic review software Review Manager 5.2, BioEstat 5.3 and StatsDirect 1.9.15.

RESULTS

Quantitative Data Synthesis

The literature search identified 116 potentially relevant studies; of these, 88 were excluded after screening the titles and abstracts. The full-text studies were retrieved for a detailed assessment. Eleven studies were excluded for specifying reasons (6 articles with overlap data of the same population resource, 2 articles with Down syndrome individuals as cases, 3 articles with insufficient data). Finally, 17 case-control studies [31-47] with a total number of 1,988 DSM and 2,739 CM, were included in the *MTR*

c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CBS* c.844ins68 and *MTHFD1* c.1958G>A (rs2236225) meta-analysis (Figure 1).

Studies were conducted in different ethnic populations: seven involved Caucasian [31-33, 35, 41, 42, 46], seven Brazilian [34, 37-40, 43, 45] and three Asian [36, 44, 47]. Some of the articles reported that CM was composed by women who had no experience with miscarriages [31, 34, 36-42, 44-47], while others articles did not bring any information about miscarriages [32, 35]. On the other hand, two studies did report CM who had previously experiences with miscarriages [33, 43]. From the seventeen studies included in this meta-analysis, only two study reports of the parental origin of the extra chromosome 21 [37, 41]. The distribution of genotypes in the control groups of all the eligible studies were in agreement with HWE except for Chango et al (χ^2 = 12.18, P = 0.0005) [33] and Ribeiro et al (χ^2 = 70.5, P < 0.0001) [38] in the *MTRR* c.66A>G (rs1801394), for Ribeiro et al (χ^2 = 4.14, P = 0.04) [38] and Liao et al (χ^2 = 4.23, P = 0.03) [47] in the *TC2* c.776G>C (rs1801198) and for Scala et al (χ^2 = 3.71, P = 0.05) [35] in the *MTHFD1* c.1958G>A (rs2236225) polymorphism. A list of the details extracted from the studies included in the meta-analysis is provided in Table 1.

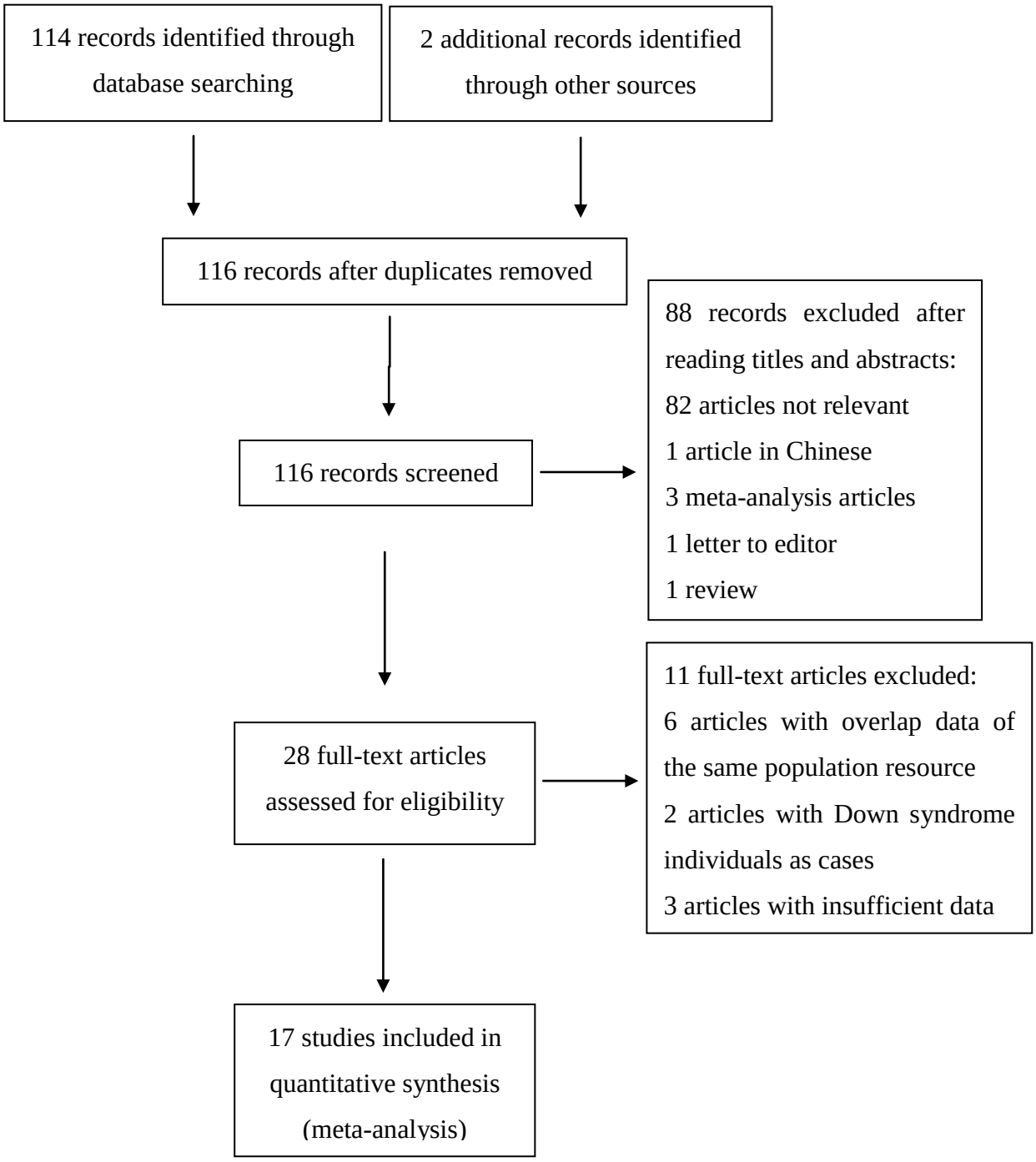

Figure 1. Flow diagram of eligible study selection process and studies excluded, with specification of reasons.

Table 1. Characteristics of the Included Studies in meta-analysis.

First Author	Year	Ethnicity	DSM[a]	CM[b]	Polymorphisms studied and included in meta-analysis	Genotype analysis
Hobbs et al [31]	2000	Caucasian	145	139	*MTRR* c.66A>G	PCR/RFLP
O'Leary et al [32]	2002	Caucasian	48	192	*MTRR* c.66A>G	PCR/RFLP
Chango et al [33]	2005	Caucasian	119	120	*MTRR* c.66A>G / *MTR* c.2756A>G / *CBS* c.844ins68	PCR/RFLP
da Silva et al [34]	2005	Brazilian	154	158	*MTRR* c.66A>G / *MTR* c.2756A>G / *CBS* c.844ins68	PCR/RFLP
Scala et al [35]	2006	Caucasian	93	257	*MTRR* c.66A>G / *MTR* c.2756A>G / *CBS* c.844ins68 / *MTHFD1* c.1958G>A	PCR/RFLP
Wang et al [36]	2008	Asian	64	70	*MTRR* c.66A>G	PCR/RFLP
Santos-Rebouças et al [37]	2008	Brazilian	103	108	*MTRR* c.66A>G	PCR/RFLP
Ribeiro et al [38]	2008	Brazilian	200	340	*MTRR* c.66A>G / *MTR* c.2756A>G / *TC2* c.776G>C / *MTHFD1* c.1958G>A	PCR/RFLP
Fintelman-Rodrigues et al [39]	2009	Brazilian	114	110	*MTR* c.2756A>G / *CBS* c.844ins68 / *TC2* c.776G>C	PCR/RFLP
Urpia et al [40]	2009	Brazilian	61	102	*MTRR* c.66A>G	PCR/RFLP
Pozzi et al [41]	2009	Caucasian	74	184	*MTRR* c.66A>G	PCR/RFLP
Coppede et al [42]	2009	Caucasian	81	111	*MTRR* c.66A>G	PCR/RFLP
Brandalize et al [43]	2010	Brazilian	239	197	*MTRR* c.66A>G / *MTR* c.2756A>G / *CBS* c.844ins68	PCR/RFLP
Neagos et al [44]	2010	Asian	26	46	*MTHFD1* c.1958G>A	PCR/RFLP
Zampieri et al [45]	2012	Brazilian	105	185	*MTRR* c.66A>G / *MTR* c.2756A>G / *CBS* c.844ins68 / *TC2* c.776G>C / *MTHFD1* c.1958G>A	PCR/RFLP
Coppede et al [46]	2013	Caucasian	286	305	*MTR* c.2756A>G	PCR/RFLP
Liao et al [47]	2014	Asian	76	115	*TC2* c.776G>C / *MTHFD1* c.1958G>A	PCR/RFLP

[a]DSM, case mothers

[b]CM, controls mothers

Meta-analyses, Test of Heterogeneity, Sensitivity and Subgroup Analyses

***MTR* c.2756A>G (rs1805087) polymorphism and the maternal risk for DS**

Firstly, we conducted meta-analysis of the effect of *MTR* c.2756A>G (rs1805087) polymorphism on the maternal risk for DS based on 8 case-control studies [33, 34, 35, 38, 39, 43, 45, 46] including 1,311 DSM and 1,674 CM. The results showed no significant association between all genetic models (Table 2). We then performed the subgroup analysis stratified by ethnicity. The pooled ORs from these analyses were also insignificant (Table 2).

***MTRR* c.66A>G (rs1801394) polymorphism and the maternal risk for DS**

We conducted meta-analysis based on 13 case-control studies [31-38, 40-43, 45], including 1,486 DSM and 2,163 CM. Overall, there was evidence for an association between the *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS in all genetic models (Table 2 and Figure 2), except in the allelic comparison (G vs A). However, there was significant heterogeneity among the studies (Table 2).

A reanalysis was carried out in order to exclude the studies whose control groups were not in HWE [33, 38] to assess the stability of the current analysis. The overall results did not change significantly after removing such studies, except to the AG vs AA comparison (OR = 1.20, 95% CI = 0.99 to 1.47) (Table 2). Additionally, the results showed that there still was heterogeneity among studies for the comparisons of GG / AG vs AA, GG vs AG + AA and GG vs AA in all in HWE (Table 2). Subsequently, we performed subgroup analysis based on different ethnicities. Increased maternal risk for DS was observed in the Caucasian population (GG / AG vs AA: OR = 1.42, 95% CI =

1.08 to 1.88 and GG vs AG / AA: OR = 1.43, 95% CI = 1.13 to 1.83) (Table 2). No association was observed in any of the genetic models in Brazilians (Table 2). Subgroup analysis was not performed for *MTRR* c.66A>G (rs1801394) in Asians because there was only one study included [36]. For the subgroup analysis by Caucasians, no relevant changes in the results emerged from the exclusion of the study whose control group was not in HWE [33].

Sensitivity analysis was also performed and consisted of the analysis of every subgroup obtained by the exclusion of one single study at a time. It focused on checking the effect of each individual study, since the exclusion of a given article may isolate the remaining subgroup from the article's particular effect. None individual study significantly induced the inter-studies heterogeneity observed in the *MTRR* c.66A>G (rs1801394) polymorphism (data not shown). Additionally, the results indicated that no single study influenced the pooled OR qualitatively (data not shown). It suggested that the results of this meta-analysis were stable. For the subgroup analysis by Caucasians, after eliminating the results of O'Leary et al [32] and Scala et al [35], heterogeneity decreased, which indicated that these studies contribute to the heterogeneity in Caucasians. However, despite eliminating the data of these studies, our results did not change (data not show).

TC2 c.776G>C (rs1801198) polymorphism and the maternal risk for DS

Four case-control studies [38, 39, 45, 47] with a total number of 495 DSM and 743 CM were included in this meta-analysis. No significant association between *TC2* c.776G>C (rs1801198) polymorphism and maternal risk for DS was found, neither for

all population nor for all population in HWE (Table 2). Subgroup analysis was not performed due to limited number of studies included.

CβS c.844ins68 polymorphism and the maternal risk for DS

We conducted meta-analysis of the effect of *CβS* c.844ins68 polymorphism on the maternal risk for DS based on 6 case-control studies [33-35, 39, 43, 45], including 825 DSM and 1,034 CM. As presented in Table 2, no significant association was found, neither when considering all population nor for Brazilian subgroup analysis. Subgroup analysis was not performed in Caucasians because there were only two studies included.

MTHFD1 c.1958G>A (rs2236225) polymorphism and the maternal risk for DS

The association between *MTHFD1* c.1958G>A (rs2236225) polymorphism and maternal risk for DS was investigated in 5 studies [35, 38, 44, 45, 47] including 497 DSM and 930 CM. Overall, there was no significant association between *MTHFD1* c.1958G>A (rs2236225) polymorphism and maternal risk for DS when all population is considered. However, the polymorphic heterozygote genotype GA was associated with significantly maternal risk for DS (OR 1.33, 95% CI 1.01 - 1.75), compared with the wild-type homozygote genotype GG when we limit the analysis by HWE (Table 2). Subgroup analysis was not performed due to limited number of studies included.

Publication Bias

The symmetry of funnel plots was examined visually by funnel plot and statistically by Begg's and [29] Egger's tests [30]. Appearances of the shapes of funnel plots were seemed symmetrical in all comparisons. Additionally, Egger's tests also

showed that there was no publication bias ($P > 0.05$) in all comparisons for all polymorphisms analyzed (Table 2).

Table 2. Pooled estimates and stratified analysis for the associations between *MTR* c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CBS* c.844ins68 and *MTHFD1* c.1958G>A (rs2236225) polymorphisms and maternal risk for Down syndrome.

Polymorphism	Comparison	Population	Study (n)	Test of Association		Test of Heterogeneity			Tests of Publication Bias (P-value)	
				OR (95%CI)	P-value (Z test)	χ^2	I^2	P-value	Rank test (Begg & Mazumdar)	Linear regression (Egger et al)
MTR c.2756A>G	GG / AG vs AA	All	8	1.14 (0.97 - 1.33)[b]	0.11	7.01	0%	0.43	0.27	0.20
		Caucasian	3	1.08 (0.84 – 1.40)[b]	0.55	2.69	26%	0.26	-	-
		Brazilian	5	1.17 (0.96 – 1.42)[b]	0.12	4.11	3%	0.39	0.48	0.65
	GG vs AG / AA	All	8	1.18 (0.80 - 1.76)[b]	0.41	3.69	0%	0.81	0.27	0.34
		Caucasian	3	0.80 (0.35 – 1.82)[b]	0.60	0.72	0%	0.70	-	-
		Brazilian	5	1.34 (0.85 – 2.12)[b]	0.21	1.92	0%	0.75	0.23	0.18
	AG vs AA	All	8	1.13 (0.96 - 1.33)[b]	0.15	9.26	24%	0.23	0.06	0.16
		Caucasian	3	1.10 (0.85 – 1.44)[b]	0.46	3.24	38%	0.20	-	-
		Brazilian	5	1.14 (0.93 – 1.40)[b]	0.21	5.99	33%	0.20	0.48	0.58
	GG vs AA	All	8	1.25 (0.84 - 1.88)[b]	0.27	2.97	0%	0.89	0.54	0.43
		Caucasian	3	0.85 (0.37 – 1.94)[b]	0.70	0.61	0%	0.74	-	-
		Brazilian	5	1.42 (0.89 – 2.27)[b]	0.14	1.28	0%	0.86	0.48	0.19
	G vs A	All	8	1.11 (0.97 - 1.26)[b]	0.14	5.33	0%	0.62	0.27	0.29
		Caucasian	3	1.04 (0.83 – 1.31)[b]	0.71	1.82	0%	0.40	-	-
		Brazilian	5	1.14 (0.96 – 1.34)[b]	0.12	3.18	0%	0.53	0.81	0.73
MTRR c.66A>G	GG / AG vs AA	All	13	**1.29 (1.09 - 1.53)[b]**	0.003	18.68	36%	0.10	0.25	0.08
		All in HWE	11	**1.28 (1.00 - 1.65)[a]**	0.05	18.42	46%	0.05	0.21	0.06
		Caucasian	6	**1.42 (1.08 – 1.88)[b]**	0.01	9.39	47%	0.09	0.46	0.31
		Brazilian	6	1.14 (0.91 - 1.42)[b]	0.25	4.87	0%	0.43	0.46	0.89
	GG vs AG / AA	All	13	**1.33 (1.03 - 1.71)[a]**	0.03	26.11	54%	0.01	0.59	0.27
		All in HWE	11	**1.37 (1.00 - 1.88)[a]**	0.05	25.82	61%	0.004	0.44	0.34
		Caucasian	6	**1.43 (1.13 – 1.83)[b]**	0.003	9.44	47%	0.09	0.46	0.64
		Brazilian	6	1.09 (0.86 - 1.37)[b]	0.47	8.35	40%	0.14	0.71	0.44

	AG vs AA	All	13	**1.22 (1.01 - 1.47)[b]**	0.04	12.47	4%	0.41	0.36	0.17
		All in HWE	11	1.20 (0.99 – 1.47)[b]	0.07	12.11	17%	0.28	0.44	0.13
		Caucasian	6	1.31 (0.98 – 1.75)[b]	0.07	7.89	37%	0.16	0.46	0.36
		Brazilian	6	1.11 (0.86 - 1.43)[b]	0.44	2.98	0%	0.70	0.46	0.60
	GG vs AA	All	13	**1.57 (1.07 - 2.31)[a]**	0.02	28.81	58%	0.004	0.30	0.07
		All in HWE	11	**1.61 (1.02 - 2.53)[a]**	0.04	28.72	65%	0.001	0.35	0.07
		Caucasian	6	1.65 (0.95 – 2.88)[a]	0.08	10.87	54%	0.05	> 0.99	0.41
		Brazilian	6	1.16 (0.83 - 1.62)[b]	0.37	9.88	49%	0.08	> 0.99	0.33
	G vs A	All	13	1.18 (0.99 - 1.40)[a]	0.07	35.50	66%	0.0004	0.20	0.12
		All in HWE	11	1.20 (0.96 – 1.49)[a]	0.10	35.38	72%	0.0001	0.28	0.12
		Caucasian	6	1.26 (0.96 – 1.66)[a]	0.10	13.85	64%	0.02	> 0.99	0.84
		Brazilian	6	1.00 (0.88 – 1.14)[b]	0.97	8.26	39%	0.14	0.71	0.64
CβS c.844ins68	Ins +/+ + Ins -/+ vs Ins -/-	All	6	1.03 (0.80 - 1.31)[b]	0.84	2.60	0%	0.76	0.13	0.22
		Brazilian	4	1.10 (0.83-1.45)[b]	0.51	1.37	0%	0.71	0.75	0.79
	Ins +/+ vs Ins -/+ + Ins-/-	All	6	1.07 (0.50 – 2.28)[b]	0.86	2.96	0%	0.56	0.75	0.73
		Brazilian	4	1.17 (0.53 – 2.56)[b]	0.70	2.42	0%	0.49	0.75	0.75
	Ins -/+ vs Ins -/-	All	6	1.02 (0.79 – 1.32)[b]	0.87	3.33	0%	0.65	0.27	0.36
		Brazilian	4	1.09 (0.81 – 1.45)[b]	0.57	2.47	0%	0.48	0.75	0.99
	Ins +/+ vs Ins -/-	All	6	1.10 (0.51 – 2.34)[b]	0.81	2.69	0%	0.61	0.75	0.74
		Brazilian	4	1.20 (0.54 – 2.64)[b]	0.65	2.10	0%	0.55	0.33	0.76
	Ins + vs Ins -	All	6	1.07 (0.86 – 1.34)[b]	0.54	1.33	0%	0.93	0.71	0.65
		Brazilian	4	1.07 (0.83 – 1.37)[b]	0.61	0.51	0%	0.92	0.33	0.21
MTHFD-1 c.1958G>A	AA + GA vs GG	All	5	1.22 (0.96 – 1.55)[b]	0.10	1.86	0%	0.76	0.48	0.38
		All in HWE	4	1.28 (0.98 – 1.67)[b]	0.07	1.16	0%	0.76	0.33	0.21
	AA vs GA + GG	All	5	0.96 (0.71 – 1.30)[b]	0.79	1.61	0%	0.81	0.08	0.43
		All in HWE	4	0.96 (0.68 – 1.37)[b]	0.84	1.60	0%	0.66	0.33	0.53
	GA vs GG	All	5	1.26 (0.98 – 1.62)[b]	0.07	2.62	0%	0.62	0.48	0.37
		All in HWE	4	**1.33 (1.01 – 1.75)[b]**	0.04	1.86	0%	0.60	0.75	0.27
	AA vs GG	All	5	1.05 (0.74 – 1.50)[b]	0.77	0.66	0%	0.96	0.81	0.81
		All in HWE	4	1.09 (0.73 – 1.62)[b]	0.67	0.54	0%	0.91	0.75	0.85
	A vs G	All	5	1.08 (0.92 – 1.27)[b]	0.35	1.30	0%	0.86	0.48	0.40

		All in HWE	4	1.11 (0.93 – 1.33)[b]	0.26	0.87	0%	0.83	0.75	0.39
TC2 c.776G>C	GG + CG vs CC	All	4	1.27 (0.83 – 1.93)[a]	0.27	8.03	63%	0.05	0.33	0.53
		All in HWE	2	0.92 (0.64 – 1.32)[b]	0.66	0.38	0%	0.53	-	-
	GG vs CG + CC	All	4	0.94 (0.70 – 1.27)[b]	0.70	5.65	47%	0.13	0.75	0.91
		All in HWE	2	1.12 (0.40 – 3.16)[a]	0.83	4.32	77%	0.04	-	-
	CG vs CC	All	4	1.34 (0.82 – 2.19)[a]	0.25	9.74	69%	0.02	0.75	0.67
		All in HWE	2	0.89 (0.61 – 1.31)[b]	0.56	0	0%	0.96	-	-
	GG vs CC	All	4	1.22 (0.87 – 1.72)[b]	0.25	4.61	35%	0.20	0.75	0.57
		All in HWE	2	1.04 (0.61 – 1.78)[b]	0.88	3.60	72%	0.06	-	-
	G vs C	All	4	1.12 (0.95 – 1.32)[b]	0.19	6.17	51%	0.10	0.08	0.20
		All in HWE	2	0.97 (0.75 – 1.25)[b]	0.79	2.63	62%	0.10	-	-

Abbreviations: OR, Odds Ratio; CI, Confidence Interval.

Bold values indicate significant associations.

[a]Random-effect model.

[b]Fixed-effect model.

- insufficient strata.

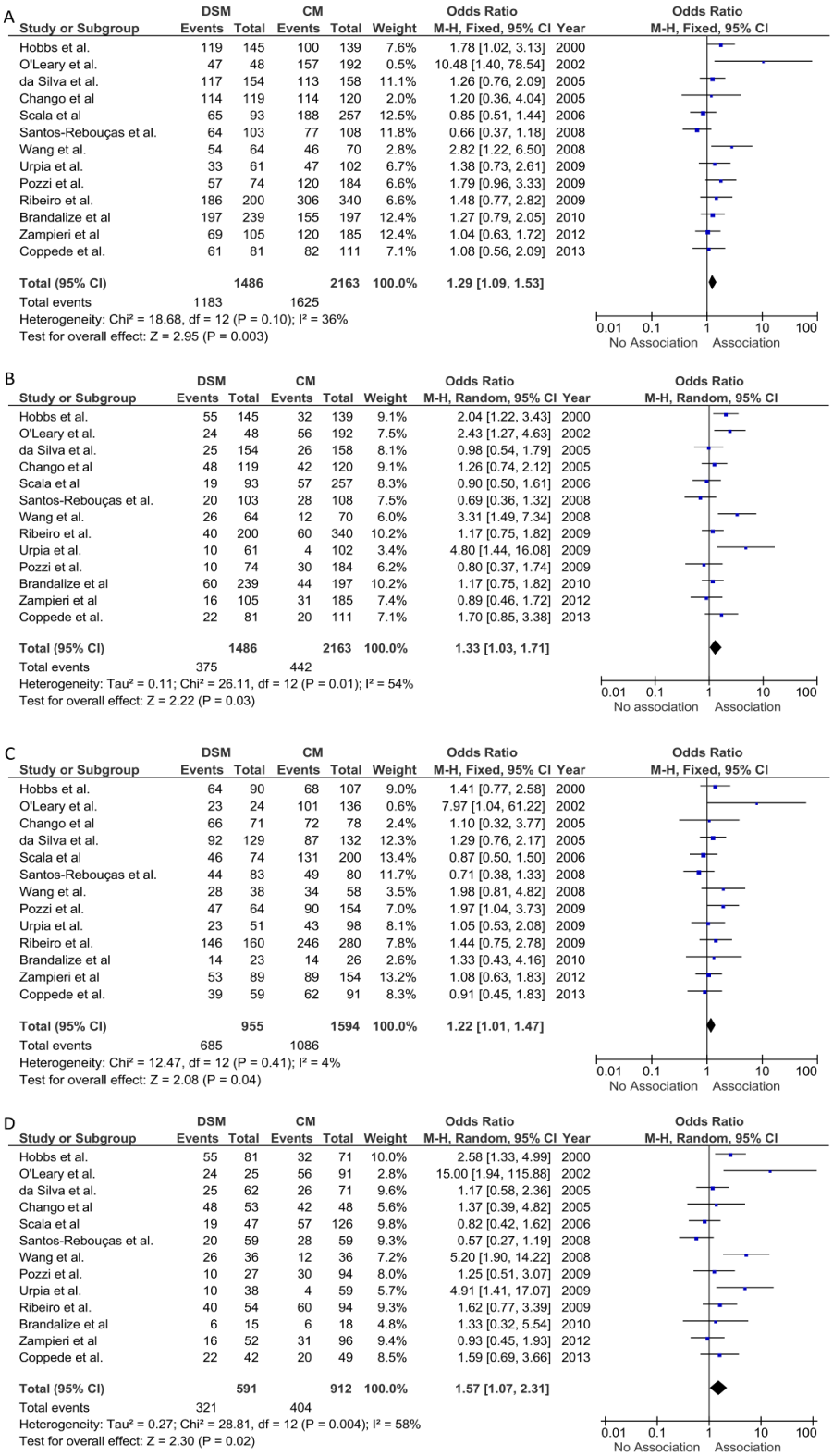

Figure 2. Forest plots showing the association between the *MTRR* A66G polymorphism and maternal risk for DS in overall population. GG/AG vs AA (A), GG vs AG/AA (B), AG vs AA (C) and GG vs AA (D) comparisons are illustrated. The squares represent odds ratios (ORs) and lines represent confidence intervals (95% CI). Abbreviations: DSM, Down syndrome mothers; CM, control mothers.

DISCUSSION

The present meta-analysis consists of an evaluation of *MTR* c.2756A>G (rs1805087), *MTRR* c.66A>G (rs1801394), *TC2* c.776G>C (rs1801198), *CβS* c.844ins68 and *MTHFD1* c.1958G>A (rs2236225) polymorphisms and maternal risk for DS. Our results show significant association between *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS in almost all genetic models when the general population is considered. Additionally, after the population was stratified by ethnicity, an increasing maternal risk for DS was observed in Caucasians. Furthermore, our results suggest that the *MTHFD1* 1958GA genotype is associated with maternal risk for DS and also that there is no significant association among *MTR* c.2756A>G (rs1805087), *TC2* c.776G>C (rs1801198) and *CβS* c.844ins68 polymorphisms and maternal risk for DS.

Folate is part of the B vitamins family and it is crucial for the synthesis of SAM, the major cellular methyl donor for DNA methylation [10, 48]. A common polymorphism reported in MTRR (66A → G) is the substitution of isoleucine by methionine on the residue 22. Such polymorphism changes the MTRR enzyme efficacy and also decreases the affinity of MTRR for MTR [14].

Based on the above, several studies were conducted in order to elucidate the association between the *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS. In our meta-analysis, we observed a significant association between the *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS in almost all genetic models, which corroborates with some of the previous case-control studies [31, 32, 36]. Although the exact mechanism is not yet determined, one possibility is related to the fact that the *MTRR* c.66A>G (rs1801394) polymorphism can lead to a decrease in the

MTRR enzyme activity [14] and, since the MTRR enzyme converts the MTR enzyme from its inactive form to its active state [10, 13, 14], such a decrease may result in plasma Hcy elevation and DNA hypomethylation.

The DNA methylation is very important to the regulation of gene expression, to genomic integrity and also to stability and chromatin organization [21, 22, 49, 50]. Several researchers have demonstrated that low folate status can affect the global methylation of DNA [51-54] and thus, increase the frequency of chromosomal breaks [55], abnormal chromatin conformation and DNA instability [56-59]. Such DNA instability may predispose to abnormal chromosome segregation [23, 60, 61], and consequently to aneuploidy [22-24, 48, 56, 57].

Since moderate heterogeneity is present in our meta-analysis, we decided to perform a stratified analyses based on HWE and on ethnicity. However, subgroup and sensitivity analyses were not able to find the source of heterogeneity. In our meta-analysis, we tried to minimize the heterogeneity between studies by performing a very careful search strategy and study selection. To accomplish that, we used an explicit inclusion criteria and performed quality data extraction and analysis. Despite all these efforts, a significant inter-study heterogeneity was present in some of the comparisons. It is necessary to point out that heterogeneity among studies is frequently observed on meta-analysis studies that report genetic associations [62], and in the present meta-analysis, the observed heterogeneity may be due to ethnic variations, environmental interactions related to folate metabolism [63] and methodological reasons. Although the sources of heterogeneity cannot be easily detected [64, 65], the sensitivity analysis did not change the pooled results, which indicates that our results were statistically robust. Finally, an optional method available to investigate this problem is the meta-regression

analysis [66]. Admittedly, one limitation of this method lies on the number of available studies with detailed covariates information, which prevents a more robust assessment of heterogeneity sources [67].

In our meta-analysis, the evidence suggested that *MTR* c.2756A>G (rs1805087), *TC2* c.776G>C (rs1801198) and *CβS* c.844ins68 polymorphisms did not contribute as an independent risk factor for DS. Our current data agrees with several previous performed case–control studies [33, 35, 39, 45, 47]. For the subgroup analysis based on ethnicity, we did not observe any effect modification. Some explanations might be responsible for the lack of association among *MTR* c.2756A>G (rs1805087), *TC2* c.776G>C (rs1801198) and *CβS* c.844ins68 polymorphisms and maternal risk for DS. First of all, the sample size of studies was relatively small. Secondly, risk factor may depend on genetic polymorphisms and potential gene-gene interaction. Several researchers showed that the polymorphisms are able to interact with each other and such interaction may modify their individual effects [34, 36, 43, 68]. Additionally, gene-environment interaction as the interaction between genotype and dietary intake, especially folate intake, may be decisive for maintaining the effects of these polymorphisms [63, 69].

To the best of our knowledge, our study was the first meta-analysis to investigate the association among *TC2* c.776G>C (rs1801198) and *MTHFD1* c.1958G>A (rs2236225) polymorphisms and maternal risk for DS. Our result suggests that the presence of the *MTHFD1* 1958GA genotype might be associated with maternal risk for DS. Previous studies have supported that *MTHFD1* c.1958G>A (rs2236225) polymorphism is able to reduce the activity and stability of the MTHFD1 enzyme and has been associated with an increased risk of neural tube defects [70] and unexplained

second semester pregnancy loss [71]. Moreover, some studies reported that the combined *MTHFR* 677CT/TT and *MTHFD* 1958AA/GA [47] and *MTHFD* 1958AA/*RFC1* 80GG genotypes [35] were significantly associated with the maternal risk for DS. Since the number of included studies on *MTHFD1* c.1958G>A (rs2236225) meta-analysis was only 5, larger sample studies should be conducted in order to confirm this result.

To the best of our knowledge, there are three meta-analyses papers that reported the association between genetic polymorphisms involved in folate metabolism and maternal risk for DS [46, 72, 73]. Such meta-analyses reported distinct results and their included studies and sample sizes are different. There are some discrepancies between Yang's study [72] and our study. We performed some independent and original analyses, such as comparisons by genetic models. We demonstrated important results in those analyses, since the dominant, recessive and codominant comparisons showed significant associations between *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS. Moreover, we were the first ones to conduct meta-analyses to evaluate the association among *TC2* c.776G>C (rs1801198) and *MTHFD1* c.1958G>A (rs2236225) polymorphisms and maternal risk for DS. Coppedè et al. [46] conducted another meta-analysis, performed almost simultaneously. In our study, the stratified analyses by codominant models were performed. However, such an analysis was not made in the Coppedè et al. [46] article. In addition, our meta-analysis included all the published studies and added another 363 DSM and 603 CM. Finally, the meta-analysis conducted by Amorim et al. [73] only observed a significant association between G allele of the *MTRR* c.66A>G (rs1801394) polymorphism and maternal risk for DS. However, we observed important results for such polymorphism: the heterozygote AG and

polymorphic homozygote GG genotypes, dominant and recessive genetic models were significantly associated with maternal risk for DS. Their study only comprised eleven case-control studies, while our study included two additional articles, which accounted for 20% of the total sample size. We also conducted stratified analyses by ethnicity, which was not made in the Amorim et al. [73] study. In conclusion, for the reasons illustrated above, we demonstrated stronger evidence and more powerful pooled results in comparison with previous meta-analyses.

There are still some limitations in this meta-analysis that need to be mentioned. Firstly, all included studies were performed as case-control studies, which prevent additional comments on a cause-effect relationship [74]. Secondly, since we only included studies written in English, Spanish and Portuguese, a potential selection bias cannot be totally excluded. Thirdly, although previous reports have suggested that genetic polymorphisms involved in folate metabolism have a synergistic effect on enzyme activity [68, 75], we could not investigate the association between the combined genotypes of these polymorphisms and maternal risk for DS due to the lack of detailed original data in the included studies. In addition, such lack of data as folate intake and maternal age at conception, in the included studies, limited our further stratified analysis. Fourthly, we did not analyze gene-gene and gene-environment interactions. It is possible that specific environmental and lifestyle factors can influence the associations between genetic polymorphisms and maternal risk for DS.

In conclusion, our meta-analysis provided evidence that *MTRR* c.66A>G (rs1801394) polymorphism was associated with maternal risk for DS, especially in Caucasians. Additionally, our result suggested that the *MTHFD1* 1958GA genotype could be associated with maternal risk for DS. Finally, the evidence demonstrated that

MTR c.2756A>G (rs1805087), *TC2* c.776G>C (rs1801198) and *CβS* c.844ins68 polymorphisms did not contribute as an independent risk factor of DS. Further larger and well-designed studies are required to confirm this conclusion; functional studies should also be conducted to fully understand the molecular mechanism of DS.

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Conclusões

3. Conclusões

1. O alelo polimórfico *MTHFR* 677T está associado ao risco materno para o nascimento de indivíduos com a SD, especialmente nas populações caucasiana e brasileira, uma vez que o tamanho amostral para a população asiática é limitado.
2. O alelo polimórfico *RFC1* 80G está associado ao risco materno para o nascimento de indivíduos com a SD, mesmo quando a análise é restringida a um grupo de mulheres com idade inferior a 35 anos. Apesar de tal associação também ter sido verificada na população asiática, os resultados devem ser interpretados com cautela visto que o tamanho amostral para esta população é limitado.
3. Os genótipos *MTRR* 66AG e 66GG estão associados ao risco materno para o nascimento de indivíduos com a SD, em especial na população caucasiana.
4. O genótipo *MTHFD1* 1958GA está associado ao risco materno para o nascimento de indivíduos com a SD. Tal resultado deve ser interpretado com cautela uma vez que o número de estudos incluídos nesta metanálise é limitado.
5. Os polimorfismos genéticos *MTHFR* A1298C, *MTR* A2756G, *TC2* C776G e *CβS* 844ins68 não estão associados ao risco materno para a SD.
6. Nossa metanálise evidencia a necessidade de realização de estudos que considerem as potenciais interações existentes entre gene-gene e gene-ambiente de modo a contribuir para um conhecimento mais profundo sobre o papel dos polimorfismos genéticos envolvidos no metabolismo do folato em relação ao risco da não-disjunção do cromossomo 21.

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4. Referências Bibliográficas

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Anexos

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Meta-analysis of *Methylenetetrahydrofolate reductase* maternal gene in Down syndrome: increased susceptibility in women carriers of the *MTHFR* 677T allele

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Abstract Because a number of data studies include some controversial results about Methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and Down syndrome (DS), we performed a meta-analysis to determine a more precise estimation of this association. Studies were searched on PubMed, EMBASE and Lilacs–Scielo, up to April 2013, and they were eligible if they included case mothers (DSM) that have gave birth to children with DS, and controls mothers (CM) that have gave birth to healthy children without chromosomal abnormality, syndrome or malformation. The combined odds ratio with 95 % confidence intervals was calculated by fixed or random effects models to assess the strength of associations. Potential sources of heterogeneity between studies were evaluated using Q test and the I^2. Publication bias was estimated using Begg's test and Egger's linear regression test. Sensitivity analyses were performed by using allelic, dominant, recessive and codominant genetic models, Hardy–Weinberg equilibrium (HWE) and ethnicity. Twenty-two studies with 2,223 DSM and 2,807 CM were included for *MTHFR* C677T and 15 studies with 1,601 DSM and 1,849 CM were included for *MTHFR* A1298C. Overall analysis suggests an association of the *MTHFR* C677T polymorphism with maternal risk for DS. Moreover, no association between the *MTHFR* A1298C polymorphism and maternal risk for DS was found. There is also evidence of higher heterogeneity, with I^2 test values ranging from 8 to 89 %. No evidence of publication bias was found. Taken together, our meta-analysis implied that the T allele carriers might carry an increased maternal risk for DS.

Keywords Down syndrome · Meta-analysis · *MTHFR* C677T · *MTHFR* A1298C · Folate

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Introduction

First described in 1866, Down syndrome (DS) is one of the most commonly identified genetic forms of intellectual disability, which affect about 1 in 660 live births [1]. The disorder is caused by a complete or partial (translocations or mosaicism) triplication of chromosome 21 resulting in multiple congenital abnormalities of variable severity [2, 3]. In the majority of DS cases (90 %), the nondisjunction event is of maternal origin, occurring primarily during meiosis I in the maturing oocyte [3].

Advanced maternal age at conception is the only well known risk factor for the great majority of DS pregnancies, as chromosome trisomies are more prevalent in children born to mothers aged 35 years and older [4, 5]. However, several children with DS are born to women younger than 35 years at conception, indicating a predisposition to chromosome nondisjunction in these women [6]. Chromosomal nondisjunction and folate metabolism have received great attention. James et al. [7] were the first to present evidence that the occurrence of chromosome 21 nondisjunction is associated with DNA hypomethylation due to abnormal folate metabolism.

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