

ARTICLE

# Reduced folate carrier polymorphism (80A → G) and neural tube defects

Patrizia De Marco<sup>1</sup>, Maria Grazia Calevo<sup>2</sup>, Anna Moroni<sup>1</sup>, Elisa Merello<sup>1</sup>, Alessandro Raso<sup>1</sup>, Richard H. Finnell<sup>3</sup>, Huiping Zhu<sup>3</sup>, Luciano Andreussi<sup>1</sup>, Armando Cama<sup>1</sup> and Valeria Capra<sup>\*,1</sup>

<sup>1</sup>Laboratorio del Servizio di Neurochirurgia, Istituto G. Gaslini, Genova, Italy; <sup>2</sup>Servizio di Epidemiologia e Biostatistica, Istituto G. Gaslini, Genova, Italy; <sup>3</sup>Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX, USA

Transport of folates in mammalian cells occurs by a carrier-mediated mechanism. The human folate carrier (*RFC-1*) gene has been isolated and characterized. Within this gene, a common polymorphism, 80A → G, changing a histidine to an arginine in exon 2 (H27R), was recently identified. Defects in folate metabolism, such as defective carrier molecules, could be implicated in the etiology of neural tube defects (NTDs). In the present case-control study, we recruited 174 Italian probands with nonsyndromic NTD, 43 mothers, 53 fathers and 156 control individuals and evaluated the impact of *RFC-1* variant on NTD risk. A statistically significant risk was calculated for the 80GG genotype of the NTD cases (OR = 2.35; 95% CI 1.21–4.58) and mothers (OR = 2.74; 95% CI 0.92–8.38). On the contrary, the heterozygous genotype of the mothers and both heterozygous and homozygous genotypes of the fathers did not seem to be significant NTD risk factors. Furthermore, according to the multifactorial inheritance of NTDs, we demonstrated that the combined genotypes for *MTHFR* 1298A → C and *RFC-1* 80A → G polymorphisms of cases resulted in greater NTD risk than heterozygosity or homozygosity for *RFC-1* 80A → G variant alone. Conversely, our data provide no evidence for an association between NTD phenotype and combined *MTHFR* C677T/*RFC-1* A80G genotypes. Moreover, here we describe the combinations of the two *MTHFR* polymorphic sites (677CT and 1298AC) with *RFC-1* genotypes. We found that both patients and controls could have at most quadruple-mutation combinations. Interestingly, 27% (7/26) of the mothers and 18.75% (30/160) of the cases genotyped presented four mutant alleles in comparison with 8.5% (11/129) of the controls. Finally, the frequency of NTD cases and mothers carrying combined heterozygosity for the two *MTHFR* polymorphisms and *RFC-1* 80GG homozygosity (677CT/1298AC/80GG) (cases = 11.3%; mothers 11.5%) was increased compared with controls (1.6%). Altogether, our findings support the hypothesis that *RFC-1* A80G variant may contribute to NTD susceptibility in the Italian population.

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## Introduction

Reduced folates are essential cofactors for the biosynthesis of purines, pyrimidines and methylated compounds. Inward transport of folates and their analogues in mammalian cells can occur by carrier-mediated as well as receptor-mediated mechanisms. The reduced-folate carrier (*RFC*) is a bidirectional anion exchanger that mediates

\*Correspondence: Dr V. Capra, Istituto G. Gaslini, Largo G. Gaslini, 5 16148 Genova, Italia. Tel: 39 010 5636712; Fax: 39 010 3993159; E-mail: capra@tin.it, valeriacapra@ospedale\_gaslini.ge.it  
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folate delivery into a variety of cells of different origin.<sup>1,2</sup> It has a much higher affinity for reduced folates, including the physiological substrate 5-methyltetrahydrofolate, than oxidized folic acid.<sup>3</sup> Furthermore, RFC-1 has been the subject of intensive studies because of its critical role in antifolate transport and resistance.<sup>4,5</sup> The molecular cloning and the primary structure of the human RFC-1 (*RFC-1*) gene (HUGO name SLC19A1) has been reported.<sup>6,7</sup> Recently, Zhao *et al* demonstrated that *RFC-1*-null embryos died *in utero* as a consequence of early failure of hematopoietic organs. Rescue of embryonic lethality was obtained by folic acid supplementation of *RFC1*<sup>+/-</sup> dams.<sup>8</sup>

Neural tube defects (NTDs) are common congenital malformations resulting from the incomplete closure of the neural tube. These birth defects have a multifactorial origin, with environmental and genetic components. The best-characterized environmental factor known to modulate NTD risk is maternal folate intake. A series of epidemiological studies have established that periconceptional folic acid supplementation can reduce a woman's risk for both the NTD occurrence and recurrence by up to 75%.<sup>9,10</sup> Furthermore, significantly elevated plasma homocysteine levels and reduced folate levels were found in mothers of NTD-affected children.<sup>11</sup> The thermolabile 677C→T and 1298A→C polymorphisms in the *MTHFR* (methylene tetrahydrofolate reductase) gene, encoding an enzyme required for the synthesis of methyltetrahydrofolate, has been implicated in the pathogenesis of hyperhomocysteinemia and NTDs.<sup>12-21</sup> These mutations can only partially explain the observed protective effect of folates against NTDs. Therefore, other defects in folate metabolism, such as defective folate receptors and carriers, could also be involved in the etiology of NTDs. Recently, our group has investigated a possible association between allelic forms of the human receptor  $\alpha$  (*FR $\alpha$* ) and increased risk of NTD and identified four unrelated NTD patients with *de novo* insertions of pseudogene-specific mutations in exon 7 and the 3' UTR of human *FR $\alpha$*  gene arising by microconversion events.<sup>22</sup> However, other investigations on the *FR $\alpha$*  gene suggest that it is unlikely to be a major factor influencing NTD risk.<sup>23,24</sup> Given its role in the folate uptake, *RFC-1* genetic variants could represent another genetic risk factor for NTDs. A common polymorphism at position 80 in exon 2 of the *RFC-1* gene has been identified. This polymorphism changes an adenine to a guanine creating a *CfoI* restriction site and introduces an arginine residue instead of a histidine (H27R).<sup>25</sup> The impact of this polymorphism separately, and in combination with the *MTHFR* 677C→T variant, on folate status and homocysteine levels has been explored. A moderate, but significant, increase in homocysteine levels was found in homozygous 80GG/677TT subjects, when compared with 80GG/677CC or 80GG/677CT individuals. In addition, individuals who were 80AA/677CT had higher plasma folate levels than individuals who were 80GG/677CT.<sup>25</sup>

In the present case-control study, we examined the impact of the *RFC-1* 80A→G polymorphism on NTD risk in the Italian population. Our data demonstrate that this variant is a genetic risk factor for NTDs. Furthermore, according to multifactorial inheritance pattern for NTDs, we were able to show that the combined polymorphic genotypes for *MTHFR* 1298A→C and *RFC-1* 80A→G results in a greater NTD risk than heterozygosity or homozygosity for the *RFC-1* 80A→G variant alone. Finally, our data provide no evidence for an association between NTD phenotype and *MTHFR* C677T/*RFC-1* A80G genotypes. Interestingly, the frequency of NTD cases and mothers carrying combined heterozygosity for the two *MTHFR* polymorphisms (677CT/1298AC) and *RFC-1* 80GG homozygosity was increased compared with controls.

## Materials and methods

### Probands and controls

The study population has been described before<sup>26</sup> and consisted of 174 (73 male and 101 females) unrelated Italian NTD children, 43 mothers and 53 fathers who were recruited from the Spina Bifida Center of the Gaslini Hospital, Genoa. A total of 156 healthy individuals (62 males and 94 females) were used as control group. The selection criteria included no individual history of NTD and frequency matching on gender and age distribution of the case group. Informed consent was obtained from patients, parents and control individuals.

### Genetic analysis

DNA was isolated from peripheral leukocytes obtained from blood draws using standard procedures. The presence of the *RFC-1* 80A→G polymorphism was investigated using the previously described PCR-diagnostic test.<sup>26</sup> The detection of the *MTHFR* C677T and 1298A→C mutations was carried out by PCR/RFLP screening, as described by Frosst *et al*<sup>27</sup> and by van der Put *et al*<sup>28</sup> respectively.

The digested PCR products were separated using 3.5–4% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA) gel electrophoresis and stained by ethidium bromide. Direct sequencing of PCR fragments was utilized to confirm the presence of the mutations. The sequencing reactions were manually performed using the Thermosequenase Cycle Sequencing Kit (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions. The forward PCR primer for the *MTHFR* 1298A→C and the reverse PCR primer for *RFC-1* 80A→G were utilized as sequencing primers.

### Statistical analysis

The odds ratio (OR) with an associated 95% confidence interval (CI) were calculated to estimate the relative risk of the different genotype combinations. Genotype combinations in cases and control groups were compared by  $\chi^2$

analysis. Exact methods were considered preferable, whenever expected numbers in any cell were less than five, and any results reported herein are based on exact methods. Bonferroni correction was applied to the positive findings in the multiple tests. *P*-value  $\leq 0.05$  were considered statistically significant, and all *P*-values were based upon two-tailed tests. The SPSS statistical software program was used for all analysis.

The transmission disequilibrium test (TDT) was applied to analyze transmission disequilibrium of *RFC-1* 80G allele in 17 family trios.<sup>29</sup>

## Results

*RFC-1* genotype distributions and allele frequencies for Italian NTD patients, parents and controls have just been reported.<sup>26</sup> In order to evaluate the impact of the *RFC-1* 80A→G variant on NTD risk, we calculated the odds ratio (OR) and 95% confidence interval (CI) associated with the 80A/G and 80G/G genotypes (Table 1). The estimated OR for heterozygous 80A/G NTD cases was 1.72 and increased to 2.35 for homozygous 80G/G NTD cases. Furthermore, the NTD risk significantly increased if both homozygous and heterozygous NTD children were compared with control individuals (OR = 1.93, *P* ≤ 0.01). Similarly, significant risk estimates resulted for the mothers: an increased risk of 2.74 was found for the association of the 80G/G homozygous genotype. On the contrary, OR both for the heterozygous genotype of the mothers and for the heterozygous and homozygous genotype of the fathers were not statistically significant.

To further confirm the obtained data from the case-control association study, we attempted to detect transmission disequilibrium for the *RFC-1* 80G allele. Unfortunately, only 17 completed families (mother, father and affected NTD child) were available. In addition, of the 34 parents, 11 were homozygous and thus not informative.

**Table 1** ORs and 95% CI of *RFC-1* genotype in NTD cases, mothers and fathers

<i>RFC-1</i> 80A→G	OR (95% CI)	<i>P</i>
Controls–patients		
AA/GG	2.35 (1.21–4.58)	<b>0.006</b>
AA/AG	1.72 (0.96–3.11)	<b>0.05</b>
AA/AG-GG	1.93 (1.11–3.35)	<b>0.01</b>
Controls–mothers		
AA/GG	2.74 (0.92–8.38)	<b>0.04</b>
AA/AG	1.86 (0.68–5.27)	0.18
AA/AG-GG	2.15 (0.86–5.73)	0.08
Controls–fathers		
AA/GG	1.28 (0.48–3.38)	0.58
AA/AG	1.29 (0.57–2.95)	0.51
AA/AG-GG	1.29 (0.60–2.80)	0.48

Statistically significant *P*-values are in bold.

From a total of 23 heterozygous parents, the 80G allele is transmitted 14 times, whereas the 80A allele is transmitted only nine times. This bias in 80G allele transmission is only suggestive of an effect (*P* = 0.75).

Since our previous study has shown that the *MTHFR* 1298A→C polymorphism is a genetic determinant for NTD risk in Italian cases,<sup>21</sup> the potential interaction between the *RFC-1* 80A→G and *MTHFR* 1298A→C polymorphisms on NTD risk was examined. In this light, we genotyped for the 1298A→C mutation in 161 NTD cases and 143 controls, who were previously analyzed for *RFC-1* 80A→G mutation. Table 2 shows combined *MTHFR* 1298A→C/*RFC-1* 80A→G genotype distributions, along with the frequencies of single *RFC-1* and *MTHFR* alleles. The *MTHFR* 1298C allele frequencies are those previously reported in our Italian population study.<sup>21</sup> All possible genotypic combinations were observed in NTD cases and controls. Furthermore,

**Table 2** Combined *MTHFR* 1298A→C/*RFC-1* 80A→G genotype combinations of NTD children and associated NTD risk

	NTD cases % (N=161)	Controls % (N=143)	OR	<i>P</i>
1298AA/80AA	8.7 (N=14)	14.7 (N=21)	ref.	
1298AA/80AG	18.6 (N=30)	24.5 (N=35)	1.29 (0.52–3.22)	0.55
1298AA/80GG	11.8 (N=19)	15.4 (N=22)	1.30 (0.47–3.58)	0.58
1298AC/80AA	6.8 (N=11)	12.6 (N=18)	0.92 (0.30–2.83)	0.87
1298AC/80AG	22.4 (N=36)	18.2 (N=26)	2.08 (0.82–5.28)	0.08
1298AC/80GG	18.0 (N=29)	6.3 (N=9)	4.83 (1.58–15.16)	<b>0.002</b>
1298CC/80AA	2.5 (N=4)	1.4 (N=2)	3.00 (0.36–36.41)	0.22
1298CC/80AG	8.1 (N=13)	4.2 (N=6)	3.25 (0.87–12.62)	<b>0.04</b>
1298CC/80GG	3.1 (N=5)	2.8 (N=4)	1.88 (0.33–11.08)	0.46
<i>MTHFR</i> 1298A	0.61	0.75		
<i>MTHFR</i> 1298C	0.39	0.25		
<i>RFC-1</i> 80A	0.425	0.53		
<i>RFC-1</i> 80G	0.575	0.47		

ref.=reference group; statistically significant *P*-values are in bold.

**Table 3** Combined *MTHFR* 677C→T/*RFC-1* 80A→G genotype combinations of NTD children and associated NTD risk

	NTD cases % (N=170)	Controls % (N=138)	OR	P
677CC/80AA	10.0 (N=17)	8.7 (N=12)	ref.	
677CC/80AG	22.9 (N=37)	17.4 (N=24)	1.15 (0.43–3.09)	0.76
677CC/80GG	11.2 (N=19)	10.9 (N=15)	0.89 (0.29–2.74)	0.82
677CT/80AA	2.9 (N=5)	13.0 (N=18)	0.20 (0.05–0.77)	<b>0.008</b>
677CT/80AG	21.2 (N=36)	21.7 (N=30)	0.85 (0.32–2.24)	0.71
677CT/80GG	17.6 (N=30)	8.7 (N=12)	1.76 (0.58–5.39)	0.26
677TT/80AA	5.3 (N=9)	6.5 (N=9)	0.71 (0.18–2.70)	0.56
677TT/80AG	4.7 (N=8)	8.7 (N=12)	0.47 (0.12–1.74)	0.56
677TT/80GG	4.1 (N=7)	4.3 (N=6)	0.82 (0.18–3.73)	0.77
<i>MTHFR</i> 677C	0.68	0.67		
<i>MTHFR</i> 677T	0.32	0.33		
<i>RFC-1</i> 80A	0.425	0.53		
<i>RFC-1</i> 80G	0.575	0.47		

ref.=reference group; statistically significant *P*-values are in bold.

combined genotypes 1298AA/80AG, 1298AA/80GG were observed at lower allelic frequency among cases when compared to the control group. On the contrary, 1298AC/80AG, 1298AC/80GG, 1298CC/80AG and 1298CC/80GG genotypes are more frequent among cases than controls, demonstrating that genotypes with 2, 3 and 4 of these nucleotide changes could be risk combination for NTD cases. Using a genotype of either homozygous wild type as the reference, a significant risk was associated with 1298AC/80GG genotype (OR=4.83, *P*=0.002). Even if the Bonferroni's correction for multiple tests was applied, this positive result was significant (*P*=0.008). Moreover, ORs associated with the 1298AC/80AG (two mutations), with the 1298CC/80AG (three mutations) and 1298CC/80GG (four mutations) combined genotypes are suggestive of an increased risk, but they are not statistically significant. The association of the 1298CC/80AG genotype with an increased NTD risk (OR=3.25, *P*=0.04) became not significant (*P*=0.15) after the Bonferroni's correction. Finally, the frequency of the 1298CC/80AA genotype was increased in NTD cases when compared with controls. ORs associated with the 1298CC/80AA genotype is 3.00 (*P*=0.22). This result, also not statistically significant, would confirm that the homozygosity for 1298A→C polymorphism is itself a genetic determinant for NTD risk, as we have demonstrated in our previous study.<sup>21</sup> Therefore, the lack of statistical significance is possibly due to the small cases (*N*=5) and controls (*N*=4) samples who carried the 1298CC/80AA genotype.

Homozygosity for the *MTHFR* 677T polymorphism has been associated with NTD risk in some populations.<sup>30</sup> The potential interaction between *RFC-1* 80A/G and *MTHFR* 677C/T genotypes on NTD risk was also investigated in this report. Table 3 analyzes the combined *MTHFR* C677T/*RFC-1* 80A/G genotype distributions of 170 NTD cases and 138 controls and their contribution to NTD risk. The frequency of the mutated T allele of the *MTHFR* C677T polymorphism

was 0.33 for controls and 0.32 for NTD cases. Thus, we observed no increased frequency of the mutated allele in the NTD patients when compared with controls. Furthermore, all possible *MTHFR/RFC-1* genotype combinations were found, although there was no link between these two folate-pathway polymorphisms and NTD risk.

Finally, the combinations of the two *MTHFR* C677T and A1298C polymorphic sites with *RFC-1* genotypes of 160 NTD cases, 26 mothers and 129 controls are shown in Table 4. In our study population, both *MTHFR* 677CT/1298CC and 677TT/1298CC genotypes were absent.<sup>31</sup> We also observed that 677TT/1298AC combination seems to be rare and only a patient presented this haplotype in combination with heterozygosity for the *RFC-1* A80G variant. Interestingly, the frequency of NTD cases and mothers carrying combined heterozygosity for the two *MTHFR* polymorphisms and *RFC-1* 80GG homozygosity (677CT/1298AC/80GG) (cases=11.3%; mothers 11.5%) was increased compared with controls (1.6%). In addition, cases and mothers with the *MTHFR* genotypes 677CC/1298AC and 677CC/1298CC more frequently carried additional *RFC1*-80AG and -80GG alleles than controls. These data further confirm the above results of an interaction between *MTHFR* A1298C and *RFC-1* A/80G genotypes interactions on the occurrence of NTD. Moreover, we found that both patients and controls could have at most quadruple-mutation combinations. It is intriguing to note that 27% (7/26) of the mothers and 18.75% (30/160) of the cases genotyped presented four mutant alleles in comparison with 8.5% (11/129) of the controls. This finding would suggest a substantial contribution of maternal genotype on NTD phenotype. However, owing to sparse data, we were unable to adequately explore the potential risk of genotypes with quadruple-mutations among mothers and NTD children.

**Table 4** Combinations of MTHFR 677CT/1298AC genotypes with RFC-1 80A→G genotypes in 60 NTD children, 26 mothers and 129 controls

MTHFR		AA	RFC-1 AG	GG	Total
677CC/ 1298AA	Patients	3.1% (n=5)	6.9% (n=11)	1.9% (n=3)	19
	Mothers	—	3.8% (n=1)	3.8% (n=1)	2
	Controls	4.7% (n=6)	5.4% (n=7)	3.1% (n=4)	17
677CC/1298AC	Patients	5.0% (n=8)	9.4% (n=15)	6.9% (n=11)	34
	Mothers	3.8% (n=1)	11.5% (n=3)	3.8% (n=1)	5
	Controls	4.7% (n=6)	7.8% (n=10)	5.4% (n=7)	23
677CC/1298CC	Patients	2.5% (n=4)	7.5% (n=12)	3.1% (n=5)	21
	Mothers	3.8% (n=1)	11.5% (n=3)	15.4% (n=4)	8
	Controls	—	3.1% (n=4)	2.3% (n=3)	7
677CT/1298AA	Patients	0.6% (n=1)	7.5% (n=12)	6.3% (n=10)	23
	Mothers	—	3.8% (n=1)	—	1
	Controls	5.4% (n=7)	11.6% (n=15)	7.8% (n=10)	32
677CT/1298AC	Patients	1.9% (n=3)	12.5% (n=20)	11.3% (n=18)	41
	Mothers	3.8% (n=1)	11.5% (n=3)	11.5% (n=3)	7
	Controls	7.8% (n=10)	10.1% (n=13)	1.6% (n=2)	25
677TT/1298AA	Patients	5.0% (n=8)	4.4% (n=7)	3.8% (n=6)	21
	Mothers	% (n=2)	3.8% (n=1)	—	3
	Controls	5.4% (n=7)	8.5% (n=11)	4.7% (n=6)	24
677TT/1298AC	Patients	—	0.6% (n=1)	—	1
	Mothers	—	—	—	0
	Controls	0.8% (n=1)	—	—	1

## Discussion

The aim of this study was to evaluate the role of a common polymorphism of *RFC-1* gene, the 80A→G variant, on NTD risk in the Italian population. Since the allele frequencies for the two *RFC-1* alleles are virtually identical,<sup>26</sup> it is unclear which allele would contain the ancestral human *RFC-1* sequence. Nevertheless, we found that the homozygous 80GG genotype itself may be associated with increased NTD risk, by 2.35-fold, in children. A lower increase in risk was observed when heterozygous genotype of cases was taken into account (OR = 1.72). The impact of homozygous *RFC-1* 80GG genotype on NTD risk could be substantial, given that the *RFC-1* polymorphic alleles are highly prevalent in this study population. A family-based TDT that directly compares frequencies of transmitted and nontransmitted alleles was applied to confirm that the 80GG is an 'at-risk' genotype. Our data showed a preferential transmission of the *RFC-1* 80G allele to the affected children, although the transmission rate did not deviate significantly from the expected values. At present, this preliminary family-based approach was limited in its effect estimation owing to small sizes of informative parents. Therefore, recruitment of a large number of families is needed for either genotype association studies or the allele TDT to have sufficient statistical power to

enable definitive conclusions regarding the causative role of the *RFC-1* 80GG genotype. Furthermore, since the calculated OR for the homozygous mutant genotype of the mother approached statistical significance, we do not absolutely conclude that the maternal 80GG genotype may have a direct effect on NTD risk. Because of the small number of mothers in the current study, this possibility should be viewed with caution. Moreover, the significance of the association between maternal 80GG genotype and NTD risk disappeared after the Bonferroni correction ( $P=0.15$ ). The question of the extent, if any, to which the maternal *RFC-1* 80GG genotype actively contributes to NTD outcome should be addressed with large number of mother-NTD case pairs in future studies.

It is well established that *RFC-1* is a very efficient transporter with a high affinity for the physiological folate substrate 5-methyltetrahydrofolate; this carrier is of particular importance during the embryonic development, because it is responsible for folate transfer across the placenta.<sup>32</sup> The 80A→G mutation affect residue 27 of the protein and substitute a histidine (or H; CAG codon) with an arginine (or R; CGG codon). This residue is not conserved between species, because the equivalent codon in the mouse and the hamster *RFC-1* gene is a cysteine (C). However, the 80A→G mutation is located in the predicted

cytoplasmic amino terminus of the protein, where the signal sequence responsible for insertion in membrane resides. Thus, the changing of the positively charged histidine (CAG) with an arginine (CGG) at residue 27 could be critical for targeting and integration of protein to the plasma membrane. On the other hand, the functional role of arginine mutations has been demonstrated for other anion transporters.<sup>33,34</sup> In this light, our finding of elevated risk of NTD for subjects with *RFC-1* 80GG homozygous genotype could be biologically plausible.

Another limitation of the present study is that we did not have access to plasma sample for evaluation of the impact of *RFC-1* variant on folate and homocysteine levels in our cases and mothers. Moreover, further work is required to determine whether the effect of *RFC-1* 80A → G polymorphism on blood folate and homocysteine levels is influenced by the amount of dietary and/or supplemental folates in a similar way to the effect of the *MTHFR* 677C → T polymorphism. Recently, Shaw *et al*<sup>35</sup> reported evidence for a gene–nutrient interaction between infant homozygosity for the *RFC-1* 80G/80G genotype and maternal folate periconceptional intake on NTD risk. In that study, the authors did not observe an increased NTD risk among infants who were heterozygous or homozygous for the *RFC-1* polymorphism; however, NTD risk was higher among cases homozygotes for *RFC-1* 80G/80G genotype whose mothers did not use periconceptional vitamin supplements containing folic acid. Although both research groups used the RFLP method for determining the *RFC-1* A80G polymorphisms, our finding that the *RFC-1* A80G variant confers a higher risk for homozygous cases, is in apparent contradiction with those reported by Shaw *et al*. In our opinion, the main reason for the conflicting results may be that the population investigated by Shaw *et al* was ethnically heterogeneous (Hispanics of Mexican descent and US individuals of European descent) and of different descent compared with the one studied by us (Italian population). Further association studies will have to confirm whether the prevalence of this common polymorphism is population dependent, and whether the *RFC-1* A80G variant may not be in every country associated with an increased NTD risk.

One hypothesis that relates to the multifactorial origin of NTDs is the possibility that common variants in more than one gene involved in folate and homocysteine metabolism could interact to increase a given infant's NTD risk. Recently, we have demonstrated that *MTHFR* 1298A → C polymorphism is a genetic determinant for NTD risk in Italy.<sup>21</sup> Therefore, the potential interaction between the *MTHFR* 1298A → C and *RFC-1* 80A → G polymorphisms on NTD risk was examined. Interestingly, combined heterozygotes carrying two mutations (*MTHFR* 1298AC/*RFC-1* 80AG) do not have a significantly increased NTD risk (OR = 2.08; *P* = 0.08). A significant effect on NTD risk was found when *RFC-1* 80A → G and *MTHFR* 1298A → C com-

bined genotypes with at least three mutations were taken into account. Instead, the ORs of children that carried *MTHFR* 1298CC/*RFC-1* 80AG and *MTHFR* 1298AC/*RFC-1* 80GG genotypes were 3.25 and 4.83, respectively. These data indicate that three variants in both *MTHFR* and *RFC-1* confer a level of risk higher than heterozygosity and homozygosity for the *RFC-1* variant alone. However, it should be noted that positive association between the *MTHFR* 1298CC/*RFC-1* 80AG became not significant (*P* = 0.15) after the Bonferroni correction for multiple analysis. Nevertheless, the fact of doubly homozygous genotypes with four mutations (1298CC/80GG) have no statistically significant OR is probably due to the low number of individuals who carried this genotype combination both among controls (2.8%) and cases (3.1%). On the whole, our findings of *RFC-1* A80G and *MTHFR* A1298C genotype interactions on NTD risk are intriguing and merit further investigations with larger samples.

In contrast to the published data,<sup>25</sup> our study did not provide evidence of statistical interaction between *MTHFR* C677T and *RFC-1* polymorphisms. The 677-T allele frequency was not different in cases and controls. This finding could reflect a lack of association with an increased NTD risk among cases with the combined *MTHFR* C677T and *RFC-1* A80G mutant genotypes. Studies from Europe also report that the C677T mutation does not result in a significant risk factor for NTD either independently or in association with other polymorphic alleles involved in folate metabolism.<sup>36–38</sup>

Moreover, we are the first to present data on *MTHFR* C677T/A1298C haploid genotypes in combination with *RFC-1* A80G variant. Examination of combined genotypes revealed that haplotypes 677CT/1298CC and 677TT/1298CC were not observed in our NTD families and controls. It is interesting to note that *MTHFR* 677TT/1298AC genotypes were represented in both groups, although at low frequencies. Complete linkage disequilibrium between the *MTHFR* 677T and 1298C mutations has been suggested, because the majority of studies examining these mutations have identified them in *trans* positions only. However, our results are in agreement with other investigators who provided evidence that *cis* configurations of *MTHFR* mutations do occur.<sup>19,31</sup> In accordance with them, we postulate that, if linkage disequilibrium between *MTHFR* mutations is present, it is incomplete. Moreover, since 677T and 1298C mutations are known to interact *in vivo*,<sup>18,19</sup> it is possible that 677TT/1298AC genotype could result in selective disadvantage. This selection effect could explain the under-representation of 677TT/1298AC genotype in our study population. The *MTHFR* risk genotype 677CT/1298AC, known to be associated with decreased enzyme activity and increased homocysteine levels,<sup>18</sup> was found in association with homozygous mutant *RFC-1* 80GG more often in patients (11.3%) and mothers (11.5%) than in controls (1.6%).

Whether statistically confirmed in larger case-control studies, this result would demonstrate that combined influence of both intra- and intergenic mutations may play a role on NTD etiology. Furthermore, the finding that mothers and cases presented quadruple-mutation genotypes more frequently than controls highlighted the importance of assessing both maternal and fetal genotypic effects. Two previous reports establishing that homocysteine and folate status of the mother has an impact on NTD outcome have highlighted the need to verify whether the maternal genotype has a direct effect on NTD risk.<sup>39,40</sup> In fact, it is plausible that maternal genotype could play a particular pathogenic role in NTDs, either by limiting the supply of folate to the embryo or by facilitating the accumulation and increased transfer to the embryo of homocysteine, high concentrations of which can disrupt neural tube closure in experimental models. Unfortunately, owing to sparse data, we are unable to estimate potential NTD risk among mothers carrying four mutations in genes involved in folate-related pathways. The identification and characterization of such gene-gene interactions would require large enough sample sizes. There were too few mothers in our study who have been genotyped for both *MTHFR* and *RFC-1* polymorphisms to investigate adequately potential interactive effects of combined genotypes on NTD risk.

In conclusion, this case-control study demonstrates a significant association between the *RFC-1* 80A→G polymorphism and NTD risk, providing further validation for the hypothesis that low folate intake or impaired folate metabolism may play a role in NTD etiology. Since this is the first report on association between this variant and NTD susceptibility, additional studies on the role of gene-environment (folate intake) and gene-gene interactions are needed to confirm our data. Furthermore, since folate supplementation may overcome the effects of genetically determined reduction of folate intake, our results suggest a potential role of folate supplementation in prevention of NTD in at-risk population carrying the *RFC-1* variant alleles.

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