677T allele frequencies have been reported in Canadian Inuit (6.1%),[14] Manitoba neonates (24.97%),[15] French Canadians (38%)[16] and our neonatal (27.3%)[8] and adult (40%) control and study groups.[9] Considering the regional variations in MTHFR allele distributions, it is not completely surprising that some studies have recognised the common MTHFR mutations only in *trans* positions.[1–4] It is quite possible that in certain populations, such as on the island of Crete recombinant events between MTHFR 677T and 1298C mutations are very rare occurrences and identification of any *cis* mutations would require genotyping of a large number of individuals.

Although Zetterberg *et al*.[1] investigated early spontaneous abortions, with all foetal deaths occurring before 6 and 20 weeks of gestation, the inherent limitation of their study, as well as other studies investigating foetal viability,[8] is that very early spontaneous abortions are not examined in study populations. The true contribution of MTHFR mutations to reduced foetal viability may be difficult to determine, as the majority of pregnancies that spontaneously terminate before 6 weeks of gestation is not identified clinically. It is evident that additional large-scale population studies are required to fully elucidate the role of MTHFR mutations in contributing to decreased foetal viability. It also appears that regionally based studies are necessary to assess risks for specific populations.

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Reply to 'MTHFR C677T and A1298C polymorphisms and mutated sequences occurring in *cis*'

European Journal of Human Genetics (2002) **10,** 579-582. doi:10.1038/sj.ejhg.5200863

Isotalo and Donnelly comment in their letter (2002, in this issue) on the conflicting reports regarding the prevalence of MTHFR 677T and 1298C alleles occurring in *cis*. In our study we did not find any triple or quadruple MTHFR mutation combinations either in the spontaneous abortion group (n=80) or in the control group which

consisted of randomly chosen healthy blood donors (n=125), suggesting complete linkage disequilibrium between the two polymorphisms in these two groups of Cretan descent.[1] Moreover, we have until now analysed >500 individuals of Swedish descent and only detected 677T and 1298C alleles in *trans* configuration (unpublished data). Most studies in the field have reported no or few cases with 677T and 1298C alleles in the *cis* configuration.[2–9] We have recently analysed an Italian population consisting of 335 individuals (155 patients with arterial or venous thrombosis and 180 controls) using the minisequencing technique employed in our spontaneous abortion study.[1] We found two individuals from Southern and one from Northern Italy with 677TT/1298AC genotypes (Figure 1). The genotypes were verified by dideoxy sequencing using the ABI PRISM Big Dye™ Terminator Cycle Sequencing kit (PE Applied Biosystems). No 677CT/1298CC or 677TT/1298CC genotypes were detected. These data, together with the studies mentioned above, demonstrate that 677T and 1298C alleles in *cis* are possible, but indeed very rare, and support the hypothesis that triple mutation combinations probably reflect recombinant events having occurred either *de novo* or in the relatively late ancestral history of specific sub-populations. Obviously, it is not excluded that specific subpopulations, such as the ones studied by Isotalo *et al*.[10,11] may display higher frequencies of triple (and possibly quadruple) mutation combinations. In these sub-populations it is also possible that triple and quadruple mutation combinations may have a negative influence on embryonic survival. This would be in agreement with our results which demonstrated a negative influence of MTHFR mutated alleles on foetal viability in the absence of folic acid supplementation;[1] the more 677T and 1298C alleles in one individual, the worse clinical effects during times of folate deficiency, such as pregnancy.

In their letter (2002, in this issue), Isotalo and Donnelly clarify the ethnicity of their Canadian study and control populations in which a very high frequency of 677TT/1298AC genotypes was detected (9.2 and 12.5% for cases and controls, respectively).[11] The populations consisted of Canadians with a predominantly European (Celtic) descent. Following this clarification, it seems possible that the conflicting results between the studies by Weisberg *et al*.[3] and Isotalo *et al*.[11] with regard to the prevalence of triple mutation combinations might be explained by differences in ethnicity. However, Volcik *et al*.[12] also studied a Canadian population of European descent, composed of patients with spina bifida and their parents. Still, Volcik *et al*.[12] reported lower frequencies of 677T and 1298C alleles (32 and 35%, respectively, compatible with most European populations) than Isotalo *et al*.[11] (40 and 48%, respectively, of healthy controls). If anything, one would expect a higher frequency of 677T and 1298C alleles in a group consisting of spina bifida patients and their relatives. The Celtic influence in the study by Isotalo and Donnelly[11] does not likely explain

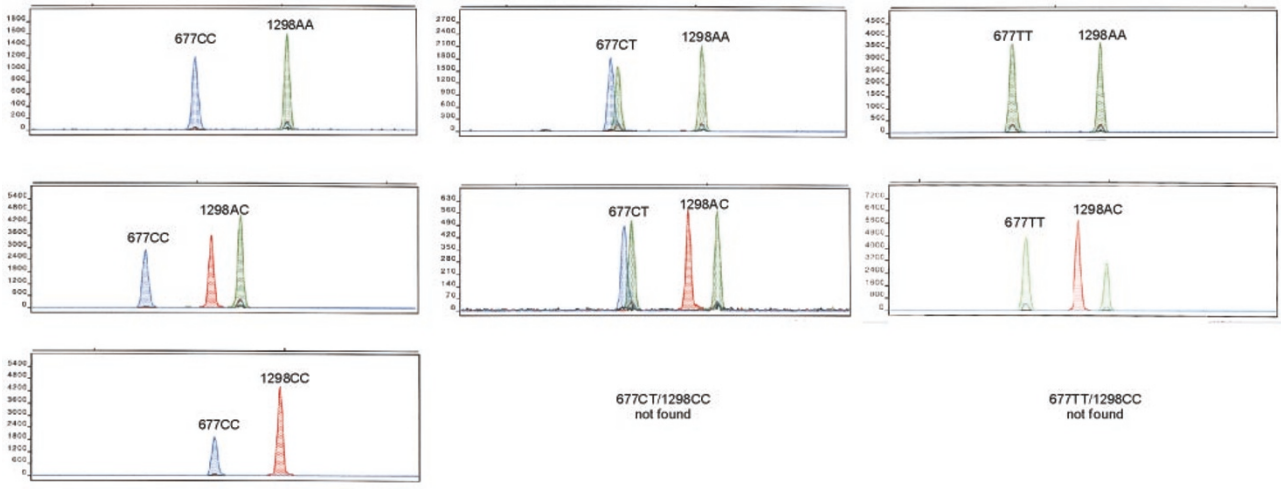

Figure 1 Multiplex minisequencing electropherograms for seven different MTHFR C677T and A1298C genotypes. The technique has been previously described in detail.[1] A blue peak represents a C nucleotide (wild type) at position 677 in the MTHFR gene. A green peak to the left in the electropherogram represents a T nucleotide at the same position. A green peak to the right in the electropherogram represents an A nucleotide (wild type) at position 1298 in the MTHFR gene. A red peak represents a C nucleotide at the same position. Three individuals with 677TT/1298AC genotypes were identified in an Italian population consisting of 335 individuals. No 677CT/1298CC or 677TT/1298CC genotypes were found.

the difference, since the 677T allele frequency in Celtic populations is lower (approximately 33%).[13] It should be remembered that patterns generated with the MTHFR C677T and A1298C restriction fragment length polymorphism (RFLP) techniques may be difficult to interpret. It is not excluded that the very diverse MTHFR C677T and A1298C allele frequencies reported in the literature, even in closely related or essentially identical populations with regard to ethnicity, to some extent could be explained by misidentification of genotype. Furthermore, one inherent problem with RFLP is its sensitivity to silent polymorphisms, which may affect restriction enzyme recognition sites and give false genotype results.

It is unfortunate that the reports of triple and quadruple MTHFR mutation combinations, especially the ones by Isotalo *et al*.[10,11] were not confirmed by independent techniques, such as DNA sequencing. We suggest that DNA sequencing should be included in the MTHFR genotyping algorithm when RFLP patterns compatible with 677TT/1298AC, 677CT/1298CC or 677TT/1298CC genotypes are detected. Future studies using this approach will most likely be helpful in revealing the true prevalence of mutated MTHFR alleles in *cis* configuration in different populations. Lastly, and this we are inclined to think is the most important element of our study,[1] the strong association between combined MTHFR C677T and A1298C mutated alleles and compromised foetal survival suggests that periconceptional folic acid supplementation may lower the incidence of spontaneous abortion and possibly increase the frequency of mutated alleles in adults. Compatible with this hypothesis, mandatory use of folic acid during pregnancy has been reported to increase the prevalence of 677T homozygosity in a Spanish population.[14]

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