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Association of *PEMT* rs4244593 Polymorphism with Non-syndromic Cleft Lip and Palate in the Indian Population

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ABSTRACT

Introduction: Non-syndromic cleft lip with or without cleft palate (NSCLP) is a multifactorial threshold trait (MFT) involving both genetic and environmental factors. Choline, methionine and folate metabolism are interrelated in converting the homocysteine to methionine. Phosphatidylethanolamine N-methyltransferase *(PEMT)* is involved in biosynthesis of choline. **Methods:** We studied the *PEMT* rs4244593 SNP to assess its effect on NSCLP risk in the South Indian population. Blood samples of 142 cases with NSCLP and 141 controls were collected and genotyped using PCR-RFLP. Statistical analysis of the results was performed by calculating OR, and 95% CI via χ^2 test. **Results:** Proportions of genotypes were 16.9 % AA, 64.8 % AC, 16.9 % CC in cases and 35.5 % AA, 47.5 % AC, 17.0 % CC in controls. The C allele frequency was 50.7% for cases and 40.8% for controls. An increased risk was found for co-dominant (AC vs. AA: OR =2.86, 95% CI =1.60 to 5.11, p<0.001; CC vs. AA: OR =2.26, 95% CI =1.08 to 4.72, p=0.029), dominant (AC+CC vs. AA: OR =2.70, 95% CI =1.55 to 4.72, p<0.001) and allelic models (C vs. A: OR =1.49, 95% CI =1.07 to 2.08, p=0.018). **Conclusions:** Although our results indicate that the *PEMT* rs4244593 polymorphism is one of the important genetic determinants of NSCLP risk in South Indian subjects, in the absence of mechanistic studies, this polymorphism cannot be considered as a determinant of NSCLP risk. Additional studies with fully validated functional SNPs and larger sample sizes are needed to confirm our findings.

Key words: Choline orofacial cleft, *PEMT* protein, SNP

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a multifactorial threshold trait (MFT) involving both genetic and environmental factors (Murthy & Bhaskar, 2009). Higher methionine trans-sulfuration and trans-methylation found respectively in the first and third trimesters during pregnancy suggest a higher requirement for methionine and folate during these periods (Dasarathy *et al.*, 2010). Furthermore, pregnant women in the absence of folate supplementation showed increased levels of homocysteine during the second and third trimesters pregnancies, probably due to increased need for folate, and declining circulating folate levels (McNulty *et al.*, 2013). Choline, a water soluble essential nutrient, plays an important role in the production of the

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phospholipid of cell membranes. Choline is the major source of labile methyl-groups; one of its metabolites betaine mediates homocysteine methylation to form methionine (Zeisel, 2006). Folate metabolism, methionine and choline are interrelated at the methionine formation step (Finkelstein, 2000). According to the Institute of Medicine (IOM), dietary reference intake (DRI) for choline is 550 mg/day and 425 mg/day for men and women respectively (Otten, Hellwig & Meyers, 2006).

The availability of choline is crucial throughout the fetal development and has a vital role in foetal brain development (Niculescu, Craciunescu & Zeisel 2005; Cheng *et al.*, 2008). Higher demand for choline by a developing foetus during pregnancy could put the mother and, subsequently, the embryo and its extraembryonic membranes at risk for choline deficiency (Zeisel *et al.*, 1995). Presence of some inhibitors of choline uptake and metabolism, produced neural tube defects in neurulating mouse embryos grown in vitro (Fisher *et al.*, 2002). This indicates that the maternal genotype determines the phenotype of the offspring. Maternal diets low in choline influence neural tube development (Fisher *et al.*, 2002) and increase the risk of having a new born with birth defects (Fisher *et al.*, 2002; Shaw *et al.*, 2006). Although choline and choline esters can be obtained through food (Otten *et al.*, 2006), the human body is capable of synthesising choline through de novo phosphatidylcholine biosynthesis catalysed by phosphatidylethanolamine N-methyltransferase(*PEMT*, EC 2.1. 1.17) (Hirata *et al.*, 1978; Schneider & Vance 1979). The gene encoding phosphatidylethanolamine N-methyltransferase is located on chromosome 17p11.2. As polymorphisms in the human *PEMT* gene alter biosynthesis of choline, some studies have been conducted to investigative the association of diseases related to choline deficiency and *PEMT* gene polymorphisms (Zhang *et al.*, 2006; Bi *et al.*, 2012; Li *et al.*, 2009, Romeo, Cohen & Hobbs, 2006). In this study, we examined the association between cleft lip and palate and *PEMT* rs4244593 (intron 3) genotypes in a South Indian population.

METHODS

Subjects

We evaluated 142 unrelated patients with NSCLP [123 cleft lip and palate (CLP) and 19 cleft palate only (CPO)] and 141 individuals as control from Cleft and Craniofacial Centre, Sri Ramachandra University, Chennai, India. All cases were enrolled in the study from May 2012 to April 2014 and were evaluated by two different surgeons for their individual phenotypic features; this was cross-verified through their medical records. Unrelated individuals without clefts or family history of clefting for three generations were considered as controls. The subjects who presented with congenital malformations or major developmental disorders were excluded from the study. This study was approved by the Institutional Ethics Committee of Sri Ramachandra University, Chennai, India, and written informed consent was obtained from all study subjects. As many of the children were minors, consent was obtained from their parents or legal guardians.

Genotyping

Blood samples (3 ml) were collected into an EDTA vaccutainer (Becton Dickinson, Franklin Lakes, NJ) for DNA extraction. Genomic DNA was isolated from leucocytes using phenol-chloroform extraction and ethanol precipitation (Sambrook & Russell, 2001). *PEMT* rs4244593 SNP genotyping was performed following polymerase chain reaction-restriction fragment length polymorphism method (Mostowska *et al.*, 2010b). Briefly, 637 basepair (bp) fragment

Table 1. Genotype distribution and allele frequencies of the *PEMT* rs4244593 in cleft lip and palate

	Control (%)	*Overall clefts (%)*	*CLP (%)*	*CPO (%)*
	Genotype distribution			
AA	50 (35.5)	24 (16.9)	19 (15.5)	5 (26.3)
AC	67 (47.5)	92 (64.8)	81 (65.9)	11 (57.9)
CC	24 (17.0)	26 (18.3)	23 (18.7)	3 (15.8)
	Allele frequency			
A allele	167 (59.2)	140 (49.3)	119 (48.4)	21 (55.3)
C allele	115 (40.8)	144 (50.7)	127 (51.6)	17 (44.7)
	Test for HWE			
Chi-square	0.037	12.44	12.48	0.555
P-value	0.847	<0.001	<0.001	0.456

of *PEMT* rs4244593 region was amplified with the primers of 5′-CTG CCT CCT CAC GAC CTG TA-3′ and 5′-GCG TGG TCC TCC ACT CTT TC-3′. The PCR products were digested with restriction enzyme TaqI (Fermentas Life Sciences, Germany) at 65°C for 4 h for *PEMT* rs4244593 genotyping and detected by electrophoresis on 2% agarose gel. Upon digestion, 637-bp PCR product remained uncleaved for the C allele and but was cleaved into smaller fragments of 401-bp and 236-bp in the case of the A allele. The digested products were stained with ethidium bromide and visualised under UV light.

Statistical analysis

Allele frequencies were calculated based on the observed number of the two different alleles, A and C. Hardy-Weinberg equilibrium (HWE) analysis was performed by comparison of observed and expected genotype frequencies using chi-squared goodness-of-fit test. Association between *PEMT* rs4244593 polymorphism and different cleft phenotypes (NSCLP, CLP and CPO) was analysed by χ^2-test. To compare all three cases, we considered the same control group (n=141). The risk estimates were examined in four hypothetical risk models, co-dominant, dominant (AC+CC vs. AA), recessive (AC+AA vs. CC) and allelic (C vs. A) models. Statistical analysis was performed using SPSS statistical software version 16.0 (SPSS Inc, Chicago, Illinois) for Windows. A two-sided p-value <0.05 was considered to be statistically significant. From the HapMap 20kb up and downstream SNPs around rs4244593 were extracted and LD maps were constructed using Haploview (Barrett *et al.*, 2005).

RESULTS

Genotype and allelic distribution for the *PEMT* rs4244593 polymorphism are presented in Table 1. Proportions of genotypes were 16.9 % AA, 64.8 % AC, 16.9 % CC in cases and 35.5 % AA, 47.5 % AC, 17.0 % CC in controls. The C allele frequency was 50.7% for cases and 40.8% for controls. The HardyWeinberg equilibrium test showed that the genotypic distribution was in equilibrium in the controls

Table 2. Results of association tests with *PEMT* rs4244593 SNP in cleft lip and palate

PEMT rs4244593 A/C	*OR (95% CI)*	*P-value*
	Overall clefts	
AA	Reference	
AC	2.86 (1.60-5.11)	<0.001
CC	2.26 (1.08-4.72)	0.029
AC+CC vs. AA	2.70 (1.55-4.72)	<0.001
AC+AA vs. CC	0.91 (0.50-1.69)	0.776
A allele	Reference	
C allele	1.49 (1.07-2.08)	0.018
	CLP	
AA	Reference	
AC	3.18 (1.71-5.91)	<0.001
CC	2.52 (1.16-5.49)	0.018
AC+CC vs. AA	3.01 (1.65-5.47)	<0.001
AC+AA vs. CC	0.89 (0.47-1.68)	0.722
A allele	Reference	
C allele	1.55 (1.10-2.19)	0.013
	CPO	
AA	Reference	
AC	1.64 (0.54-5.03)	0.381
CC	1.25 (0.28-5.67)	0.772
AC+CC vs. AA	1.54 (0.52-4.52)	0.431
AC+AA vs. CC	1.09 (0.29-4.05)	0.893
A allele	Reference	
C allele	1.18 (0.59-2.33)	0.642

(P=0.847). The frequency of the C allele of the *PEMT* rs4244593 polymorphism was 40.8% among control individuals. The genotype and allele frequencies were significantly different between cases and controls (Table 2). Table 2 represents the OR and 95% CI that were calculated to assess the effect of the *PEMT* rs4244593 variant on NSCLP risk. Both heterozygous (OR=2.86; 95% CI: 1.60-5.11; $p<0.001$) and homozygous (OR=2.26; 95% CI: 1.08-4.72; p=0.029) genotypes significantly increased NSCLP risk. In the dominant model, the risk of NSCLP remained the same as for the co-dominant model but the level of significance increased (OR=2.70; 95% CI: 1.55-4.72; $p<0.001$). Whereas in the recessive model (AC+AA vs. CC), no significant risk for this variant (OR=0.91; 95% CI: 0.50-1.69; P=0.776) was observed. The allelic model also showed a significant positive association between C allele and susceptibility to oral clefts, but this effect was modest (OR=1.49; 95% CI: 1.07-2.08; p<0.018). In subgroup analysis, the rs4244593 variant showed a similar trend of association for CLP group (Table 2). The *PEMT* rs4244593 polymorphism did not show a significant association with CPO risk in three different models (Table 2). The *PEMT* rs4244593 variant allele frequencies collected from various populations are presented in Figure 1. Data revealed that the frequency of the *PEMT* rs4244593

Reference	2N	AA	AC	CC	HWEp
Present study	141	50	67	24	0.847
GIH (Asian)	176	46	96	34	0.371
HCB (Asian)	86	20	46	20	0.655
JPT (Asian)	172	38	94	40	0.403
CHB (Asian)	82	24	34	24	0.294
CHD (Asian)	170	58	86	26	0.655
AFD_CHN (Asian)	38	6	22	10	0.479
EGP_ASIAN (Asian)	42	6	28	8	0.15
CEU (European)	226	40	102	84	0.527
AFD_EUR (European)	46	12	24	10	1.0
EGP_CEPH (European)	34	12	8	14	0.05
MEX (Mexican)	100	20	54	26	0.584
TSI (Italians)	176	36	96	44	0.403
Mostowska et al. 2010b (Polish)	166	33	89	44	0.321
EGP_HISP (Hispanic)	36	8	22	6	0.343
YRI (Sub-Saharan African)	226	10	80	136	1.0
ASW (African American)	98	8	36	54	0.752
LWK (African)	180	2	34	144	1.0
MKK (African)	282	18	102	162	1.0
AFD_AFR (African American)	44	0	18	26	0.479
EGP_AD (African American)	26	0	10	16	0.655

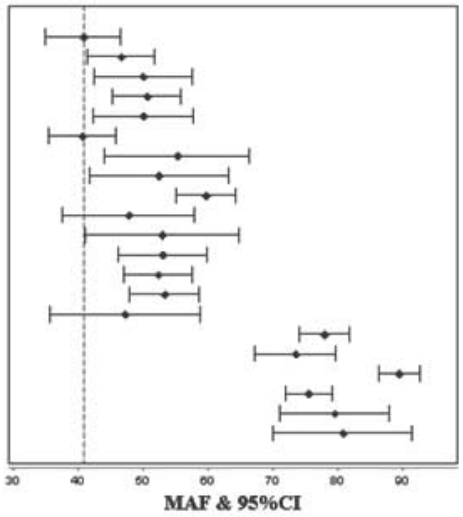


Figure 1. The frequency of *PEMT* rs4244593 SNP in the current study compared to the world populations.

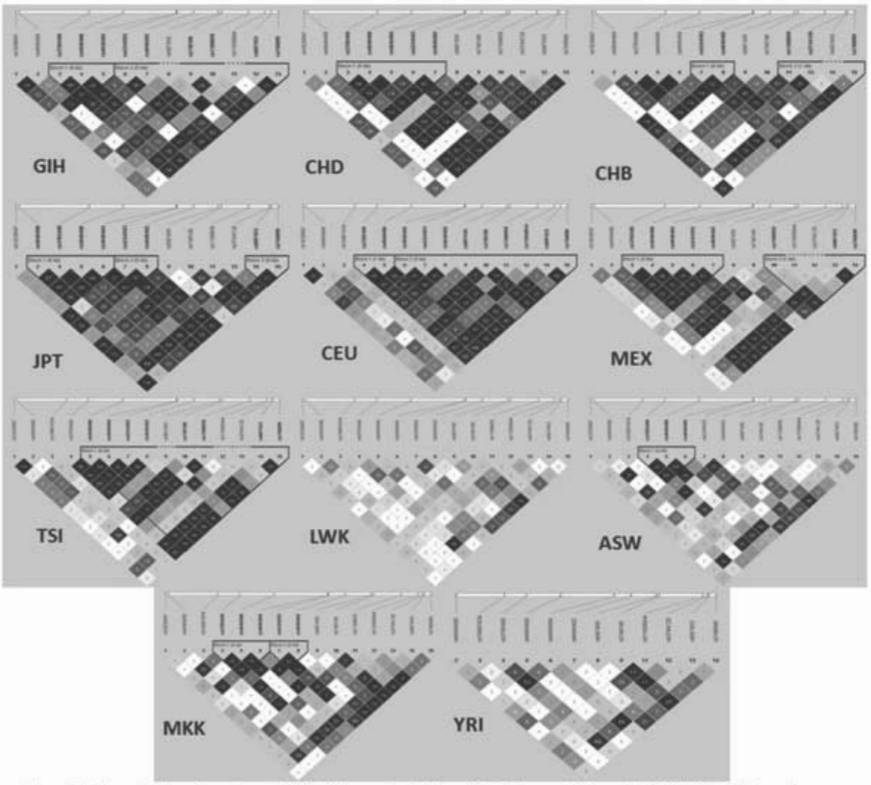
Note: **Forest plot rep resent minor allele frequency (MAF) with 95% confidence interval (CI). Data from previous studies is denoted by the citations. N: sample size; GG, AG and AA are genotypes; HWp: Hardy-Weinberg equilibrium *p* value.**

minor allele in Indian populations are showing slight propinquity with the frequencies of East Asian ($p>0.05$) and European populations ($p>0.05$), but it is fairly dissimilar from African populations ($p<0.001$). Calculation of LD in the 20 kb up and downstream regions from *PEMT* rs4244593 loci in HapMap populations revealed that the East Asian populations showed larger LD blocks followed by the European populations. These blocks are very small in African populations (Figure 2).

DISCUSSION

The *PEMT* rs4244593 minor allele frequency (C allele) in our controls was 40.8%, which is slightly less than that (47%) found in the GIH population of HapMap (*http://hapmap.ncbi.nlm.nih.gov*). This discrepancy may be due to the variations in source of samples included in the HapMap. The highest minor allele frequency of C allele was observed in African populations followed by European and Asian populations of HapMap. The *PEMT* rs4244593 polymorphism is significantly associated with cleft lip and palate in co-dominant, dominant and allelic models.

Some of the enzymes like cystathionine beta-synthase (CBS), methionine synthase reductase (MTR) and 5,10-methylenetetrahydrofolate reductase (MTHFR) are directly involved in folate-Hcy metabolism, while *PEMT* is indirectly coupled to folate-Hcy metabolism, by modulating its metabolite profile through the choline pathway. Previous studies showed higher dietary intakes of choline decreased risk of CLP (Shaw *et al.*, 2006), in contrast to this higher plasma choline increased CLP risk (Shaw *et al.*, 2009) and neural tube defects (Shaw *et al.*, 2004). *PEMT* knockout mice showed substantially diminished concentrations of docosahexaenoic acid (DHA) in plasma



Gujarati Indians in Houston, Texas (GIH), Chinese in Metropolitan Denver, Colorado (CHD), Han Chinese in Bejing, China (CHB), Japanese in Tokyo, Japan (JPT), Utah residents with Northern and Western European ancestry from the CEPH collection (CEU), Mexican ancestry in Los Angeles, California (MEX), Toscans in Italy (TSI), Luhya in Webuye, Kenya (LWK), African ancestry in Southwest USA (ASW), Maasai in Kinyawa, Kenya (MKK) and Yoruba in Ibadan, Nigeria (YRI).

Figure 2. Linkage disequilibrium profiles in different world populations studied in International HapMap Project.
Note: Colour coding represents the D′/LOD values and the values in cells are r2 multiplied by 100.

and of liver phosphatidylcholine (PtdCho) (Watkins, Zhu & Zeisel, 2003).

PEMT gene is highly polymorphic; direct sequencing of *PEMT* gene in 48 Japanese individuals revealed 98 SNPs (Saito *et al.*, 2001). The rs12325817 (-744 G/C) SNP is located in the promoter region of *PEMT* gene and is associated with increased risk to choline deficiency syndrome in women (da Costa *et al.*, 2006). The exact function of this SNP is not known because it does not fall within a mapped transcription factor binding site nor is it assessed in the HapMap study(Figure 2) (Frazer *et al.*, 2007). A functionally significant SNP (rs7946: Val175Met) in *PEMT* exon 6 region results in a 30% loss of function and is associated with increased risk of non-alcoholic fatty liver disease (Song *et al.*, 2005; Dong *et al.*, 2007). Polymorphisms of *PEMT* were not independently correlated with NSCLP risk in the Polish population. However, considerable epistatic interaction between the polymorphisms of MTHFR (Ala222Val), MTR (A2756G) and *PEMT* (rs4646406), indicate interaction between choline and folate metabolisms in the pathogenesis of NSCLP (Mostowska *et al.*, 2010b). Similar epistatic interaction between rs1801133

of MTHFR and rs4244593 of *PEMT* in endometriosis-associated infertility has been reported (Szczepanska *et al.*, 2011). Another Polish population study, the gene-gene interaction analysis revealed a significant epistatic interaction of BHMT2 (rs673752), *PEMT* (rs12325817), and PCYT1A (rs712012) with maternal NSCLP susceptibility (Mostowska *et al.*, 2010a). In contrast to the present study, the above two studies (Mostowska *et al.*, 2010b, Szczepanska *et al.*, 2011) considered "A" allele as risk allele in their analysis because it is a minor allele in the Polish population. Further, the *PEMT* rs4244593 is an A to C transversion, and C allele is the minor allele and therefore the risk allele in this study.

In conclusion, our analyses suggest that *PEMT* rs4244593 may contribute to the risk of NSCLP in the Indian population. In the absence of mechanistic studies, additional studies with fully validated functional SNPs and larger sample sizes are needed to confirm our findings.

Conflict of interest

There is no conflict of interest.

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REFERENCES

Barrett JC, Fry B, Maller J, Daly MJ *et al.* (2005). Haploview: analysis and visualisation of LD and haplotype maps. *Bioinformatics* 21(2): 263-265.

Bi XH, Zhao HL, Zhang ZX & Zhang JW *et al.* (2012). PEMT G523A (V175M) is associated with sporadic Alzheimer's disease in a Chinese population. *J Mol Neurosci* 46(3): 505-508.

Cheng RK, MacDonald CJ, Williams CL, Meck WH *et al.* (2008). Prenatal choline supplementation alters the timing, emotion, and memory performance (TEMP) of adult male and female rats as indexed by differential reinforcement of low-rate schedule behavior. *Learn Mem* 15(3): 153-162.

da Costa KA, Kozyreva OG, Song J, Galanko JA *et al.* (2006). Common genetic polymorphisms affect the human requirement for the nutrient choline. *FASEB J* 20(9): 1336-1344.

Dasarathy J, Gruca LL, Bennett C, Parimi PS *et al.* (2010). Methionine metabolism in human pregnancy. *Am J Clin Nutr* 91(2): 357-365.

Dong H, Wang J, Li C, Hirose A, Nozaki Y et al. (2007). The phosphatidylethanolamine N-methyltransferase gene V175M single nucleotide polymorphism confers the susceptibility to NASH in the Japanese population. *J Hepatol* 46(5): 915-920.

Finkelstein, JD (2000). Pathways and regulation of homocysteine metabolism in mammals. *Semin Thromb Hemost* 26(3): 219-225.

Fisher MC, Zeisel SH, Mar MH & Sadler TW (2002). Perturbations in choline metabolism cause neural tube defects in mouse embryos in vitro. *FASEB J* 16(6): 619-621.

Frazer KA, Ballinger DG, Cox DR, Hinds DA *et al.* (2007). A second generation human haplotype map of over 3.1 million SNPs. *Nature* 449(7164): 851-861.

Hirata F, Viveros OH, Diliberto EJ Jr, Axelrod J *et al.* (1978). Identification and properties of two methyltransferases in conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc Natl Acad Sci USA* 75(4): 1718-1721.

Li H, Zhang H, Liu L, Ju G *et al.* (2009). No association of the rs4646396 SNP in the PEMT locus with schizophrenia in a Chinese case-control sample. *Psychiatry Res* 169(2): 176-177.

McNulty B, McNulty H, Marshall B, Ward M *et al.* (2013). Impact of continuing folic acid after the first trimester of pregnancy: Findings of a randomised trial of folic acid supplementation in the second and third trimesters. *Am J Clin Nutr* 98(1): 92-98.

Mostowska A, Hozyasz KK, Wojcicki P, Biedziak B *et al.* (2010a). Association between genetic variants of reported candidate genes or regions and risk of cleft lip with or without cleft palate in the polish population. *Birth Defects Res A Clin Mol Teratol* 88(7): 538-545.

Mostowska A, Hozyasz KK, Wojcicki P, Dziegelewska M *et al.* (2010b). Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. *J Med Genet* 47(12): 809-815.

Murthy J & Bhaskar L (2009). Current concepts in genetics of nonsyndromic clefts. *Indian J Plast Surg* 42(1): 68-81.

Niculescu MD, Craciunescu CN & Zeisel SH (2005). Gene expression profiling of choline-deprived neural precursor cells isolated from mouse brain. *Brain Res Mol Brain Res* 134(2): 309-322.

Otten JJ, Hellwig JP & Meyers LD (2006). DRI, Dietary Reference Intakes : The Essential Guide to Nutrient Requirements, Washington, DC: National Academies Press.

Romeo S, Cohen JC & Hobbs HH (2006). No association between polymorphism in PEMT (V175M) and hepatic triglyceride content in the Dallas Heart Study. *FASEB J* 20(12): 2180; author reply 2181-2182.

Saito S, Iida A, Sekine A, Miura Y *et al.* (2001). Identification of 197 genetic variations in six human methyltranferase genes in the Japanese population. *J Hum Genet* 46(9): 529-537.

Sambrook J & Russell DW 2001. Molecular Cloning : A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schneider WJ & Vance DE (1979). Conversion of phosphatidylethanolamine to phosphatidylcholine in rat liver. Partial purification and characterisation of the enzymatic activities. *J Biol Chem* 254(10): 3886-3891.

Shaw GM, Carmichael SL, Laurent C, Rasmussen SA *et al.* (2006). Maternal nutrient intakes and risk of orofacial clefts. *Epidemiology* 17(3): 285-291.

Shaw GM, Carmichael SL, Yang W, Selvin S et al. (2004). Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. *Am J Epidemiol* 160(2): 102-109.

Shaw GM, Vollset SE, Carmichael SL, Yang W *et al.* (2009). Nested case-control study of one-carbon metabolites in mid-pregnancy and risks of cleft lip with and without cleft palate. *Pediatr Res* 66(5): 501-506.

Song J, da Costa KA, Fischer LM, Kohlmeier M *et al.* (2005). Polymorphism of the PEMT gene and susceptibility to nonalcoholic fatty liver disease (NAFLD). *FASEB J* 19(10): 1266-1271.

Szczepanska M, Mostowska A, Wirstlein P, Lianeri M *et al.* (2011). Polymorphic variants of folate and choline metabolism genes and the risk of endometriosis-associated infertility. *Eur J Obstet Gynecol Reprod Biol* 157(1): 67-72.

Watkins SM, Zhu X & Zeisel SH (2003). Phosphatidylethanolamine-N-methyltransferase activity and dietary choline regulate liver-plasma lipid flux and essential fatty acid metabolism in mice. *J Nutr* 133(11): 3386-3391.

Zeisel SH (2006). Choline: critical role during fetal development and dietary requirements in adults. *Annu Rev Nutr* 26(229-250.

Zeisel SH, Mar MH, Zhou Z, da Costa KA *et al.* (1995). Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. *J Nutr* 125(12): 3049-3054.

Zhang J, Zhu H, Yang W, Shaw GM *et al.* (2006). Phosphatidylethanolamine N-methyltransferase (PEMT) gene polymorphisms and risk of spina bifida. *Am J Med Genet A* 140(7): 785-789.