

Methylenetetrahydrofolate reductase gene polymorphism as a risk factor for diabetic nephropathy: a meta-analysis

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Abstract Investigations into the association between diabetic nephropathy (DN) and MTHFR C677T gene polymorphism in several case–control studies has yielded contradictory results. To shed light on these inconclusive findings, a meta-analysis of all available studies relating the C677T polymorphism to the risk of developing DN was conducted. The PubMed database was searched, and case–control studies investigating the association between MTHFR C677T gene polymorphism and DN were included in the meta-analysis. The meta-analysis included 15 studies, of which 8 involved Caucasians and 5 East Asians; 11 studies involved subjects with type 2 diabetes and 4

with type 1 diabetes. The main analysis (all studies) revealed significant heterogeneity between the studies ($P_Q < 0.01$) and a marginal association between the 677T allele and the risk of developing DN; the random effects (RE) pooled odds ratio (OR) was 1.30 (1.03–1.64). However, the sensitivity analysis (exclusion of studies not in Hardy–Weinberg equilibrium) produced non-significant results. The recessive model derived significant results in main analysis [fixed effects (FE) OR = 1.32 (1.10–1.58), $P_Q = 0.27$], and in type 2 diabetes [FE OR = 1.30 (1.06–1.60), $P_Q = 0.38$]. The additive model produced significant association in main analysis [RE OR = 1.65 (1.13–2.42), $P_Q < 0.01$] in Caucasians [FE OR = 1.48 (1.11–1.98), $P_Q = 0.17$] and in type 2 diabetes [RE OR = 1.65 (1.03–2.67), $P_Q < 0.01$]. However, sensitivity analysis diminished the significant results in type 2 diabetes. There is no differential magnitude of effect in large versus small studies. In conclusion, although there is some evidence of association between MTHFR C677T gene polymorphism and DN, the above findings reinforce the need for further and more rigorous association studies.

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Introduction

Diabetic nephropathy (DN) is a serious chronic microangiopathic complication of both type 1 and type 2 diabetes, and is the primary cause of end-stage renal failure (Gross et al. 2005). The etiology of DN is multifactorial and involves both environmental and genetic factors (Shpichinetsky et al. 2000; Zintzaras and Stefanidis 2005).

A familial clustering of DN indicated that a genetic predisposition is implicated in the pathogenesis of DN in both types of diabetes (Borch-Johnsen et al. 1992; Quinn et al. 1996; Strojek et al. 1997). Methylenetetrahydrofolate reductase (MTHFR) catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for transmethylation of homocysteine to methionine. An elevated plasma homocysteine level has been identified as an independent risk factor for macroangiopathy (cerebrovascular or coronary artery disease), and for arterial or venous thrombosis (Bostom et al. 2001; Boushey et al. 1995; McCully 1996). A C677T transition is a common ubiquitous missense mutation in the coding region of the MTHFR gene (chromosome 1p36.3) (Goyette et al. 1994), causing an alanine to valine (Al222Val) amino acid substitution located in the anticipated catalytic domain of the enzyme (Frosst et al. 1995) and resulting in a thermo-labile MTHFR variant with reduced catalytic activity. Homozygosity for the mutation (TT genotype) predisposes to significantly elevated plasma homocysteine levels (Frosst et al. 1995; Kang et al. 1991). Thus, MTHFR C677T gene polymorphism associated with a predisposition to increased plasma homocysteine levels may present a genetic risk factor for DN (Sun et al. 2004), a form of diabetic microangiopathy.

Case-control studies investigating the association between C677T gene polymorphism and DN have so far provided conflicting or inconclusive results. Each study involved only a small number of cases and controls, and data interpretation was complicated by the fact that different populations and sampling strategies were used. In order to shed some light on these controversial results, as well as to decrease the uncertainty of the effect size of estimated risk, a meta-analysis of all available studies relating the C677T polymorphism of the MTHFR gene to the risk of developing DN was carried out. In this meta-analysis, a pooled estimate of the risk was obtained for the allele contrast, and the additive and dominance models. In addition, the heterogeneity between studies and the existence of bias were investigated.

Materials and methods

Selection of studies

All studies published before January 2007 were identified by extended computer-based searches of the PubMed database. The following search criterion was used: (“MTHFR” or “Methylenetetrahydrofolate reductase” or “C677T”) and “diabetic nephropathy”. The retrieved studies were then read in their entirety to assess their appropriateness for inclusion in the meta-analysis. All

references cited in the studies were also reviewed to identify additional published work not indexed by the PubMed database. Case reports, editorials and review articles were excluded. The search was restricted to articles in English.

Case-control studies that determine the genotype distribution of the C677T polymorphism in cases with diabetes and nephropathy, and in controls with diabetes and free of diabetic nephropathy were eligible for inclusion in the meta-analysis. Cases with diabetes were considered as diabetic nephropathy on the basis of a persistent albuminuria (i.e. macroalbuminuria, equivalent to an overt glomerular proteinuria) or a persistent microalbuminuria with or without chronic renal insufficiency and in the absence of non-diabetic renal disease. Case groups that consisted exclusively of subjects with microalbuminuria were excluded from the meta-analysis. The control groups consisted of subjects with diabetes and free of diabetic kidney disease, i.e. normoalbuminuria (urinary albumin excretion < 20 mg/24 h or <20 µg/min) and normal renal function. The different methods used in the studies to determine urinary albumin excretion and the respective cut-offs to define macro- or microalbuminuria were all equivalent and previously clinically validated (Gross et al. 2005) (Table 1). Only studies in human subjects and having used validated genotyping methods were considered (Frosst et al. 1995). Genome scans were excluded since they investigate linkage (Zintzaras and Ioannidis 2005a), and family-based studies association studies were also not considered because of different design considerations.

Data extraction

From each study the following information was extracted: first author, journal, year of publication, ethnicity of the study population, demographics, clinical characteristics, matching, validity of the genotyping method, and the number of cases and controls. The frequencies of the alleles and the genotypic distributions were extracted or calculated for both cases and controls. In addition, whether the genotyping in each study was blinded to clinical status was recorded.

Meta-analysis

The meta-analysis examined the overall association of the T allele with the risk of DN relative to the C allele, the recessive model for T allele (TT vs TC + CC), the dominant model for T allele (TC + TT vs CC), the additive model for allele T (TT vs CC), and the co-dominance model (TC vs TT + CC). All associations were indicated as odds ratios (OR) with the corresponding 95% confidence

Table 1 Characteristics of the studies considered in the meta-analysis. *DM* diabetes mellitus, *SD* standard deviation, *AER* albumin excretion rate, *ACR* albumin/creatinine ratio, *ESRD* end-stage renal disease, *CRF* chronic renal failure, *BMI* body mass index

First author, year	Ethnicity	Type of DM	Cases			Controls				
			No. (M/F)	Age (years) ^a	DM duration (years) ^a	Selection criteria	No. (M/F)	Age (years) ^a	DM duration (years) ^a	Matched
Neugebauer 1998	East Asian	2	67	59 (57–61)	15 (14–16)	Persistent proteinuria (ACR > 230 mg/g creatinine)	63	53 (51–55)	14 (13–15)	–
Shcherbak 1999	Caucasian	1	51 (23/28)	32 ± 12	9.98 ± 3.22	Treatment for renal disease, persisting proteinuria (AER > 300 mg/24 h) or albuminuria (AER > 30 mg/24 h)	90 (41/49)	28 ± 9	8.97 ± 3.68	–
Fujita 1999	East Asian	2	105 (62/43)	60 ± 12	>15	Persistent macroalbuminuria (AER > 200 µg/min)	68 (32/36)	62 ± 10	NP ^b	Age, BMI, HbA1c
Bluthner 1999	Caucasian	2	147 (72/75)	64.1 ± 9.6	19.9 ± 7.4	Persistent albuminuria (AER > 20 µg/min)	146 (67/79)	62.7 ± 9.3	17.0 ± 7.0	Age–gender
Bluthner 1999	Caucasian	1	102 (56/46)	44.5 ± 13.9	23.7 ± 8.6	Persistent albuminuria (AER > 20 µg/min)	148 (79/69)	44.2 ± 14.2	21.8 ± 8.2	Age–gender
Smyth 1999	Caucasian	1	131	NP	>10	Persistent proteinuria (>0.5 g/24 h)	119	NP	>20	–
Odawara 1999	East Asian	2	143 (77/66)	59.4 ± 12.2	15.5 ± 10.8	Macroalbuminuria (>300 mg/24 h)	131 (86/45)	57.4 ± 10.6	13.7 ± 13.0	Age, gender, DM duration, age at onset, BP
Shpichnetsky 2000	Mixed	2	55 (26/29)	62.0 ± 6.6	10.8 ± 7.7	Persistent macro- or microalbuminuria (>30 mg/24 h)	43 (15/28)	62.9 ± 8.4	11.8 ± 9.3	Gender, age, DM duration
Makita 2003	Caucasian	1	88 (46/42)	40 ± 7	24.0 ± 7	Persistent proteinuria or ESRD	310 (143/167)	35 ± 9	23.0 ± 8.0	–
Moczulski 2003	Caucasian	2	121 (56/65)	62.4 ± 9.0	11.6 ± 7.12	Overt proteinuria (ACR ≥ 28.2 and ≥40.2 mg/mmol creatinine for men and women respectively) or CRF (creatinine ≥1.5 mg/dl) or dialysis	159 (52/107)	64.2 ± 7.7	15.8 ± 5.5	Age at diagnosis, BMI
Sun 2004	E. Asian	2	124 (55/69)	56.8 ± 11.3	8.8 ± 4.2	Persistent macroalbuminuria (AER > 200 µg/min) or microalbuminuria (AER > 20 µg/min)	96 (44/52)	58.9 ± 10.7	9.7 ± 3.8	Age
Ksiazek 2004	Caucasian	2	171 (78/93)	60.2 ± 11.2	11 ± 6.7	Persistent proteinuria (>300 mg/24 h)	155 (72/83)	58.1 ± 9.3	9.8 ± 5.1	–
Yoshioka 2004	East Asian	2	40	65.2 ± 11.8	14.9 ± 9.0	Macroalbuminuria (ACR > 300 mg/g creatinine)	207	59.3 ± 10.2	10.5 ± 8.0	–
Mitraoui 2007	Arab	2	93 (47/46)	59.3 ± 10.7	12.7 ± 3.0	AER > 30 mg/24 h and/or high plasma creatinine (>176 µmol/L)	267 (118/149)	57.3 ± 10.4	12.3 ± 4.1	–
Boger 2007	Caucasian	2	439 (241/198)	65 ± 8.3	15.4 ± 9.5	ESRD and dialysis	148 (69/79)	63.4 ± 10.5	15.2 ± 8.5	–

^a Given as mean ± SD or as mean(min–max)

^b Data not provided

interval (CI). A pooled OR was then estimated based on the individual ORs.

The heterogeneity between studies was tested using the Q -statistic, which is a weighted sum of squares of the deviations of individual study OR estimates from the overall estimate (Ioannidis et al. 2006; Zintzaras and Ioannidis 2005b). Heterogeneity was considered statistically significant if $P_Q < 0.10$. Heterogeneity was quantified with the I^2 metric, which is independent of the number of studies in the meta-analysis (Zintzaras and Hadjigeorgiou 2004). I^2 takes values between 0 and 100%, with higher values denoting a greater degree of heterogeneity ($I^2 = 0$ –25%: no heterogeneity; $I^2 = 25$ –50%: moderate heterogeneity; $I^2 = 50$ –75%: large heterogeneity; $I^2 = 75$ –100%: extreme heterogeneity; when $I^2 < 0$ then $I^2 = 0$).

The pooled OR was estimated using fixed effects (Mantel-Haenszel) and random effects (DerSimonian and Laird) models (DerSimonian and Laird 1986). The calculation of pooled OR and Q used as a weighting factor (w_i), the inverse variance of $\theta_i = \ln\text{OR}$ [i.e. $w_i = 1/\text{var}(\theta_i)$] of each study i . Random effects modelling assumes a genuine diversity in the results of various studies and incorporates a between-study variance into the calculations. Therefore, when there is heterogeneity between studies, the pooled OR is estimated using the RE model (Whitehead 2002).

A cumulative and recursive cumulative meta-analysis was carried out for each polymorphism to evaluate the trend of RE OR in time for the genetic contrast under investigation (Lau et al. 1992; Zintzaras et al. 2006a). In cumulative meta-analysis, studies were chronologically ordered by publication year; the pooled ORs were then obtained at the end of each year, i.e. at each information step. In recursive cumulative meta-analysis, the relative change in pooled OR in each information step (OR in next year/OR in current year) was calculated (Zintzaras et al. 2006b). Thus, cumulative and recursive cumulative meta-analysis provide a framework for updating a genetic effect from all studies and a measure of how much the genetic effect changes as evidence accumulates (Zintzaras 2007).

A differential magnitude of effect in large versus small studies (Zintzaras 2006a; Zintzaras and Hadjigeorgiou 2005) for the genetic contrast under investigation was checked using the Egger regression test for funnel plot asymmetry (Egger et al. 1997a, b) and the Begg–Mazumdar test, which is based on Kendall's tau (Begg and Mazumdar 1994). Given that these tests are underpowered, they were always considered statistically significant for $P < 0.10$ rather than for $P < 0.05$. Whether the OR of the allele contrast in the first study versus the pooled OR of subsequent studies were different beyond chance ($P < 0.05$) was assessed using the Z -statistic (Zintzaras et al. 2005).

In addition to the main (or overall) analysis, which included all available data, subgroup analysis for each

ethnic group and diabetes type was also performed. The distribution of the genotypes in the control group was tested for Hardy–Weinberg equilibrium (HWE) using an exact test ($P \geq 0.05$) (Weir 1996). Studies with controls not in HWE were subjected to a sensitivity analysis (Zintzaras 2006c). Lack of HWE indicates possible genotyping errors and/or population stratification (Zintzaras and Hadjigeorgiou 2004). In sensitivity analysis, the effect of excluding specific studies was examined. Analyses were performed using Meta-Analyst (Joseph Lau, Boston, MA, USA 1998) and CVF90 with IMSL library (Zintzaras et al. 2006d; Zintzaras 2006b).

Results

Eligible studies

The literature review identified 29 titles in PubMed that met the search criterion. The full articles of the retrieved studies were read to assess their appropriateness for meta-analysis according to the inclusion criteria. Data from 14 articles that investigated the association C677T and DN met the inclusion criteria and were included in the meta-analysis. One article (Bluthner et al. 1999) provided data on both type 1 and type 2 diabetes separately. Thus, data were obtained from 15 studies. Data were extracted by two investigators (G.K. and K.U.) and disagreements were resolved by discussion. The studies were published between 1998 and 2007.

In all studies, validated genotyping methods were used for the determination of the genetic polymorphism; namely, polymerase chain reaction (PCR) analysis followed by *Hinf*I digestion (Frosst et al. 1995), except one study that used PCR with allele-specific probes (Makita et al. 2003). Seven studies stated that the controls were age-, sex-, HbA1c-, body mass index (BMI)- or diabetes duration-matched (Shpichinetsky et al. 2000; Sun et al. 2004; Bluthner et al. 1999; Fujita et al. 1999; Odawara and Yamashita 1999; Moczulski et al. 2003). Studies were conducted in populations of various ethnicity: eight involved Caucasians (Bluthner et al. 1999; Makita et al. 2003; Moczulski et al. 2003; Boger et al. 2007; Ksiazek et al. 2004; Shcherbak et al. 1999; Smyth et al. 1999), five East Asians (Sun et al. 2004; Fujita et al. 1999; Odawara and Yamashita 1999; Neugebauer et al. 1998; Yoshioka et al. 2004), one a mixed population (Shpichinetsky et al. 2000), and one Arabs (Mtiraoui et al. 2007). Eleven studies involved cases with type 2 diabetes (Shpichinetsky et al. 2000; Sun et al. 2004; Bluthner et al. 1999; Fujita et al. 1999; Odawara and Yamashita 1999; Moczulski et al. 2003; Boger et al. 2007; Ksiazek et al. 2004; Neugebauer et al. 1998; Mtiraoui et al. 2007; Yoshioka et al. 2004), and

Table 2 The distribution of the MTHFR C677T genotypes for cases and controls and the allele frequencies (in parenthesis are the respective percentages). The significance level (*P*-value) for Hardy–Weinberg equilibrium (HWE) testing for controls is shown

First author, year	TT		TC		CC		T		C		<i>P</i> -value HWE
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	
Neugebauer 1998	12 (17)	8 (12)	31(46)	18 (28)	24 (35)	37 (58)	55 (41)	34 (26)	79 (58)	92 (73)	0.03
Shcherbak 1999	11(21)	5 (5)	21(41)	29 (32)	19 (37)	56 (62)	43 (42)	39 (21)	59 (57)	141 (78)	0.63
Fujita 1999	17 (16)	9 (13)	57(54)	39 (57)	31 (29)	20 (29)	91 (43)	57 (41)	119 (56)	79 (58)	0.14
Bluthner (type 2) 1999	23 (15)	18 (12)	50 (34)	65 (44)	74 (50)	63 (43)	96 (32)	101 (34)	198 (67)	191 (65)	0.85
Bluthner (type 1), 1999	13 (12)	18 (12)	54 (52)	67 (45)	35 (34)	63 (42)	80 (39)	103 (34)	124 (60)	193 (65)	0.98
Smyth 1999	16 (12)	15 (12)	62 (47)	57 (47)	53 (40)	47 (39)	94 (35)	87 (36)	168 (64)	151 (63)	0.72
Odawara 1999	26 (18)	25 (19)	65 (45)	68 (51)	52 (36)	38 (29)	117 (40)	118 (45)	169 (59)	144 (54)	0.57
Shpichinetsky 2000	10 (18)	6 (13)	22 (40)	16 (37)	23 (41)	21 (48)	42 (38)	28 (32)	68 (61)	58 (67)	0.32
Makita 2003	13 (14)	32 (10)	35 (39)	143 (46)	40 (45)	135 (43)	61 (34)	207 (33)	115 (65)	413 (66)	0.51
Moczulski 2003	76 (62) ^a	84 (52) ^a			45 (37)	75 (47)					NA ^b
Sun 2004	26 (20)	16 (16)	53 (42)	23 (23)	45 (36)	57 (59)	105 (42)	55 (28)	143 (57)	137 (71)	0.00
Ksiazek 2004	29 (16)	15 (9)	65 (38)	58 (37)	77 (45)	82 (52)	123 (35)	88 (28)	219 (64)	222 (71)	0.32
Yoshioka 2004	6 (15)	29(14)	13 (32)	107 (51)	21 (52)	71 (34)	25 (31)	165 (39)	55 (68)	249 (60)	0.26
Mtiraoui 2007	26 (27)	36(13)	56 (60)	79 (29)	11 (11)	152(56)	108(58)	151 (28)	78 (41)	383 (71)	0.00
Boger 2007	188 (42)	64(43)	219 (49)	69 (46)	32 (7)	15(10)	595(67)	197 (66)	283 (32)	99 (33)	0.56
Total	492 (26)	380(17)	803 (42)	838 (38)	582 (31)	932(43)	1,635(46)	1,430 (35)	1,877 (53)	2,552 (64)	

^a Data concerned (TC + TT)

^b Non-applicable

four type 1 diabetes (Bluthner et al. 1999; Makita et al. 2003; Shcherbak et al. 1999; Smyth et al. 1999). One study provided data for males and females separately (Moczulski et al. 2003). Three studies (Smyth et al. 1999; Neugebauer et al. 1998; Yoshioka et al. 2004) did not specify gender. One study (Bluthner et al. 1999) did not specify what proportion of cases had microalbuminuria.

Sensitivity analyses were performed for studies that may involve cases with microalbuminuria (Shcherbak et al. 1999; Bluthner et al. 1999; Shpichinetsky et al. 2000; Sun et al. 2004; Mtiraoui et al. 2007), and for studies involving cases with chronic renal failure (CRF) and/or end stage renal disease (ESRD) (Makita et al. 2003; Moczulski et al. 2003; Mtiraoui et al. 2007; Boger et al. 2007). A list of details abstracted from the studies included in the meta-analysis is provided in Table 1.

Summary statistics

The studies provided 1,877 cases and 2,150 controls. The allele C was the most common. The frequencies of the CC and TC genotypes were the highest in controls and in cases, respectively, while that for genotype TT was the lowest (Table 2). One study (Moczulski et al. 2003) did not provide data for all genotypes separately; it provided data only

for T-carriers. In three studies (Sun et al. 2004; Neugebauer et al. 1998; Mtiraoui et al. 2007), the distribution of the genotypes in the control group was not in HWE (*P* < 0.05) (Table 2).

Main results, subgroup and sensitivity analyses

Table 3 and Fig. 1 show the results for the association between the C677T gene polymorphism and the risk of DN.

The main analysis for investigating the association between C677T allele T and the risk of developing DN relative to the allele C revealed significant heterogeneity ($P_Q < 0.01, I^2 = 80\%$) between the 14 studies; the random effects pooled OR was marginally significant: RE OR = 1.30 (1.03–1.64). However, the sensitivity analysis for HWE (exclusion of studies not in HWE) yielded non-significant results. The sensitivity analyses for microalbuminuria (exclusion of studies involving cases with microalbuminuria), and for CRF/ESRD (exclusion of studies involving cases with CRF/ESRD) also yielded non-significant results. In subgroup analysis, there was significant heterogeneity in Caucasians and East Asians ($P_Q = 0.03, I^2 = 57\%$ and $P_Q < 0.01, I^2 = 75\%$, respectively), and the associations were not significant: RE OR = 1.19 (0.97–1.46) and RE OR = 1.16 (0.79–1.17), respectively. In type 1 and 2

Table 3 Summary estimates for the odds ratio (OR) of MTHFR C677T in various allele/genotype contrasts, the significance level (*P*-value) of heterogeneity test (*Q*-test) and the I^2 metric (when negative was set to zero): overall analysis, subgroup analyses and sensitivity analysis

Genetic contrasts	Studied population	Studies (<i>N</i>)	Fixed effects OR (95% CI)	Random effects OR (95% CI)	I^2 (%)	<i>P</i> -value <i>Q</i> -test	
T vs C	All	14	1.27 (1.15–1.40)	1.30 (1.03–1.64)	80	<0.01	
	Excluded HWE ^a	11	1.08 (0.97–1.21)	1.10 (0.93–1.30)	51	0.03	
	Excluded microalbuminuria ^b	8	1.07 (0.94–1.22)	1.07 (0.90–1.28)	43	0.09	
	Excluded CRF/ESRD ^c	11	1.18 (1.5–1.34)	1.22 (0.99–1.51)	66	<0.01	
	Caucasian	7	1.16 (1.01–1.32)	1.19 (0.97–1.46)	57	0.03	
	East Asian	5	1.13 (0.94–1.37)	1.16 (0.79–1.170)	75	<0.01	
	East Asian in HWE	3	0.86 (0.68–1.09)	0.86 (0.68–1.10)	0	0.04	
	Type1 diabetes	4	1.21 (1.00–1.47)	1.29 (0.90–1.86)	71	0.02	
	Type2 diabetes	10	1.29 (1.15–1.45)	1.30 (0.95–1.76)	84	<0.01	
	Type2 diabetes in HWE	7	1.02 (0.89–1.18)	1.02 (0.86–1.21)	28	0.22	
	Recessive model for allele T	All	14	1.32 (1.10–1.58)	1.35 (1.10–1.66)	17	0.27
		Excluded HWE ^a	11	1.21 (0.99–1.50)	1.22 (0.99–1.50)	2	0.42
		Excluded microalbuminuria ^b	8	1.16 (0.92–1.45)	0.16 (0.92–1.45)	0	0.73
		Excluded CRF/ESRD ^c	11	1.32 (1.05–1.67)	1.31 (1.04–1.66)	0	0.58
Caucasian		7	1.26 (1.00–1.59)	1.34 (0.98–1.83)	36	0.06	
East Asian		5	1.17 (0.83–1.65)	1.17 (0.82–1.65)	0	0.92	
East Asian in HWE		3	1.05 (0.67–1.63)	1.05 (0.67–1.63)	0	0.86	
Type1 diabetes		4	1.40 (0.95–2.07)	1.48 (0.83–2.61)	50	0.11	
Type2 diabetes		10	1.30 (1.06–1.60)	1.31 (1.06–1.63)	7	0.38	
Type2 diabetes in HWE		7	1.16 (0.91–1.47)	1.15 (0.91–1.46)	0	0.73	
Dominant model for allele T		All	15	1.38 (1.20–1.59)	1.41 (1.01–1.97)	81	<0.01
		Excluded HWE ^a	12	1.09 (0.93–1.27)	1.09 (0.87–1.38)	52	0.02
		Excluded microalbuminuria ^b	9	1.10 (0.92–1.31)	1.09 (0.83–1.44)	56	0.02
		Excluded CRF/ESRD ^c	11	1.19 (1.01–1.41)	1.23 (0.89–1.69)	71	<0.01
	Caucasian	8	1.27 (1.01–1.44)	1.24 (0.96–1.59)	47	0.06	
	East Asian	5	1.17 (0.90–1.53)	1.17 (0.61–2.27)	83	<0.01	
	East Asian in HWE	3	0.70 (0.50–1.00)	0.70 (0.48–1.03)	14	0.31	
	Type1 diabetes	4	1.23 (0.94–1.60)	1.30 (0.84–2.00)	61	0.06	
	Type2 diabetes	11	1.45 (1.23–1.70)	1.45 (0.93–2.25)	85	<0.01	
	Type2 diabetes in HWE	8	1.02 (0.84–1.23)	1.01 (0.76–1.34)	51	0.05	
	Additive model	All	14	1.61 (1.30–2.00)	1.65 (1.13–2.42)	66	<0.01
		Excluded HWE ^a	11	1.29 (1.01–1.64)	1.30 (0.97–1.73)	26	0.19
		Excluded microalbuminuria ^b	8	1.24 (0.94–1.64)	1.24 (0.93–1.65)	3	0.41
		Excluded CRF/ESRD ^c	11	1.39 (1.08–1.78)	1.41 (1.02–1.96)	38	0.10
Caucasian		7	1.48 (1.11–1.98)	1.50 (1.05–2.15)	33	0.17	
East Asian		5	1.22 (0.84–1.77)	1.24 (0.76–2.02)	38	0.17	
East Asian in HWE		3	0.84 (0.51–1.37)	0.84 (0.51–1.37)	0	0.69	
Type1 diabetes		4	1.51 (1.00–2.28)	1.64 (0.83–3.22)	59	0.28	
Type2 diabetes		10	1.65 (1.29–2.11)	1.65 (1.03–2.67)	71	<0.01	
Type2 diabetes in HWE		7	1.19 (0.89–1.60)	1.19 (0.88–1.61)	0	0.48	
Co-dominance model		All	14	1.13 (0.99–1.30)	1.15 (0.87–1.52)	74	<0.01
		Excluded HWE ^a	11	0.93 (0.79–1.08)	0.93 (0.77–1.11)	22	0.23
		Excluded microalbuminuria ^b	8	0.95 (0.79–1.13)	0.94 (0.74–1.19)	40	0.12
		Excluded CRF/ESRD ^c	11	1.03 (0.87–1.22)	1.06 (0.81–1.39)	59	0.01
	Caucasian	7	0.99 (0.83–1.19)	1.00 (0.81–1.22)	20	0.27	
	East Asian	5	1.07 (0.82–1.39)	1.09 (0.61–2.00)	78	<0.01	
	East Asian in HWE	3	0.71 (0.51–0.99)	0.71 (0.50–1.01)	8	0.33	
	Type1 diabetes	4	1.05 (0.81–1.37)	1.06 (0.79–1.42)	15	0.32	
	Type2 diabetes	10	1.16 (0.99–1.37)	1.17 (0.80–1.72)	80	<0.01	
	Type2 diabetes in HWE	7	0.87 (0.72–1.05)	0.86 (0.68–1.07)	25	0.24	

^a Exclusion of studies with the controls not in HWE

^b Exclusion of studies that may involve cases with microalbuminuria

^c Exclusion of studies that involve cases with CRF and/or ESRD

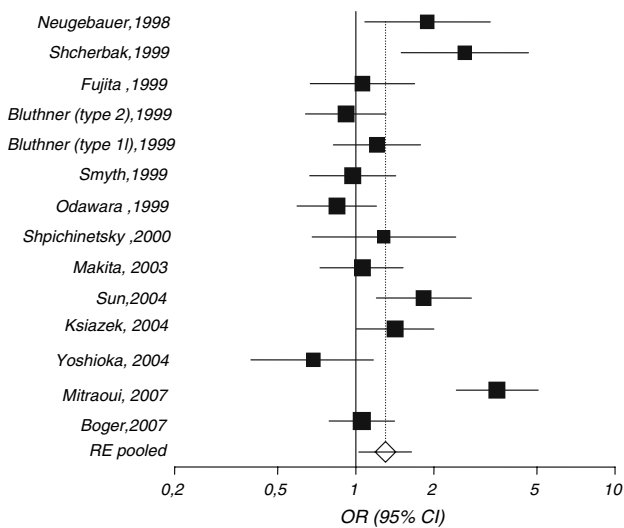


Fig. 1 Random effects (RE) odds ratio (OR) estimates with the corresponding 95% confidence interval (CI) for the allele contrast T vs C of MTHFR C677T gene polymorphism. The OR estimate of each study is marked with a solid black square. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond. The horizontal axis is plotted on a log scale

diabetes, the heterogeneity was again significant ($P_Q < 0.01$, $I^2 = 80\%$ and $P_Q < 0.01$, $I^2 = 80\%$, respectively), and the associations were not significant: RE OR = 1.29 (0.90–1.86) and RE OR = 1.30 (0.95–1.76), respectively.

The recessive model derived significant results overall [FE OR = 1.32 (1.10–1.58), $P_Q = 0.27$, $I^2 = 17\%$], and in type 2 diabetes [FE OR = 1.30 (1.06–1.60), $P_Q = 0.38$, $I^2 = 7\%$]; however, sensitivity analysis for HWE diminished the latter result. The sensitivity analysis for CRF/ESRD did not alter the overall significance [FE OR = 1.32 (1.05–1.67), $P_Q = 0.58$, $I^2 = 0\%$]. The dominant model produced a marginal significant association overall [RE OR = 1.41 (1.01–1.97), $P_Q < 0.01$, $I^2 = 81\%$]. Then, overall, the additive model produced significant association [RE OR = 1.65 (1.13–2.42), $P_Q < 0.01$, $I^2 = 66\%$], whereas the co-dominant model did not [RE OR = 1.15 (0.87–1.52), $P_Q < 0.01$, $I^2 = 74\%$], as it was anticipated. A similar pattern of results was found for studies not involving cases with CFR/ESRD, Caucasians and type 2 diabetes; however, sensitivity analysis for HWE diminished the latter result.

Potential bias

None of the studies included in the meta-analysis stated that genotyping was performed blinded to clinical status. Overall, the cumulative and recursive meta-analyses for the allelic contrast showed that the RE OR declined from 1.88

in 1998 to 1.20 in 1999 (relative change = 64%), and remained fairly constant in the period 1999–2004, and then increased to 1.30 in 2007 (relative change = 108%) (Fig. 2). There is statistical difference between the OR of the first study versus the pooled OR of subsequent studies ($P = 0.04$). The pooled OR without the first study was RE OR = 1.27 (0.99–1.62). The Egger test and the Begg–Mazumdar test indicated that there is no differential magnitude of effect in large versus small studies ($P = 0.57$ and $P = 0.23$, respectively).

Discussion

Methylenetetrahydrofolate reductase (MTHFR) is involved in the transmethylation of homocysteine to methionine and a C677T polymorphism in the MTHFR gene yields a thermo-labile MTHFR variant with reduced enzymatic activity. This mutation is a genetic determinant of hyperhomocysteinemia in healthy subjects (Frosst et al. 1995; Kang et al. 1991) and also in patients with diabetes (Buysschaert et al. 2004). Hyperhomocysteinemia induces endothelial dysfunction (Constans et al. 1999) and it has been implicated as a risk factor not only for atherosclerosis and arterial or venous thrombosis (Frosst et al. 1995; McCully et al. 1969) but also for nephropathy in type 2 diabetes patients (Miraoui et al. 2007; Chico et al. 1998). Generally, hyperhomocysteinemia promotes the atherosclerotic and thrombotic process by modulating vascular cell proliferation and prothrombotic activities in the vascular wall (McCully et al. 1996; Constans et al. 1999). In addition, recent in vitro studies indicate that homocysteine and other thiol-containing reductive compounds (i.e. thio-lactone) increase the expression of vascular endothelial growth factor (VEGF) in cell cultures via activation of VEGF gene transcription (Maeda et al. 2003; Roybal et al. 2004). VEGF is a pro-angiogenic factor known to play a role in the pathogenesis of DN (Sung et al. 2006). For these reasons, it may be readily postulated that the C677T MTHFR gene polymorphism as a determinant of hyperhomocysteinemia might be involved in the development of DN.

This is the first meta-analysis to examine C677T polymorphisms of the MTHFR gene and their relationship to susceptibility for DN. The meta-analysis involved 15 studies, which provided 1,877 cases and 2,150 controls. The strength of the present analysis was based on the accumulation of published data giving greater information to detect significant differences. Non-English, non-indexed and non-published studies in the literature were not reviewed, thus suggesting bias (Egger et al. 1997; Zintzaras and Kaditis 2007). Studies demonstrating significant results are more likely to be published, especially in

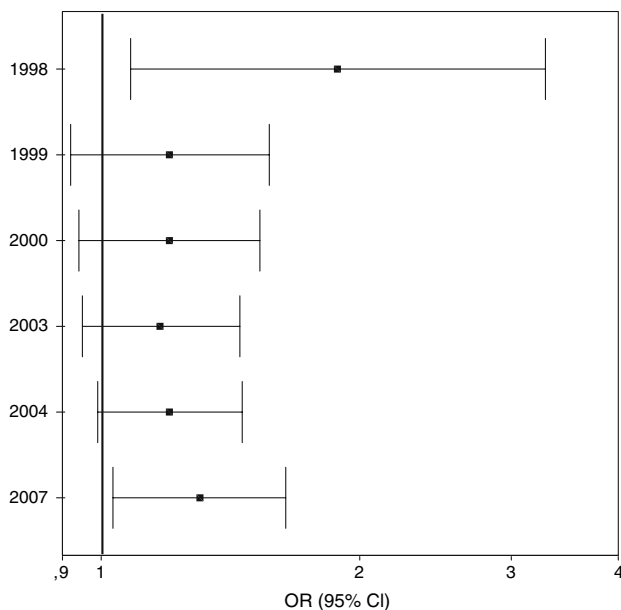


Fig. 2 Cumulative meta-analysis: the RE pooled OR with the corresponding 95% CI at the end of each year-information step is shown

English language indexed journals, as opposed to studies presenting negative findings, which are more likely to be published in a local journal, often non-indexed. In the present study, the effect of allele frequency and the effects of the dominant, recessive, additive and co-dominance models were estimated. In addition, the consistency of genetic effects across populations from different ethnicities was investigated. Ethnicity was categorised into two main groups: (1) Caucasian descent, and (2) East Asian descent (Zintzaras et al. 2005, 2006c). However, the consistency of genetic effects across these traditionally defined ethnicities does not necessarily mean that ethnicity-specific genetic effects are exactly the same. Subgroup analysis by diabetes type, and sensitivity for studies not in HWE was carried out.

The overall data showed an excess of heterozygotes for cases, and an excess of homozygous CC alleles in controls. The main analysis (all studies) produced significant results for the allele contrast, the contrast of the extreme homozygotes, and the recessive and dominant models. The co-dominance model produced non-significant association. Overall, the meta-analysis results indicated that homozygosity in allele T was associated with a 65% increased risk of DN compared with homozygosity in allele C, and it can be excluded with 95% certainty that heterozygotes would have less than 52% odds for developing DN and more than 15% for preventing DN. Caucasians and East Asians produced no significant associations, with similar estimated risks in all genetic contrasts. Type 2 diabetes showed significant results; however, sensitivity analysis diminished

their significance, and thus, any interpretation must be cautious. In general, there is some indication of consistency of genetic effects between type 1 and 2 diabetes. The lack of association in subgroup analyses could be due to lack of power to detect existing significant associations and to other loci that are probably in linkage disequilibrium and that may affect (or mask) MTHFR susceptibility to DN (Imperatore et al. 1998). Cumulative meta-analysis showed a trend for stabilising the association as evidence accumulates, although the first published study showed an exaggerated association. There are no potential sources of bias, and there is no differential magnitude of effect in large versus small studies.

Sampling variability and stratification in case-control study design could be a possible confounding factor on the role of genetic markers. The meta-analysis included studies with a case group consisting of patients with persistent macro- or microalbuminuria, and thus the pooled estimated risk of DN may underestimate the true risk. However, the meta-analysis excluded case groups that consisted exclusively of patients with microalbuminuria. Although the presence of microalbuminuria may be an early finding in DN it is not invariably equivalent to it. A major limitation of using a meta-analytic approach for population-based observational studies is the confounding factors (e.g. age, sex, and lifestyle) that influence the estimates of associations. In this meta-analysis, only unadjusted pooled ORs could be calculated since data for each level of possible confounding factors were not provided. The cases and controls of each study were well defined and had similar inclusion criteria, although they unavoidably cover a wide spectrum of disease in terms of duration, demographics and other clinical manifestations. In addition, the risk effect may depend on the interaction with other risk factors, such as lifestyle (i.e. smoking, alcohol consumption, control of diabetes and body mass index), all of which directly modulate the development of DN (Passaro et al. 2003; Ma et al. 1997).

The present meta-analysis provided evidence that the TT genotype of the MTHFR C677T polymorphism contributes to susceptibility to DN. DN is a complex disease with a multifactorial etiology and therefore the contributing pathogenetic role of lifestyle factors and dietary intake should also be considered. The existence of gene-environment interactions may explain the discrepancy of results obtained in individual genetic association studies, and thus case-control studies that investigate gene-environment interactions (Clayton and McKeigue 2001) might help further elucidate the genetics of DN.

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References

- Begg CB, Mazumdar M (1994) Operating characteristics of a rank correlation test for publication bias. *Biometrics* 50:1088–1101
- Bluthner M, Bruntgens A, Schmidt S, Strojek K, Grzeszczak W, Ritz E (1999) Association of methylenetetrahydrofolate reductase gene polymorphism and diabetic nephropathy in type 2 diabetes? *Nephrol Dial Transplant* 14:56–57
- Boger CA, Stubanus M, Haak T, Götz AK, Christ J, Hoffmann U, Riegger GA, Krämer BK (2007) Effect of MTHFR C677T genotype on survival in type 2 diabetes patients with end-stage diabetic nephropathy. *Nephrol Dial Transplant* 22:154–162
- Borch-Johnsen K, Norgaard K, Hommel E, Mathiesen ER, Jensen JS, Deckert T, Parving HH (1992) Is diabetic nephropathy an inherited complication? *Kidney Int* 41:719–722
- Bostom AG, Selhub J, Jacques PF, Rosenberg IH (2001) Power shortage: clinical trials testing the “homocysteine hypothesis” against a background of folic acid-fortified cereal grain flour. *Ann Intern Med* 135:133–137
- Boushey CJ, Beresford SA, Omenn GS, Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* 274:1049–1057
- Buysschaert M, Gala JL, Bessomo A, Hermans MP (2004) C677T methylene-tetrahydrofolate reductase mutation in type 2 diabetic patients with and without hyperhomocysteinaemia. *Diabetes Metab* 30:349–354
- Chico A, Perez A, Cordoba A, Arcelús R, Carreras G, de Leiva A, González-Sastre F, Blanco-Vaca F (1998) Plasma homocysteine is related to albumin excretion rate in patients with diabetes mellitus: a new link between diabetic nephropathy and cardiovascular disease? *Diabetologia* 41:684–693
- Clayton D, McKeigue PM (2001) Epidemiological methods for studying genes and environmental factors in complex diseases. *Lancet* 358:1356–1360
- Constans J, Blann AD, Resplandy F, Parrot F, Seigneur M, Renard M, Amiral J, Guérin V, Boisseau MR, Conri C (1999) Endothelial dysfunction during acute methionine load in hyperhomocysteinaemic patients. *Atherosclerosis* 147:411–413
- DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. *Control Clin Trials* 7:177–188
- Egger M, Davey SG, Schneider M, Minder C (1997a) Bias in meta-analysis detected by a simple, graphical test. *BMJ* 315:629–634
- Egger M, Zellweger-Zahner T, Schneider M, Junker C, Lengeler C, Antes G (1997b) Language bias in randomised controlled trials published in English and German. *Lancet* 350:326–329
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 10:111–113
- Fujita H, Narita T, Meguro H, Ishii T, Hanyu O, Suzuki K, Kamoi K, Ito S (1999) No association between MTHFR gene polymorphism and diabetic nephropathy in Japanese type II diabetic patients with proliferative diabetic retinopathy. *J Diabetes Complicat* 13:284–287
- Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, Rozen R (1994) Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. *Nat Genet* 7:195–200
- Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T (2005) Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* 28:164–176
- Imperatore G, Hanson RL, Pettitt DJ, Kobes S, Bennett PH, Knowler WC (1998) Sib-pair linkage analysis for susceptibility genes for microvascular complications among Pima Indians with type 2 diabetes. Pima diabetes genes group. *Diabetes* 47:821–830
- Ioannidis JP, Trikalinos TA, Zintzaras E (2006) Extreme between-study homogeneity in meta-analyses could offer useful insights. *J Clin Epidemiol* 59:1023–1032
- Kang SS, Wong PW, Bock HG, Horwitz A, Grix A (1991) Intermediate hyperhomocysteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. *Am J Hum Genet* 48:546–551
- Ksiazek P, Bednarek-Skublewska A, Buraczynska M (2004) The C677T methylenetetrahydrofolate reductase gene mutation and nephropathy in type 2 diabetes mellitus. *Med Sci Monit* 10:BR47–BR51
- Lau J, Antman EM, Jimenez-Silva J, Kupelnick B, Mosteller F, Chalmers TC (1992) Cumulative meta-analysis of therapeutic trials for myocardial infarction. *N Engl J Med* 327:248–254
- Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, Fuchs C, Willett WC, Selhub J, Hennekens CH, Rozen R (1997) Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res* 57:1098–1102
- Maeda M, Yamamoto I, Fujio Y, Azuma J (2003) Homocysteine induces vascular endothelial growth factor expression in differentiated THP-1 macrophages. *Biochim Biophys Acta* 1623:41–46
- Makita Y, Moczulski DK, Bochenski J, Smiles AM, Warram JH, Krolewski AS (2003) Methylenetetrahydrofolate reductase gene polymorphism and susceptibility to diabetic nephropathy in type 1 diabetes. *Am J Kidney Dis* 41:1189–1194
- McCully KS (1969) Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol* 56:111–128
- McCully KS (1996) Homocysteine and vascular disease. *Nat Med* 2:386–389
- Moczulski D, Fojcik H, Zukowska-Szczechowska E, Szydłowska I, Grzeszczak W (2003) Effects of the C677T and A1298C polymorphisms of the MTHFR gene on the genetic predisposition for diabetic nephropathy. *Nephrol Dial Transplant* 18:1535–1540
- Mtiraoui N, Ezzidi I, Chaieb M, Marmouche H, Aouni Z, Chaieb A, Mahjoub T, Vaxillaire M, Almawi WY (2007) MTHFR C677T and A1298C gene polymorphisms and hyperhomocysteinemia as risk factors of diabetic nephropathy in type 2 diabetes patients. *Diabetes Res Clin Pract* 75:99–106
- Neugebauer S, Baba T, Watanabe T (1998) Methylenetetrahydrofolate reductase gene polymorphism as a risk factor for diabetic nephropathy in NIDDM patients. *Lancet* 352:454
- Odawara M, Yamashita K (1999) A common mutation of the methylenetetrahydrofolate reductase gene as a risk factor for diabetic nephropathy. *Diabetologia* 42:631–632
- Passaro A, Calzoni F, Volpato S, Nora ED, Pareschi PL, Zamboni PF, Fellin R, Solini A (2003) Effect of metabolic control on homocysteine levels in type 2 diabetic patients: a 3-year follow-up. *J Intern Med* 254:264–271
- Quinn M, Angelico MC, Warram JH, Krolewski AS (1996) Familial factors determine the development of diabetic nephropathy in patients with IDDM. *Diabetologia* 39:940–945
- Roybal CN, Yang S, Sun CW, Hurtado D, Vander Jagt DL, Townes TM, Abcouwer SF (2004) Homocysteine increases the expression of vascular endothelial growth factor by a mechanism involving endoplasmic reticulum stress and transcription factor ATF4. *J Biol Chem* 279:14844–14852
- Shcherbak NS, Shutskaia ZV, Sheidina AM, Larionova VI, Schwartz EI (1999) Methylenetetrahydrofolate reductase gene polymorphism as a risk factor for diabetic nephropathy in IDDM patients. *Mol Genet Metab* 68:375–378

- Shpichinetsky V, Raz I, Friedlander Y, Goldschmidt N, Wexler ID, Ben-Yehuda A, Friedman G (2000) The association between two common mutations C677T and A1298C in human methylenetetrahydrofolate reductase gene and the risk for diabetic nephropathy in type II diabetic patients. *J Nutr* 130:2493–2497
- Smyth JS, Savage DA, Maxwell AP (1999) MTHFR gene polymorphism and diabetic nephropathy in type 1 diabetes. *Lancet* 353:1156–1157
- Strojek K, Grzeszczak W, Morawin E, Adamski M, Lacka B, Rudzki H, Schmidt S, Keller C, Ritz E (1997) Nephropathy of type II diabetes: evidence for hereditary factors? *Kidney Int* 51:1602–1607
- Sun J, Xu Y, Zhu Y, Lu H (2004) Genetic polymorphism of methylenetetrahydrofolate reductase as a risk factor for diabetic nephropathy in Chinese type 2 diabetic patients. *Diabetes Res Clin Pract* 64:185–190
- Sung SH, Ziyadeh FN, Wang A, Pyagay PE, Kanwar YS, Chen S (2006) Blockade of vascular endothelial growth factor signaling ameliorates diabetic albuminuria in mice. *J Am Soc Nephrol* 17:3093–3104
- Weir BS (1996) Genetic data analysis II: methods for discrete population genetic data. Sinauer, Sunderland
- Whitehead A (2002) Meta-analysis of controlled clinical trials. Wiley, Chichester
- Yoshioka K, Yoshida T, Umekawa T, Kogure A, Takakura Y, Toda H, Yoshikawa T (2004) Methylenetetrahydrofolate reductase gene polymorphism is not related to diabetic nephropathy in Japanese Type 2 diabetic patients. *Diabet Med* 21:1051–1052
- Zintzaras E (2006a) Association of methylenetetrahydrofolate reductase (MTHFR) polymorphisms with genetic susceptibility to gastric cancer: a meta-analysis. *J Hum Genet* 51:618–624
- Zintzaras E (2006b) Methylenetetrahydrofolate reductase gene and susceptibility to breast cancer: a meta-analysis. *Clin Genet* 69:327–336
- Zintzaras E (2006c) C677T and A1298C methylenetetrahydrofolate reductase gene polymorphisms in schizophrenia, bipolar disorder and depression: a meta-analysis of genetic association studies. *Psychiatr Genet* 16:105–115
- Zintzaras E (2007) Brain-derived neurotrophic factor gene polymorphisms and schizophrenia: a meta-analysis. *Psychiatr Genet* 17:69–75
- Zintzaras E, Hadjigeorgiou GM (2004) Association of paraoxonase 1 gene polymorphisms with risk of Parkinson's disease: a meta-analysis. *J Hum Genet* 49:474–481
- Zintzaras E, Hadjigeorgiou GM (2005) The role of G196A polymorphism in the brain-derived neurotrophic factor gene in the cause of Parkinson's disease: a meta-analysis. *J Hum Genet* 50:560–566
- Zintzaras E, Ioannidis JP (2005a) Heterogeneity testing in meta-analysis of genome searches. *Genet Epidemiol* 28:123–137
- Zintzaras E, Ioannidis JP (2005b) HEGESMA: genome search meta-analysis and heterogeneity testing. *Bioinformatics* 21:3672–3673
- Zintzaras E, Kaditis AG (2007) Sleep-disordered breathing and blood pressure in children: a meta-analysis. *Arch Pediatr Adolesc Med* 161:172–178
- Zintzaras E, Stefanidis I (2005) Association between the GLUT1 gene polymorphism and the risk of diabetic nephropathy: a meta-analysis. *J Hum Genet* 50:84–91
- Zintzaras E, Chatzoulis DZ, Karabatsas CH, Stefanidis I (2005) The relationship between C677T methylenetetrahydrofolate reductase gene polymorphism and retinopathy in type 2 diabetes: a meta-analysis. *J Hum Genet* 50:267–275
- Zintzaras E, Kitsios G, Stefanidis I (2006a) Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. *Hypertension* 48:700–710
- Zintzaras E, Rodopoulou P, Koukoulis GN (2006b) *BsmI*, *TaqI*, *ApaI* and *FokI* polymorphisms in the vitamin D receptor (VDR) gene and the risk of osteoporosis: a meta-analysis. *Dis Markers* 22:317–326
- Zintzaras E, Koufakis T, Ziakas PD, Rodopoulou P, Giannouli S, Voulgarelis M (2006c) A meta-analysis of genotypes and haplotypes of methylenetetrahydrofolate reductase gene polymorphisms in acute lymphoblastic leukemia. *Eur J Epidemiol* 21:501–510
- Zintzaras E, Stefanidis I, Santos M, Vidal F (2006d) Do alcohol-metabolizing enzyme gene polymorphisms increase the risk of alcoholism and alcoholic liver disease? *Hepatology* 43:352–361