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Msc Final Paper

Perform a meta-analysis for the variant MTHFR-polymorphisms C677T in peripheral arterial disease

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Meta-analysis for the variant MTHFR-polymorphisms C677T - in peripheral arterial disease

Θα ήθελα να καταθέσω τις ολόθερμες ευχαριστίες μου στο Διευθυντή του ΠΜΣ Καθηγητή Ηλία Ζιντζαρά ο οποίος κατάφερε με την επιστημονική του ομάδα να οργανώσει ένα ενδιαφέρον, χρήσιμο για πολλές επιστήμες και πολυ υψηλού επιπέδου μεταπτυχιακό: “ΜΕΘΟΔΟΛΟΓΙΑ ΒΙΟΙΑΤΡΙΚΗΣ ΕΡΕΥΝΑΣ, ΒΙΟΣΤΑΤΙΣΤΙΚΗ ΚΑΙ ΚΛΙΝΙΚΗ ΠΛΗΡΟΦΟΡΙΚΗ”. Επίσης τον ευχαριστώ απο καρδιάς γιατί ήταν ο άνθρωπος που με καθοδήγησε έξυπνα στη συγγραφή της διπλωματικής εργασίας και μου φανέρωσε πολλά μυστικά της Βιοστατιστικής Επιστήμης. Επίσης θα ηθελα να ευχαριστήσω ολόψυχα τα μέλη της τριμελούς επιτροπής γαι τη Διπλωματική μου Εργασία Καθηγητή Νεφρολογίας – Πρόεδρο του Ιατρικού Τμήματος Πανεπιστημίου Θεσσαλίας Ιωάννη Στεφανίδη και την κ, Χρυσουλα Δοξάνη, Ιατρό, Επιστημονική συνεργάτης του τμήματος Βιοστατιστικής που αγόγγυστα μου παρείχαν τις συμβουλές τους .

Τέλος δεν θα μπορούσα να μην ευχαριστήσω όλη την οικογένειά μου (διαίταρα τη Σύζυγο Μαίρη Ρούκα και τα παιδιά μου Γιώργο και Μιχάλη) που με ανέχτηκαν και με ενθάρρυναν κατά τη διάρκεια αυτού του μεταπτυχιακού προγράμματος.

Abstract

Background: Peripheral Arterial Disease (PAD) is a complex disease with genetic background. The genetic association studies (GAS) that investigated the association between PAD and the *MTHFR C677T* gene variant have produced contradictory or inconclusive results.

Materials and Methods: In order to decrease the uncertainty of estimated genetic risk effects and to explore whether there is enough evidence to claim or deny association between the variant and PAD, a meticulous meta-analysis, including cumulative and recursive cumulative meta-analyses, of all relevant published GAS was conducted. The risk effects were estimated based on the generalized odds ratio (OR_G), a genetic model-free approach.

Results: The analysis showed large heterogeneity studies (I^2=57%, P_Q<0.01) and marginal significant association for the MTHFR *C677T* variant [OR_G=1.21 (1.01-1.48)]. However, a sensitivity analysis for the studies with the controls not in Hardy-Weinberg equilibrium derived a significant association [RE OR_G=1.26 (1.09-1.45)]. The cumulative meta-analysis indicated a trend of association as evidence accumulates and the recursive cumulative meta-analysis indicated lack of sufficient evidence for claiming or denying an association.

Conclusion: The current evidence is not sufficient to draw definite conclusions regarding the association of *MTHFR* variants and development of PAD. More studies are needed to be conducted.

Meta-analysis for the variant MTHFR-polymorphisms C677T - in peripheral arterial disease

1. Introduction

Peripheral arterial disease (PAD) encompasses a large series of noncoronary arterial syndromes and affects approximately 20% of adults older than 55 years and an estimated 27 million persons in North America and Europe [(1).] The term “peripheral arterial disease” includes a diverse group of disorders that lead to progressive stenosis or occlusion, or aneurismal dilation, of the aorta and its noncoronary branch arteries, including the carotid, upper extremity, visceral and lower extremity arterial branches [(2)]. About one fifth of people with lower extremity PAD have typical symptoms of intermittent lower limb claudication, “rest pain”, ulceration, or gangrene, another third have atypical exertional leg symptoms whereas half of all people are asymptomatic[(3).]

The existence of inherited genetic predisposition to PAD has been investigated with various types of familial aggregation studies [(4-8).] Heritability estimates have shown that the contribution of genetic factors to overall variation in ankle-brachial index, which is a widely utilized measure for detecting PAD, was 21% [(8).] One main approach to disentangle the genetic etiology of complex human traits includes association studies. Genetic association studies, in particular, are central to efforts to identify and characterize genomic variants (e.g. single nucleotide polymorphisms, SNPs) underlying susceptibility to PAD. Polymorphisms may influence gene activity and mRNA conformation, alter the protein binding ability to its substrate and

change its sub-cellular localization. Therefore, polymorphisms are emerging as possible factors that may predispose to PAD and correlate with the pathogenesis of the disease [9]

Methylenetetrahydrofolate reductase (*MTHFR*) is a critical enzyme in one-carbon metabolism. *MTHFR* catalyzes the irreversible conversion of 5,10-methylenetrahydrofolate to 5-methyltetrahydrofolate, which is the predominant circulating form of folate and serves as the carbon donor for the remethylation of homocysteine to methionine (10). The *MTHFR* [11, 12] gene is localized on chromosome 1p36.3. The common polymorphism (variant) have been described in the *MTHFR* gene is the C677T (exon 4 at codon 222) which are single nucleotide substitution resulting in amino acid changes, which is a C>T substitution at position 677 resulting in an alanine to valine substitution. The polymorphism has an impairment in the enzyme activity. The 677T allele has been found to result in decreased enzyme activity [11, 12] leading to increased homocysteine levels and thus to an imbalance in plasma folate concentration. The C677T polymorphism is implicated with the development of PAD and have already been associated with different disorders/diseases (13-16) . However, other functional polymorphisms in genes associated with impaired folate metabolism, may also contribute to the risk of cancer and other disorders [17, 18]

The genetic association studies (GAS) that investigated the association between PAD and the *MTHFR C677T* variant have produced contradicted or inconclusive results, partly because the studies had limited sample sizes and their power was not adequate to demonstrate significant association. In addition, the studies involved different populations and sampling strategies making the interpretation of results difficult. Two meta-analyses[19,20] regarding the role of *MTHFR C677T* variant has been previously carried out, but they

include relative scarce information available at that time and they did not focus on whether there is enough evidence to claim and deny association between the MTHFR C677T variant and PAD.

In the present study, a meticulous meta-analysis[21], including cumulative and recursive cumulative meta-analysis [22, 23] , was performed for GAS related the *MTHFR C677T* variant with PAD. Aim of the meta-analysis was to provide an estimated pooled genetic risk effect with decreased uncertainty and to explore whether there is sufficient evidence to deny or claim association. The consistency of genetic effects across traditionally defined ethnicities was also examined.

2. Material and Methods

2.1. Identification and eligibility of relevant studies

GAS that investigated the association of the *MTHFR C677T* genetic variant with the risk of developing PAD, published in English before August 2015 were considered in the meta-analysis. The studies were identified by systematically searching the PubMed database. The following search criterion was used: ("peripheral arterial disease" OR "peripheral artery disease" OR "PAD" OR "peripheral arterial occlusive disease" OR "lower extremity arterial disease" OR "atherosclerotic vascular disease" OR "peripheral vascular disease" OR "intermittent claudication" OR "limb ischemia") AND (“methylenetetrahydrofolate reductase” or “*MTHFR*” or “*C677T*”) and (“association” or “risk” or “susceptibility”). Then, the abstracts were retrieved

and screened to assess their appropriateness for inclusion in the meta-analysis. After the abstract screening, the articles were read in their entirety in order to assess their eligibility for the meta-analysis. Finally, all the references in the eligible articles were extensively reviewed to identify additional published articles not indexed by PubMed database.

The eligible studies fulfilled the following inclusion criteria: 1) providing cases with clinically diagnosed PAD and healthy controls free of PAD, 2) providing information on the genotype distribution of C677T variant in patients with PAD and in healthy controls subjects, 3) using DNA-based analysis methods for genotyping, and 4) including subjects who were human. In this article we focus on lower extremity PAD, which is a chronic obstructive disease of the aortic, iliac and lower limb arteries.[24] Cases were thus considered as patients suffering from lower extremity PAD, with the diagnosis based on noninvasive and invasive diagnostic tools. Studies investigating progression, severity, phenotype modification, response to treatment, or survival were excluded from our study. Case reports, editorials, and review articles were also excluded. Finally, family-based studies were excluded because of different design settings.

2.2. Data extraction

From each study the following information was extracted: first author, journal, year of publication, ethnicity of study population, demographics, diagnosis criteria for PAD and genotype distribution.

2.3. Data synthesis and analysis

The meta-analysis examined the association between the MTHFR C677T variant and the risk of PAD based on the generalized odds ratio (OR_G)[25]. The OR_G provides an estimate of the overall risk effect by utilizing the complete genotype distribution. The OR_G express the probability of a subject being with PAD relative to probability of being free of disease, given that the subject with PAD has a higher mutational load than the non-diseased.[25, 26]

In synthesizing the studies, the fixed effects (FE) and random effects (RE) OR_G were used.[21, 25, 27] The RE model incorporates in the estimates the between study variability [21]. The heterogeneity between studies was tested using the Q-statistic [28, 29] and the heterogeneity was considered significant at $P_Q<0.10$. The heterogeneity was also quantified using the I^2 metric which takes values between 0% and 100% with higher values denoting greater degree of heterogeneity[30]. When the heterogeneity is large and significant the RE model might be used whereas when there is lack of heterogeneity the RE model coincides with the FE effects model [31]. In order to evaluate the trend of association, a cumulative and recursive cumulative meta-analysis was conducted based on the RE ORG [21, 23, 32]. In cumulative meta-analysis, studies were chronologically ordered by publication year and the OR is obtained at the end of each year (each information step) [21]. In recursive cumulative meta-analysis, the relative change in OR at each information step was calculated. Cumulative and recursive cumulative meta-analyses, provide the framework for updating the estimated genetic risk effect in time and they measure the changes in risk effect as evidence accumulates [21]. In particular,

the cumulative meta-analysis indicates the trend in the risk effect and recursive cumulative meta-analysis indicates the stability in the risk effect. The differential magnitude of effect in large versus small studies for the allele contrast was checked using the Harbord's test [33].

The meta-analysis consisted of the overall analysis, which includes all available data and subgroup analysis by ethnicity ("racial" descent). A sensitivity analysis which examines the effect of excluding specific studies was also considered [21]. The distribution of the genotypes in the control group was tested for conforming to the Hardy-Weinberg equilibrium (HWE) rule using an exact test [21]. Deviation from HWE indicates possible genotyping errors and/or population stratification and studies with controls deviated from HWE were subjected to a sensitivity analysis [21]. Analyses were performed using ORGGASMA (http://biomath.med.uth.gr) [25, 26].

3. Results

3.1. Eligible studies and studies' characteristics

The literature review identified 48 articles in PubMed that met the search criteria. Data from 12 articles met the inclusion criteria. Figure 1 presents a flow chart of the retrieved and excluded studies with specification of reasons. The characteristics of the individual studies included in the meta-analysis are provided in Table 1. The studies provided 1578 cases and 2580 controls. The studies were published between 1998 and 2011. The studies used validated genotyping methods for the determination of the genetic variants and the diagnosis of PAD was based on valid criteria. Studies were conducted in

various populations of different ethnicity: 10 involved Whites and two Brazilian populations. The distribution of genotypes in the control group deviated from HWE (P≥0.05) in four studies [34-37]. Then, a sensitivity analysis was carried out for these studies.

3.3. Meta-analysis results

Figure 2 and Table 2 show the meta-analysis results for the association between the MTHFR C677T variant and the risk of developing PAD. The overall analysis for investigating the association between the variant and the risk of PAD showed a large heterogeneity between studies (I^2=57%, P_Q<0.01) and the risk of PAD on the basis of the current evidence was marginally significant was also showed for Whites [FE OR_G=1.12 (1.01-1.26) and RE OR_G=1.13 (0.94-1.34)].

3.4. Potential Bias

None of the studies reported that genotyping was blinded to clinical status. The cumulative meta-analysis, revealed a trend of association as information accumulates in the studied period (1998-2011) with the association being significant at the end of this period Figure 3. The recursive cumulative meta-analysis showed that the relative change in OR_G did not stabilize around OR=1, indicating that there is no sufficient evidence for claiming or denying association Figure 4. Thus, more studies should be conducted in order to provide conclusive evidence for the association between the variant and PAD. The test by Harbord et al. for *C677T* indicated that there is no differential magnitude of effect in large versus small studies (P=0.34).

4. Discussion

Herein, we presented a comprehensive meta-analysis of GAS investigated the association between the MTHFR C677T variant and PAD susceptibility. The purpose of the meta-analysis was to provide an estimate of the genetic risk effect with a reduced uncertainty and to explore the trend of risk effect as evidence accumulates. The individual GAS included in the meta-analysis have relatively small sample sizes to detect the minor contribution of the mutant alleles in PAD susceptibility. Typically, for a modest significant risk effect (OR around 1.3) to be detected in single GAS, a sample size of more than 10,000 subjects is needed to achieve power >80% [(22)]. The meta-analysis has the advantage by synthesizing data from published GAS to provide greater power to detect significant associations than an individual GAS, especially in the absence of large heterogeneity between studies [(21)]. However, there is no established methodology for calculating the power of the meta-analysis. In addition, meta-analysis is a retrospective all inclusive synthesis of published studies and power analysis may not be applicable [(21)]. Nevertheless, type II errors are expected to be less in a meta-analysis than in the individual GAS [(21)].

The association was assessed based on the OR_G. The current practice is to analyse various genetic models, such as the dominant, recessive, additive and co-dominant models; however, these models are not independent [(25)]. In addition, there is no a priori biological justification for choice of a specific

genetic model. The OR_G is a metric that quantifies the probability of disease, given that a diseased subject has a higher, or lower, mutational load than a healthy one. The use of OR_G is a genetic-model free approach and provides an integrated way to evaluate genetic associations, by exploiting all available information in terms of disease and genotype distribution [25]. Thus, the application of OR_G in analysis and meta-analysis of individual GAS may overcome the drawbacks of multiple model testing or erroneous model specification [25].

Overall, only marginal association between the MTHFR *C677T* variant and risk of PAD was found in the meta-analysis. However, the sensitivity analysis for the studies with the controls not in HWE revealed a significant association. However, the heterogeneity between studies is large and therefore, the findings should be interpret with caution. Also, the meta-analysis indicated lack of differential magnitude of effect in large versus small studies (“publication bias”); however, inspection of funnel plot was avoided since its interpretation can be misleading [38]. Nevertheless, the conclusions reached in the present meta-analysis were based on relatively small numbers of studies and participants and thus, any inferences have to be cautious. The cumulative and the recursive cumulative meta-analyses indicated that there is a trend towards to association and that more evidence is needed to draw definite conclusion regarding the existence or absence of association for the MTHFR C677T variant.

Stratification in genetic association studies might blur the genetic effect. In the studies with the controls not conformed to HWE, the lack of HWE indicates population stratification and/or genotyping errors. Besides, deviation from HWE in a population implies continued selection, migration, mutation, and absence of random mating [39]. Hence, the validity of the genotyping method and the selection of controls are questionable for these studies [39, 40]. Moreover, the absence of reporting blindness to phenotype in genotyping laboratory personnel and the possible lack of a controlled genotyping procedure may potential bias which is difficult to assess.

The lack of strong association between the *MTHFR* C677T variant and PAD might be due to other unidentified functional variants that exist in the folate pathway and the *MTHFR* gene that are in linkage disequilibrium, and therefore, they may affect the susceptibility to PAD. However, the development of PAD might be associated with gene-gene-environment interactions and their effects should be considered in individual GAS and subsequent meta-analyses [41]. Currently, limited information is provided by the individual GAS and the available study-level genotype distribution was used in the meta-analysis, precluding the adjusted analysis for potential gene–gene–environment interactions [42]. Thus, failure to account for these interactions may have reduced the efficiency of the genetic risk estimates, but is unlikely to inflate false-positive results.

In conclusion, a marginal association between for the MTHFR C677T variant and risk of PAD has been detected in the present data synthesis. However, the results should be interpreted with caution since there is no sufficient evidence for the current published individual GAS to claim or deny association. Considering that PAD is a complex disease with multifactorial etiology, the minor contributing pathogenetic role of the *MTHFR* variants, including C677T, may not be totally excluded. The results of rigorous GAS that take into account epistatic and gene-environment interactions may provide more conclusive evidence regarding the genetic susceptibility to ALL.

Conflict of interest statement

The authors declare no conflict of interest.

Table 1: **Characteristics of the studies considered in the meta-analysis.**

Author, year	**Ethnicity**	**No of cases**	**No of controls**	**M/F with PAD**	**M/F Controls**	**Age of cases/controls meanSD median (min-max)**	**Diagnostic criteria for cases of PAD**
Fowkes, 2000	caucasian	80	300	41/39	144/156	65.9 (0.6)/ 63.2 (0.3)	ABPI was used in baseline studies and World Health Organization intermittent claudication questionnaire was used on follow-up
Mueller, 2005	caucasian	433	433	306/127	306/127	68 (59-75)/ 68 (60-75)	Clinical criteria were confirmed by ABPI and arteriography.
Sofi, 2005	caucasian	280	280	216/64	216/64	69 (35-90)/ 70 (33-95)	Fontaine stage II to IV and confirmed by ABPI
Sabino, 2007	mixed (brazilian)	44	37	26/18	17/20	68.9±9.7/ 60.9±9.2	clinical examination and measurement of the ankle/arm index
Jones, 2005	caucasian	226	282	131/95	120/162	71.6±9.1/ 69.5±7.60	Clinical criteria were confirmed by ABPI and arteriography.
Todesco,	caucasian	63	106	29/34	58/48	74±10/	(Fontaine, stage

1999						76±16	II and III/IV).
Verhoef, 1998	caucasian	48	272	ND	ND	NR	Clinical criteria confirmed by angiography and ultrasound.
Rassoul, 2000	caucasian	85	51	85/0	51/0	60 ±11 (all)	All patients were Fontaine stage III and IV
Ciccarone, 2003	caucasian	130	212	99/36	155/64	65±8/ 64±10	Clinical criteria confirmed by ultrasound. DM II patients
Pollex, 2005	caucasian	20	118	7/13	42/76	48 ±11.2/ 45.8±11.7	ABPI was used and World Health Organization intermittent claudication questionnaire. DM II patients
Folclaud, 2008	caucasian	130	457	90/40	457/0	69.7±8/ 60 (40-80)	Patients with intermittent claudication and arterial insufficiency were confirmed by ABPI
Santos, 2011	brazilian	39	32	23/16	9/23	66.89 ± 8.82/ 62.22± 12.92	Clinical examination and measurement of ABI

M: male, F: female, NR: not reported

Table 2: Fixed effects (FE) and random effects (RE) generalized odds ratios (OR_G) and heterogeneity results (I^2, P_Q-value) for the *MTHFR C677T* variant for testing its the association with PAD.

Population	Studies	FE OR_G (95% CI)	I^2, P_Q-value
		RE OR_G (95% CI)	
All	12	1.15 (1.03-1.30)	57%, <0.01
		1.21 (1.01-1.48)	
All in HWE	8	1.26 (1.09-1.45)	59%, 0.02
		1.44 (1.11-1.87)	
Whites	10	1.12 (1.01-1.26)	46%, 0.06
		1.13 (0.94-1.34)	

Figure 1: Flow chart of retrieved studies and studies excluded, with specification of reasons.

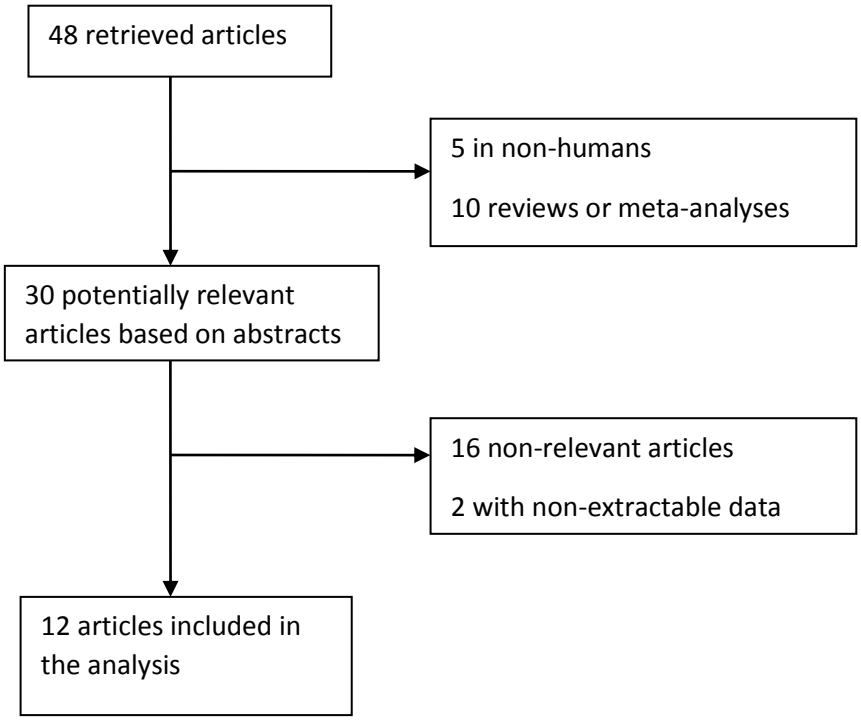

Figure 2: Fixed effects (FE) and random effects (RE) odds ratio (OR) estimates with the corresponding 95% confidence interval (CI) for the ORG of *MTHFR C677T* variant and the risk of PAD. The horizontal axis is plotted on a log scale

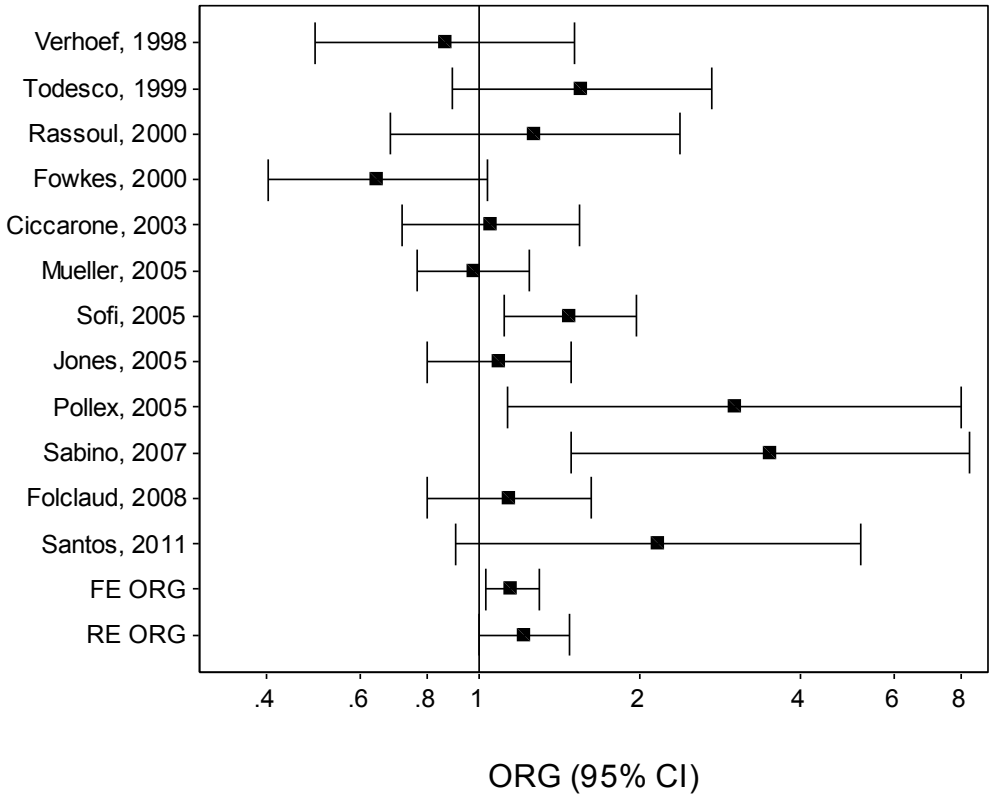

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Figure 3: Cumulative meta-analysis for *MTHFR C677T* variant in PAD: the pooled odds ratio (OR) with the corresponding 95% confidence interval (CI) at the end of each year-information step is shown

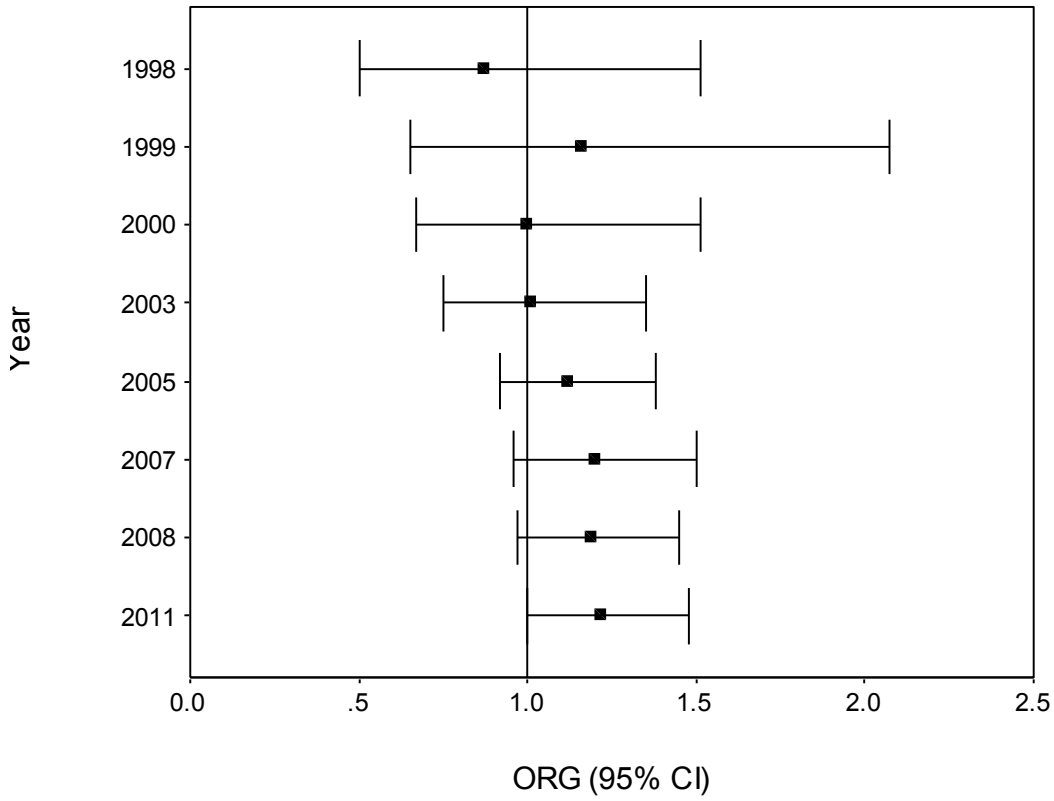

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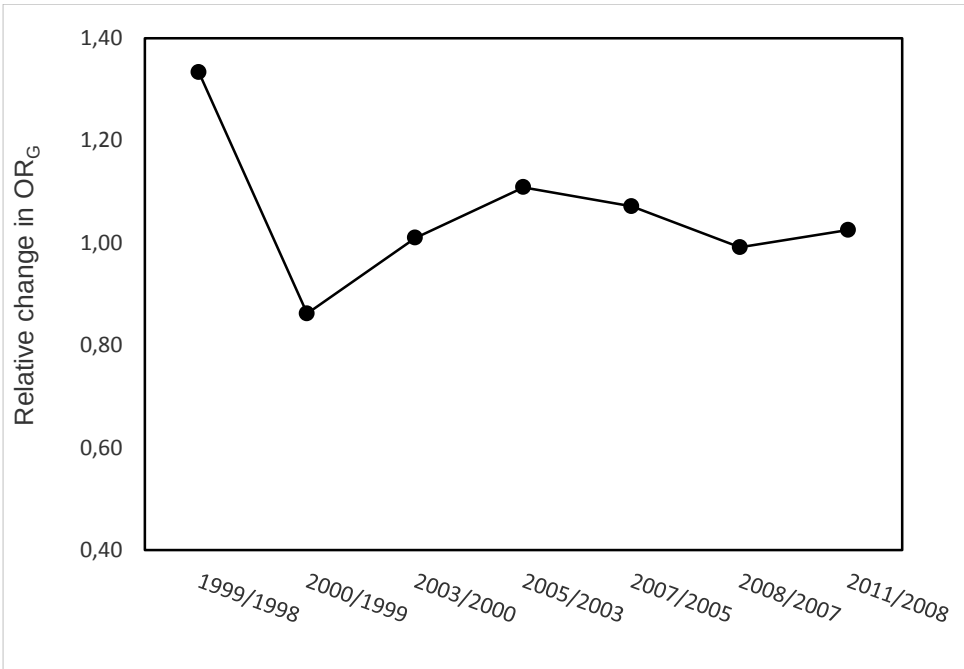
Figure 4: Recursive cumulative meta-analysis for *MTHFR C677T* variant in PAD: the relative change in random effects pooled odds ratio (OR) in each information step (OR in next year/ OR in current year) is shown.



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