



ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ  
Σχολή Επιστημών Υγείας  
Πανεπιστήμιο Θεσσαλίας

UNIVERSITY OF THESSALY, MEDICAL SCHOOL OF LARISSA  
Methodology of Biomedical Research, Biostatistics and  
Clinical Bioinformatics

UNIVERSITY OF THESSALY

MEDICAL SCHOOL OF LARISSA

MSc Program: Methodology of Biomedical Research, Biostatistics  
and Clinical Bioinformatics

Msc Final Paper

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

Stylios Koutsias

Supervisor: Professor Elias Zintzaras

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 πολύ 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 ήθελα να ευχαριστήσω ολόψυχα τα μέλη της τριμελούς επιτροπής για 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.

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



## 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 <sup>(1)</sup>. 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 <sup>(2)</sup>. 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<sup>(3)</sup>.

The existence of inherited genetic predisposition to PAD has been investigated with various types of familial aggregation studies <sup>(4-8)</sup>. 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% <sup>(8)</sup>. 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

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



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 <sup>(9)</sup>

Methylenetetrahydrofolate reductase (*MTHFR*) is a critical enzyme in one-carbon metabolism. *MTHFR* catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate 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* <sup>(11, 12)</sup> 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 <sup>(11, 12)</sup> 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 <sup>(17, 18)</sup>

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 <sup>(19,20)</sup> regarding the role of *MTHFR* C677T variant has been previously carried out, but they

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



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<sup>(21)</sup>, including cumulative and recursive cumulative meta-analysis<sup>(22, 23)</sup>, 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

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



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.<sup>(24)</sup> 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.

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



### **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 ( $OR_G$ )<sup>(25)</sup>. 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.<sup>(25, 26)</sup>

In synthesizing the studies, the fixed effects (FE) and random effects (RE)  $OR_G$  were used.<sup>(21, 25, 27)</sup> The RE model incorporates in the estimates the between study variability<sup>(21)</sup>. The heterogeneity between studies was tested using the Q-statistic<sup>(28, 29)</sup> 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<sup>(30)</sup>. 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<sup>(31)</sup>. In order to evaluate the trend of association, a cumulative and recursive cumulative meta-analysis was conducted based on the RE  $OR_G$ <sup>(21, 23, 32)</sup>. 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)<sup>(21)</sup>. 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<sup>(21)</sup>. In particular,

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





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 <sup>(33)</sup>.

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 <sup>(21)</sup>. The distribution of the genotypes in the control group was tested for conforming to the Hardy-Weinberg equilibrium (HWE) rule using an exact test <sup>(21)</sup>. Deviation from HWE indicates possible genotyping errors and/or population stratification and studies with controls deviated from HWE were subjected to a sensitivity analysis <sup>(21)</sup>. Analyses were performed using ORGGASMA (<http://biomath.med.uth.gr>) <sup>(25, 26)</sup>.

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



various populations of different ethnicity: 10 involved Whites and two Brazilian populations. The distribution of genotypes in the control group deviated from HWE ( $P \geq 0.05$ ) in four studies<sup>(34-37)</sup>. 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 ( $P=0.34$ ).

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



#### 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% <sup>(22)</sup>. 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 <sup>(21)</sup>. 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 <sup>(21)</sup>. Nevertheless, type II errors are expected to be less in a meta-analysis than in the individual GAS <sup>(21)</sup>.

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 <sup>(25)</sup>. In addition, there is no a priori biological justification for choice of a specific

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



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 <sup>(25)</sup>. 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 <sup>(25)</sup>.

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 interpreted 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 <sup>(38)</sup>. 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.

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



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 <sup>(39)</sup>. Hence, the validity of the genotyping method and the selection of controls are questionable for these studies <sup>(39, 40)</sup>. 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 <sup>(41)</sup>. 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 <sup>(42)</sup>. 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.

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



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.

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



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

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



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

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





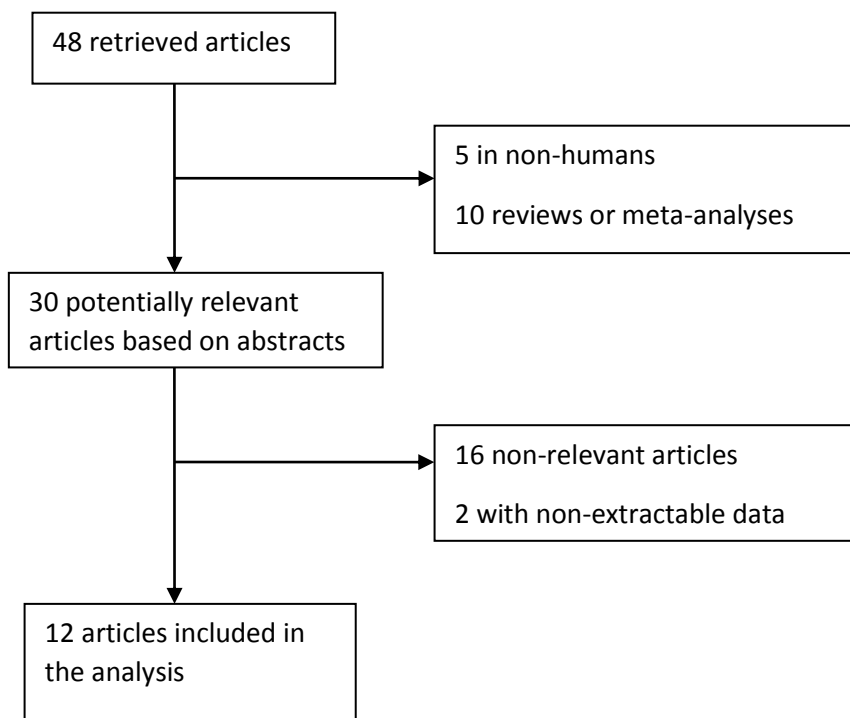
**Table 2:** Fixed effects (FE) and random effects (RE) generalized odds ratios (OR<sub>G</sub>) and heterogeneity results ( $I^2$ ,  $P_Q$ -value) for the *MTHFR C677T* variant for testing its the association with PAD.

Population	Studies	FE OR <sub>G</sub> (95% CI)	$I^2$ , $P_Q$ -value
		RE OR <sub>G</sub> (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)	

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



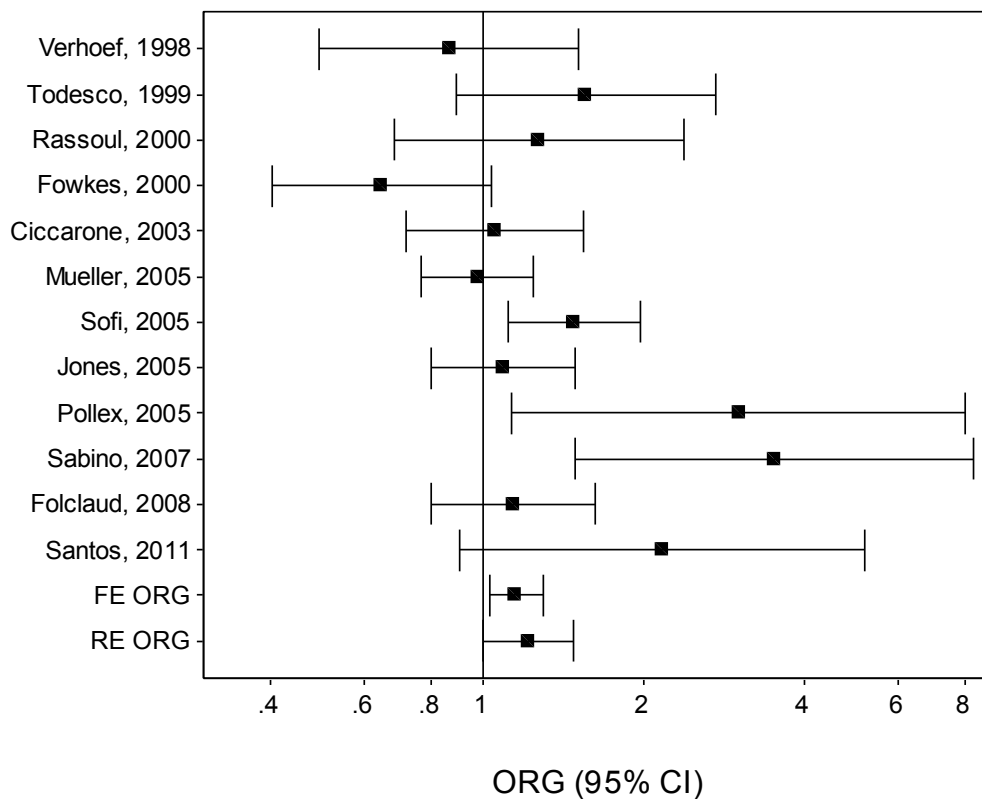
Figure 1: Flow chart of retrieved studies and studies excluded, with specification of reasons.



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



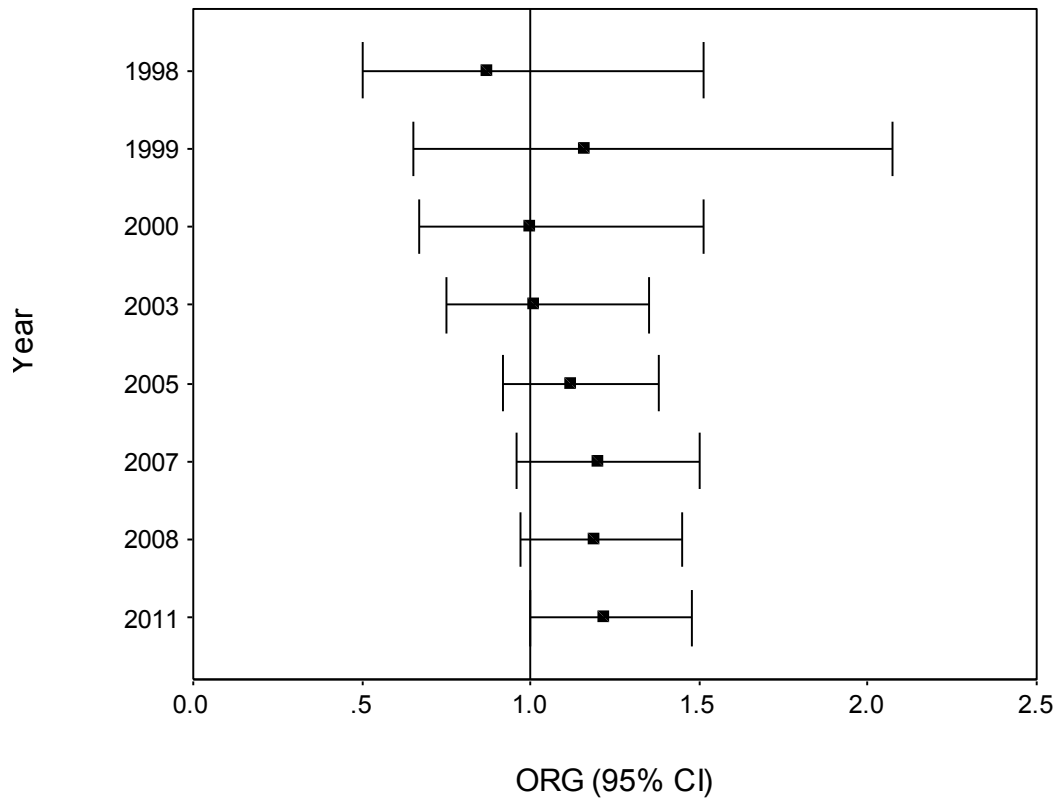
**Figure 2:** Fixed effects (FE) and random effects (RE) odds ratio (OR) estimates with the corresponding 95% confidence interval (CI) for the ORG of *MTHFR C677T* variant and the risk of PAD. The horizontal axis is plotted on a log scale



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



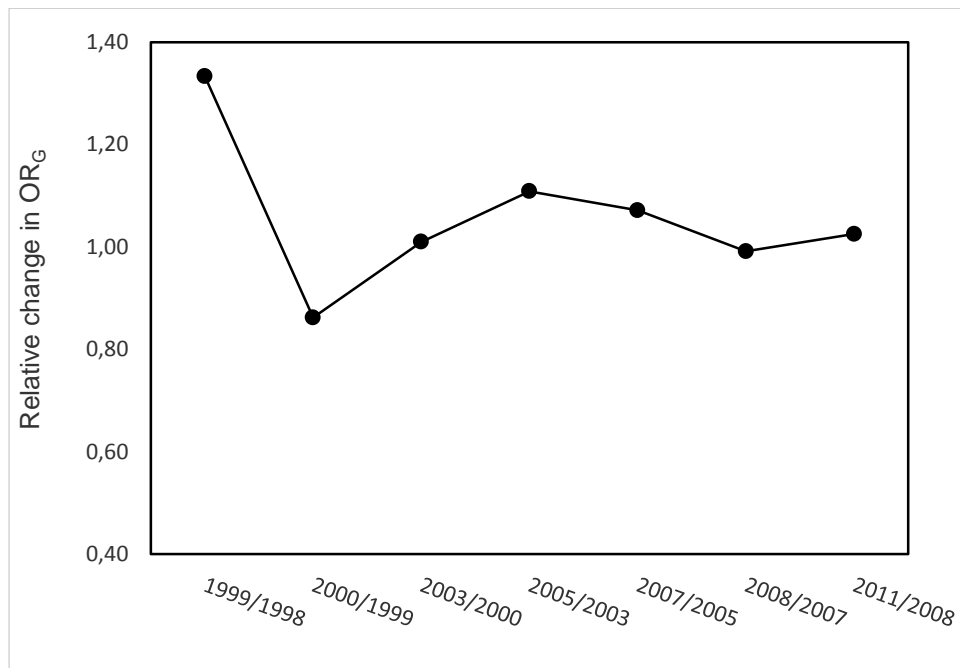
**Figure 3:** Cumulative meta-analysis for *MTHFR* C677T variant in PAD: the pooled odds ratio (OR) with the corresponding 95% confidence interval (CI) at the end of each year-information step is shown



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



**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.



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



## References:

1. Hirsch AT, Criqui MH, Treat-Jacobson D, et al. Peripheral arterial disease detection, awareness, and treatment in primary care. *JAMA*. 2001;286(11):1317-1324.
2. Hirsch AT, Haskal ZJ, Hertzler NR, et al. ACC/AHA Guidelines for the Management of Patients with Peripheral Arterial Disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Associations for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (writing committee to develop guidelines for the management of patients with peripheral arterial disease)--summary of recommendations. *J Vasc Interv Radiol*. 2006;17(9):1383-1397; quiz 1398.
3. McDermott MM, Mehta S, Greenland P. Exertional leg symptoms other than intermittent claudication are common in peripheral arterial disease. *Arch Intern Med*. 1999;159(4):387-392.
4. Valentine RJ, Verstraete R, Clagett GP, et al. Premature cardiovascular disease is common in relatives of patients with premature peripheral atherosclerosis. *Arch Intern Med*. 2000;160(9):1343-1348.

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



5. Allison MA, Criqui MH, McClelland RL, et al. The effect of novel cardiovascular risk factors on the ethnic-specific odds for peripheral arterial disease in the Multi-Ethnic Study of Atherosclerosis (MESA). *J Am Coll Cardiol.* 2006;48(6):1190-1197.
6. Carmelli D, Fabsitz RR, Swan GE, et al. Contribution of genetic and environmental influences to ankle-brachial blood pressure index in the NHLBI Twin Study. National Heart, Lung, and Blood Institute. *Am J Epidemiol.* 2000;151(5):452-458.
7. Kullo IJ, Turner ST, Kardia SL, et al. A genome-wide linkage scan for ankle-brachial index in African American and non-Hispanic white subjects participating in the GENOA study. *Atherosclerosis.* 2006;187(2):433-438.
8. Murabito JM, Guo CY, Fox CS, et al. Heritability of the ankle-brachial index: the Framingham Offspring study. *Am J Epidemiol.* 2006;164(10):963-968.
9. Clark AG. The role of haplotypes in candidate gene studies. *Genet Epidemiol.* 2004;27(4):321-333.
10. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage.

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



11. Proc Natl Acad Sci U S A 1997;94(7):3290-3295.  
Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10(1):111-113.
12. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998;64(3):169-172.
13. Zintzaras E, Koufakis T, Ziakas PD, Rodopoulou P, Giannouli S, Voulgarelis M. A meta-analysis of genotypes and haplotypes of methylenetetrahydrofolate reductase gene polymorphisms in acute lymphoblastic leukemia. Eur J Epidemiol 2006;21(7):501-510.
14. Zintzaras E. Association of methylenetetrahydrofolate reductase (MTHFR) polymorphisms with genetic susceptibility to gastric cancer: a meta-analysis. J Hum Genet 2006;51(7):618-624.
15. Zintzaras E. Methylenetetrahydrofolate reductase gene and susceptibility to breast cancer: a meta-analysis. Clin Genet 2006;69(4):327-336.
16. Zintzaras E, Uhlig K, Koukoulis GN, Papathanasiou AA, Stefanidis I. Methylenetetrahydrofolate reductase gene polymorphism as a risk factor for diabetic nephropathy: a meta-analysis. J Hum Genet 2007;52(11):881-890.

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





17. Kelemen LE, Sellers TA, Schildkraut JM, Cunningham JM, Vierkant RA, Pankratz VS, et al. Genetic variation in the one-carbon transfer pathway and ovarian cancer risk. *Cancer Res* 2008;68(7):2498-2506.
18. Zintzaras E. Genetic variants of homocysteine/folate metabolism pathway and risk of inflammatory bowel disease: a synopsis and meta-analysis of genetic association studies. *Biomarkers* 2010;15(1):69-79.
19. Zintzaras E, Zdoukopoulos N. A field synopsis and meta-analysis of genetic association studies in peripheral arterial disease: The CUMAGAS-PAD database. *Am J Epidemiol.* 2009 Jul 1;170(1):1-11. doi: 10.1093/aje/kwp094. Epub 2009 May 12. PubMed PMID: 19435865.
20. Khandanpour N, Willis G, Meyer FJ, Armon MP, Loke YK, Wright AJ, Finglas PM, Jennings BA. Peripheral arterial disease and methylenetetrahydrofolate reductase (MTHFR) C677T mutations: A case-control study and meta-analysis. *J Vasc Surg.* 2009 Mar;49(3):711-8. doi: 10.1016/j.jvs.2008.10.004. Epub 2009 Jan 21. Review. PubMed PMID: 19157768.
21. Trikalinos TA, Salanti G, Zintzaras E, Ioannidis JP. Meta-analysis methods. *Adv Genet* 2008;60:311-334.
22. Zintzaras E, Lau J. Trends in meta-analysis of genetic association studies. *J Hum Genet* 2008;53(1):1-9.

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



23. Lau J, Antman EM, Jimenez-Silva J, Kupelnick B, Mosteller F, Chalmers TC. Cumulative meta-analysis of therapeutic trials for myocardial infarction. *N Engl J Med* 1992;327(4):248-254.
24. Hankey GJ, Norman PE, Eikelboom JW. Medical treatment of peripheral arterial disease. *JAMA*. 2006;295(5):547-553.
25. Zintzaras E. The generalized odds ratio as a measure of genetic risk effect in the analysis and meta-analysis of association studies. *Stat Appl Genet Mol Biol*. 2010;9:Article21. doi: 10.2202/1544-6115.1542. Epub 2010 May 12. PubMed PMID: 20597847.
26. Zintzaras E. The power of generalized odds ratio in assessing association in genetic studies with known mode of inheritance. *J Appl. Stat*. 2012. 39(12): 2569-2581.
27. DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986;7(3):177-188.
28. Munafò MR, Flint J. Meta-analysis of genetic association studies. *Trends Genet* 2004;20(9):439-444.
29. Zintzaras E, Ioannidis JP. HELOW: a program for testing extreme homogeneity in meta-analysis. *Comput Methods Programs Biomed*. 2014 Nov;117(2):383-6. doi: 10.1016/j.cmpb.2014.06.009. Epub 2014 Jun 23. PubMed PMID: 25023534.
30. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002;21(11):1539-1558.

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



31. Zintzaras E, Lau J. Synthesis of genetic association studies for pertinent gene-disease associations requires appropriate methodological and statistical approaches. *J Clin Epidemiol*. 2008; 61(7):634-645.
- 32.: Zintzaras E. Examining the stability of genetic risk effect as evidence accumulates in the context of meta-analysis. *Comput Biol Med*. 2013 Sep;43(9):1293-9. doi: 10.1016/j.combiomed.2013.04.017. Epub 2013 May 14. PubMed PMID: 23768991.
33. Harbord RM, Egger M, Sterne JA. A modified test for small-study effects in meta-analyses of controlled trials with binary endpoints. *Stat Med* 2006;25(20):3443-3457.
34. X Giovannetti E, Ugrasena DG, Supriyadi E, Vroling L, Azzarello A, de Lange D, et al. Methylenetetrahydrofolate reductase (MTHFR) C677T and thymidylate synthase promoter (TSER) polymorphisms in Indonesian children with and without leukemia. *Leuk Res* 2008;32(1):19-24.
35. Alcasabas P, Ravindranath Y, Goyette G, Haller A, Del Rosario L, Lesaca-Medina MY, et al. 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphisms and the risk of acute lymphoblastic leukemia (ALL) in Filipino children. *Pediatr Blood Cancer* 2008;51(2):178-182.

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



36. Damjanovic T, Milicevic R, Novkovic T, Jovicic O, Bunjevacki V, Jekic B, et al. Association between the methylenetetrahydrofolate reductase polymorphisms and risk of acute lymphoblastic leukemia in Serbian children. *J Pediatr Hematol Oncol* 2010;32(4):e148-150.
37. Sood S, Das R, Trehan A, Ahluwalia J, Sachdeva MU, Varma N, et al. Methylenetetrahydrofolate reductase gene polymorphisms: association with risk for pediatric acute lymphoblastic leukemia in north Indians. *Leuk Lymphoma* 2010;51(5):928-932.
38. Lau J, Ioannidis JP, Terrin N, Schmid CH, Olkin I. The case of the m Weir B. Genetic data analysis II: methods for discrete population genetic data. Sunderland MA: Sinauer Associates, 1996. isleading funnel plot. *BMJ* 2006;333(7568):597-600.
39. Weir B. Genetic data analysis II: methods for discrete population genetic data. Sunderland MA: Sinauer Associates, 1996.
40. Xu J, Turner A, Little J, et al. Positive results in association studies are associated with departure from Hardy-Weinberg equilibrium: hint for genotyping error? *Hum Genet.* 2002;111(6):573-574.
41. Chapman J, Clayton D. Detecting association using epistatic information. *Genet Epidemiol* 2007;31(8):894-909.
42. Clayton D, McKeigue PM. Epidemiological methods for studying genes and environmental factors in complex diseases. *Lancet* 2001;358(9290):1356-1360.

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