



Qualitative assessment of previous evidence and an updated meta-analysis confirms lack of association between the *ESR1* rs2234693 (*PvuII*) variant and coronary heart disease in men and women

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ARTICLE INFO

Article history:

Received 6 March 2009

Received in revised form 15 April 2009

Accepted 28 May 2009

Available online 18 June 2009

Keywords:

Coronary disease

Estrogen Receptor alpha

Polymorphism

Genetic

Meta-analysis

ABSTRACT

Background: Coronary heart disease (CHD) is the leading cause of mortality worldwide. CHD clusters in families but this familial aggregation remains largely unexplained. *ESR1* is a candidate gene for CHD although recent meta-analyses of the rs2234693 variant reported inconsistent evidence for association with myocardial infarction (MI) in men. The objectives of this study were to perform a qualitative and a quantitative assessment of all evidence to date regarding this association.

Methods: We performed structured literature searches for studies addressing the association between the *ESR1* rs2234693 and CHD. We assessed the quality of these studies collectively and individually according to recently published guidelines on the reporting and interpretation of genetic association studies. We also performed a meta-analysis of all studies to date, including a sample of MI cases and controls from our region.

Results: The qualitative assessment indicated that many studies met a low proportion of the criteria proposed by the current guidelines. No significant association between *ESR1* rs2234693 and MI was observed in our sample or in the meta-analysis (16 studies; $N \sim 32,000$; $OR \sim 1$). Strong between-study heterogeneity was largely explained by a quality score based on the quality criteria. Studies that reported significant associations were generally of poorer quality.

Conclusion: We confirm the lack of association between the *ESR1* rs2234693 and CHD, and show that inconsistencies between previous studies is explained by differences in their quality.

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1. Introduction

Coronary heart disease (CHD) is the leading cause of mortality worldwide [1]. CHD clusters in families with estimates of heritability ranging from 38 to 57% [2]. A positive family history of CHD remains a strong risk factor even after adjustment for traditional risk factors [3], suggesting that genetic variation could play an important role in CHD risk. Although recent studies have identified some variants associated with this disease, to date the genetic architecture of CHD remains largely unexplained by known gene variants [4,5].

Using the candidate gene association study approach, many single gene variants have been reported to be associated with common diseases including CHD, although replication of such studies is often inconsistent [6]. Recently, a framework for assessing cumulative epidemiological evidence in genetic association studies [7] and a set of best practices for the design, conduct, reporting and publication of replication studies [8] have been proposed.

Among the genes that have been studied as potential modulators of CHD risk [4], *ESR1*, which encodes Estrogen Receptor α , is an interesting candidate because estrogen has beneficial effects on cardiovascular health. Premenopausal women have a lower incidence of CHD than postmenopausal women [9], in whom CHD risk approaches that observed in men. Genetic variants in *ESR1* have been reported to be associated both with CHD risk factors [10], as well as clinical endpoints like stroke [10] and myocardial infarction (MI) [11]. However, the results of these studies have been inconsistent, and further investigation is required.

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Recently two meta-analyses have been published in relation to the role of the *ESR1* rs2234693 (*PvuII*; IVS1-397 T>C) variant in risk of MI. Shearman *et al.* [11] reported a significant association between rs2234693 and MI, but this was not replicated by Kjaer-gaard *et al.* [12].

The objectives of this study were: (i) to perform a qualitative assessment of all evidence reported to date regarding the association between the *ESR1* rs2234693 variant and MI using recently published guidelines on the reporting and interpretation of genetic association studies; and, (ii) to update and expand the previously published meta-analyses, including new data from our study and other new published association studies, and extending the meta-analysis to coronary heart disease and to women.

2. Materials and methods

2.1. Candidate gene association study (REGICOR study)

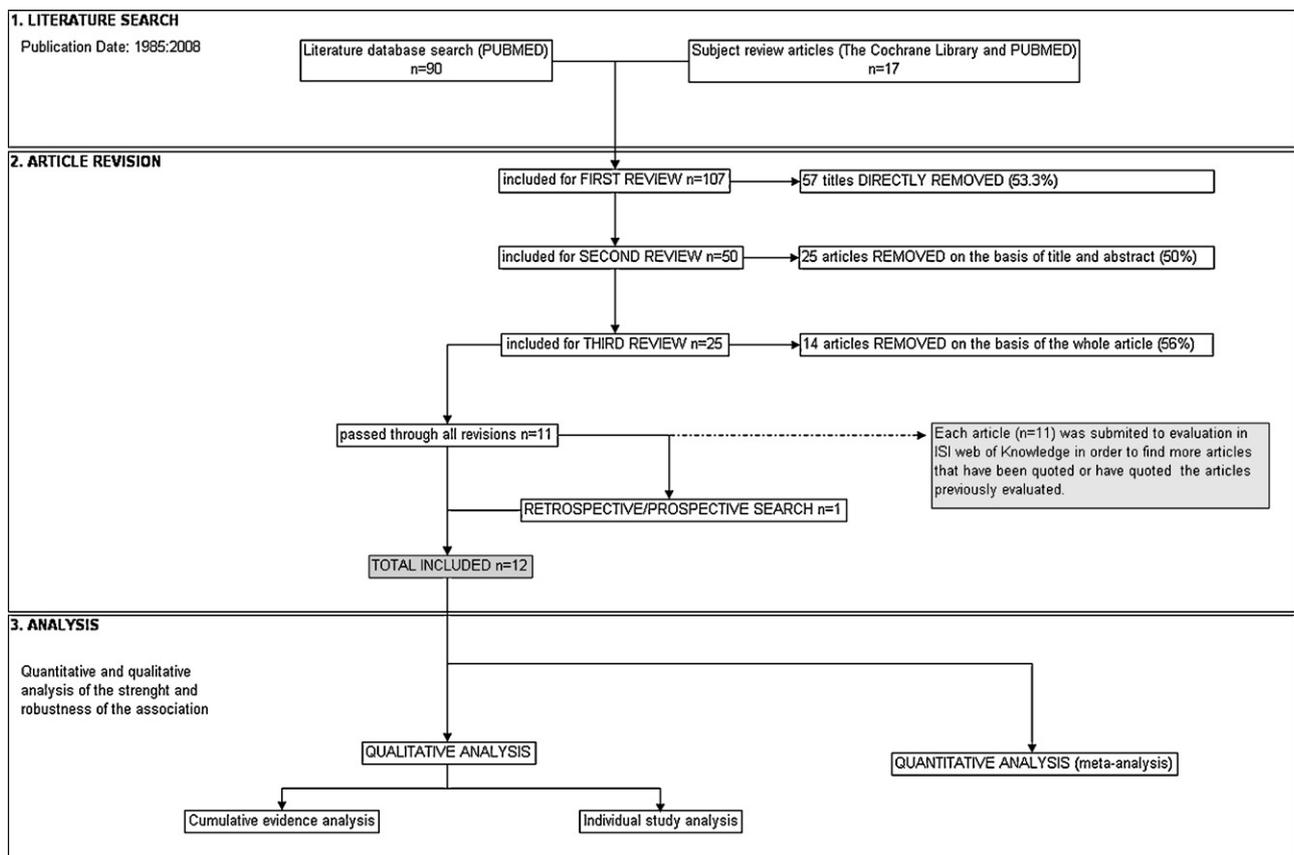
The study sample was composed of 423 cases of MI, aged 29–74, and 1269 age- and sex-matched controls (case–control ratio, 1:3). Cases were survivors of a first MI who were recruited consecutively in the reference hospital (Hospital Universitari de Girona Dr. Josep Trueta) of the REGICOR (Registre Gironí del Cor) study catchment area (Girona, Spain [13]). MI was defined on the basis of MONICA criteria [14]. Controls were randomly selected from two cross-sectional studies carried out in the province of Girona, in 1994–1996 and 1999–2001 to establish the prevalence of cardiovascular risk factors in this region [13]. Controls were free from angina and MI, as determined by clinical history, physical examination and electrocardiography. Hypertension, diabetes, dyslipidemia and family history of CHD were self-reported or confirmed by treatment. Sub-

jects were classified as current smokers if they had reported having smoked during the previous year. Barefoot weight and height measurements were used to calculate body mass index (BMI; weight in kg divided by height in m²). This study was approved by the local Ethics Committee and all participants gave written informed consent. All subjects were of European descent.

The rs2234693 variant was genotyped in these individuals by TaqMan Assay (ABI PRISM 7900HT; Applied Biosystems, Foster City, CA), using previously described primers and probes [12]. Deviation from Hardy–Weinberg equilibrium (HWE) among controls was assessed using a chi-square (χ^2) test with one degree of freedom (df). The distributions of clinical variables in each genotype group were compared using a χ^2 test or Fisher exact test for categorical variables, and analysis of variance for normally distributed continuous variables. Genotype frequencies in cases and controls were compared using a 1 df χ^2 test (two separate tests: common homozygotes (TT) versus heterozygotes (CT); common homozygotes (TT) versus rare homozygotes (CC)). Our sample had 80% power to detect an odds ratio (OR) of 1.37 at a significance level of $p < 0.05$, given the minor allele frequency among controls (MAF = 0.45). Statistical analysis was carried out using SPSS v12 (SPSS Inc. Chicago, IL), and power calculation was carried out using the *bpower* function from the R package *Hmisc*. A p -value of < 0.05 was considered statistically significant.

2.2. Qualitative assessment of previous evidence

To assess the quality of current evidence regarding the association between rs2234693 and CHD (including fatal and non-fatal acute MI, angina, angiographic CHD), we searched the published literature for studies addressing this question, and evaluated these



studies collectively and individually according to published guidelines on the reporting and interpretation of association studies [7,8] (flow chart in Fig. 1).

To identify studies of interest, we performed structured literature searches in PubMed and reviewed the bibliographies of topical review articles. To further ensure the inclusion of all relevant studies, we also performed retrospective bibliography searches and prospective cited-reference searches for all articles encountered during this process. The search strategy used is described in detail in [Supplementary Data](#). Data of interest was extracted from these articles by three independent reviewers (CL, GL, RE). This information was used to assess the quality of reported evidence for association, and also to perform a meta-analysis of these studies.

The quality of the evidence for association between rs2234693 and CHD risk was assessed cumulatively and also for each individual article.

The quality of the cumulative evidence for this association was assessed according to the classification system proposed by Ioannidis *et al.* [7]. Briefly, this classification consists of a 3-letter code, which describes (i) the quantity of evidence for a given association in terms of the total number of carriers of the rare allele from all reported studies, (ii) the extent of independent replication, and (iii) the likelihood of an important bias in the studies reported. The quality of evidence for the association can then be ranked as “strong”, “moderate” or “weak”.

Assessment of the quality of evidence at the individual article/study level was performed according to guidelines proposed by the NCI-NHGRI Working Group on Replication in Association Studies [8], a set of recommended best practices for the design, conduct and publication of studies that report an association or attempt to replicate a reported association. These guidelines identify a set of attributes that an association study should ideally possess, and are presented in the form of 55 conditions/questions related to experimental design, demography, quality control, etc. For each of these conditions, data was extracted from the articles identified in the literature searches mentioned above, and each study was scored as 1 when the requirement was met and 0 otherwise. In order to provide as objective an assessment as possible, we considered only the first 43 questions/conditions, since the remainder (44–55) were related to the subjective view of the author/reviewer. Thus we assigned a quality score (QS) from 0 to 43 for each study. The data retrieval process and scoring strategy is described in more detail in [Supplementary Data](#).

2.3. Quantitative assessment of all evidence to date (meta-analysis)

To quantitatively summarise all evidence reported to date regarding the putative association between rs2234693 and CHD, we performed a meta-analysis of studies previously meta-analysed [11,12], as well as 7 additional published association studies [15–21] and the REGICOR study data presented here. Genotype data and sample characteristics for these studies are shown in [Supplementary Table 5](#).

Genotype frequencies in cases and controls were extracted from each article. These data were used to perform two genotypic tests of association in each study (common homozygotes versus heterozygotes (CT vs. TT), and common homozygotes versus rare homozygotes (CC vs. TT)). A pooled OR was estimated by inverse-variance weighting under a random-effects model (DerSimonian/Laird method [22]). A χ^2 test for heterogeneity between studies included in this meta-analysis was performed. The meta-analysis was also carried out separately for males and females.

Statistical analysis for the meta-analysis was performed using the *meta.DSL* function from the R package *rmeta* [23]. In the pres-

ence of between-study heterogeneity, a meta-regression under a mixed-effects model [24], was also carried out to identify study characteristics that may account for this heterogeneity. To identify studies that had individually strong effects on the pooled OR, sensitivity analysis was performed by excluding one study at a time and calculating the OR for the remaining studies.

All of the analyses described here were also carried out for the rs9340799 (*Xbal*) variant in *ESR1*.

3. Results

3.1. Candidate gene association study

Demographic characteristics, distribution of clinical variables and genotype counts and frequencies for all individuals are shown in [Table 1](#), and stratified by sex in [Supplementary Table 6](#). No significant association was observed between genotype frequencies and demographic and clinical variables in cases compared to controls ($p > 0.05$). No significant difference in genotype frequencies was observed among cases compared to controls (CT vs. TT: OR 1.19, 95%CI 0.92–1.54, $p = 0.185$; CC vs. TT: OR 0.98, 95%CI 0.71–1.37, $p = 0.915$).

3.2. Qualitative assessment of previous evidence

We identified 12 articles, representing 15 distinct cohorts/samples that tested for association between rs2234693 and CHD (see [Table 2](#) for study characteristics). These included 4 cohort studies [11,12,25,26] and 11 case-control studies [11,12,15–21,27].

Table 1
Characteristics of the cases and controls participating in REGICOR study.

	Cases (N = 423)	Controls (N = 1269)	p-Value ¹
Age (years)	61.21 ± 11.16	60.72 ± 10.65	0.416
Sex (men/women)	318/105	954/315	1
Hypertension ^a	230 (54.4)	437 (34.4)	<0.001
Diabetes ^a	96 (22.7)	270 (21.3)	0.08
Dyslipemia ^a	218 (51.5)	559 (44.1)	<0.001
Smoking			
Never smokers	108 (25.5)	619 (48.8)	<0.001
Current smokers	176 (41.6)	253 (19.9)	
Ex-smokers >1 year	81 (19.1)	371 (29.2)	
Body mass index (kg/m ²)	27.70 ± 4.57	28.18 ± 4.12	0.102
Family history of CHD ^a	60 (14.2)	134 (10.6)	<0.001
rs2234693 (<i>PvuII</i>)			
TT	117 (27.7)	383 (30.2)	0.276
CT	231 (54.6)	636 (50.1)	
CC	75 (17.7)	250 (19.7)	
MAF	0.450	0.450	
OR (95% CI)			
TT		1	
CT		1.19 (0.92–1.54)	0.185
CC		0.98 (0.71–1.37)	0.915
rs9340799 (<i>Xbal</i>)			
AA	175 (41.4)	544 (42.9)	0.387
AG	202 (47.8)	563 (44.4)	
GG	46 (10.8)	162 (12.7)	
MAF	0.348	0.349	
OR (95% CI)			
GG		1	
AG		1.26 (0.86–1.85)	0.208
AA		1.13 (0.78–1.64)	0.507

Results are expressed as mean ± SD for normally distributed variables or *n* (%) for categorical variables. MAF, minor allele frequency; SD, standard deviation. Both polymorphisms were in HWE.

^a Self-reported history or treatment.

¹ To test differences between cases and controls, a Pearson χ^2 test was performed for categorical variables and a Student's *t*-test for continuous-normal distributed variables.

Table 2Characteristics of the studies included in the qualitative and quantitative evaluation of the association between *ESR1* rs2234693 (*PvuII*) and coronary heart disease.

Reference	Country	Study design	% women (cases/controls)	Clinical term	N cases	N controls	N total	QS	\overline{QS}^a
Matsubara et al. [18]	Japan	Case/control	25.3/20.2	CAD	87	94	181	9	–
Alevizaki et al. [15]	Greece	Case/control	100.0/100.0	CAD	87	70	157	13	+
Almeida and Hutz [16]	Brazil	Case/control	28.8/55.9	CAD	210	143	353	9	–
Mansur et al. [17]	Brazil	Case/control	40.0/46.0	CAD	153	142	295	6	–
Yilmaz et al. [21]	Turkey	Case/control	35.7/62.6	CAD	168	99	267	10	–
Xu et al. [20]	China	Case/control	44.8/25.9	CAD	210	174	384	9	–
Koch et al. [27]	Germany	Case/control	24.2/49.4	MI	3587	1211	4798	16	+
Shearman Framingham Heart study [11,25]	USA	Cohort	0.0/0.0	MI	154	721	875	11	–
Shearman Rotterdam [11,26]	Netherlands	Cohort	0.0/0.0	MI	303	1869	2172	11	–
Shearman Northwick Park Heart study [11]	UK	Cohort	0.0/0.0	MI	360	2349	2709	7	–
Shearman GCI-USA [11]	USA	Case/control	0.0/0.0	MI	226	414	640	7	–
Shearman GCI-Poland [11]	Poland	Case/control	0.0/0.0	MI	235	441	676	7	–
Kjaergaard et al. [12]	Denmark	Cohort	44.7/57.9	MI	1137	8044	9181	19	+
Kjaergaard et al. [12]	Denmark	Case/control	27.8/60	MI	2495	4447	6942	19	+
Morgan et al. [19]	USA	Case/control	32.2/39.4	ACS	805	656	1461	14	+
REGICOR	Spain	Case/control	25.0/25.0	MI	423	1269	1692	10	–

Shearman [11] present results from 5 studies, of which the results for rs2234693 from the Framingham Heart Study [25] and the Rotterdam Study [26] were previously reported. The QS shown for these studies corresponds to the evaluation of the original article. For the three remaining samples the QS shown corresponds to the evaluation of the article by Shearman et al. [11].

CAD, coronary artery disease; MI, myocardial infarction; ACS, acute coronary syndrome; QS, quality Score; \overline{QS} , mean QS.

^a QS less than (–) or greater than (+) the \overline{QS} (11.06).

The quality of cumulative evidence reported to date regarding this association was assessed according to the guidelines proposed by Ioannidis et al. [7]. In terms of the amount of evidence (first letter), the total number of individuals reported in the least common genetic group of interest (rare homozygote, CC) was 6964, 2163 cases, and 4801 controls (Supplementary Table 5), corresponding to classification A. In terms of the extent of replication, (second letter), this association has been extensively replicated, but without between-study consistency corresponding to classification B. Finally, in terms of potential biases in reported studies (third letter), there were no obvious biases in the studies reported, but there was considerable missing information regarding the generation of evidence, corresponding to classification B. Therefore, the cumulative evidence presented in all previously reported studies achieves a classification of ABB, corresponding to “moderate” evidence [7].

For each of these 12 articles, the quality of the evidence presented was assessed using a scoring process based on 43 of the 55 conditions cited in the NCI-NHGRI guidelines [8]. A grid of the full results of this exercise (43 conditions by 12 articles), and the resulting QS's are presented in Supplementary Table 4; the QS for each article is also shown in Table 2 and Fig. 2. The mean number of conditions met was 11.06 (median 10; range 6–19). Studies with a relatively small total sample size, e.g. <1000 subjects [11,15–18,20,21], generally met fewer conditions (mean QS 9; range 6–13). Moreover, the smaller studies generally showed more extreme, non-mutually concordant results (Fig. 2). The five larger studies [11,12,19,26,27] generally performed better (mean QS 13.7; range 7–19), and concordantly, these studies reported generally less extreme, non-significant results and narrower confidence intervals.

These results generally show broad variability in quality between studies, and a negative relationship between the reported OR and the QS, consistent with no association in the high quality, generally larger and better powered studies, and a false-positive association in the smaller, lower quality studies.

3.3. Quantitative assessment of all evidence to date, meta-analysis

A meta-analysis was performed using data from the 15 studies identified in the literature searches and the REGICOR data

reported here (32,783 individuals; Table 2). No significant association between this variant and CHD was observed [pooled OR 1.06, 95%CI 0.96–1.18, $p=0.243$ (CT vs. TT test) and 1.17, 95%CI 1.00–1.32, $p=0.055$ (CC vs. TT test)], nor when the analysis was stratified by gender (Fig. 2a and Supplementary Fig. 2) or by phenotype (MI/CAD) (data not shown).

The meta-analysis showed substantial between-study heterogeneity ($\chi^2=29.04$, $p=0.0159$ and $\chi^2=47.24$, $p=0.0000337$, for the CT vs. TT and CC vs. TT tests, respectively). Meta-regression analysis showed that most of this heterogeneity was explained by QS (heterogeneity, after adjusting for QS: $\chi^2=24.37$, $p=0.0413$ and $\chi^2=35.21$, $p=0.0014$, for CT vs. TT and CC vs. TT, respectively). Heterogeneity could not be accounted for by clinical outcome (MI/CAD), study design, gender or sample size, either individually or in combination.

Having identified QS as accounting for the majority of between-study heterogeneity, we stratified the meta-analysis by the mean QS (QS; 11.06; Fig. 2b). A significant association was observed (CC vs. TT test) for studies with QS < \overline{QS} (pooled OR 1.37, 95%CI 1.08–1.74, $p=0.01$), while no association was observed for studies with QS > \overline{QS} (pooled OR 0.93, 95%CI 0.82–1.05, $p=0.248$). Between-study heterogeneity persisted for studies with QS < \overline{QS} , but disappeared for studies with QS > \overline{QS} . The results for the CT vs. TT test remained non-significant after stratification by \overline{QS} .

In the sensitivity analysis (Fig. 2a), the pooled OR for the CC vs. TT meta-analysis (range 1.10–1.22) was most strongly affected by the removal of the Shearman study [11] (pooled OR (95%CI) reduced from 1.17 (1.00–1.32) to 1.10 (0.95–1.27)). The results of the CT vs. TT meta-analysis were not significantly affected by the removal of any study.

Results of these analyses carried out for the rs9340799 (XbaI) variant are shown in the Supplementary Data. No significant association was found for any of the tests performed.

4. Discussion

In this study, we performed a qualitative and quantitative evaluation of all reported evidence regarding a widely studied putative association between the rs2234693 variant in Intron 1 of the *ESR1* gene and CHD. We also present data from the REGICOR study to address this question in our population. We have updated and

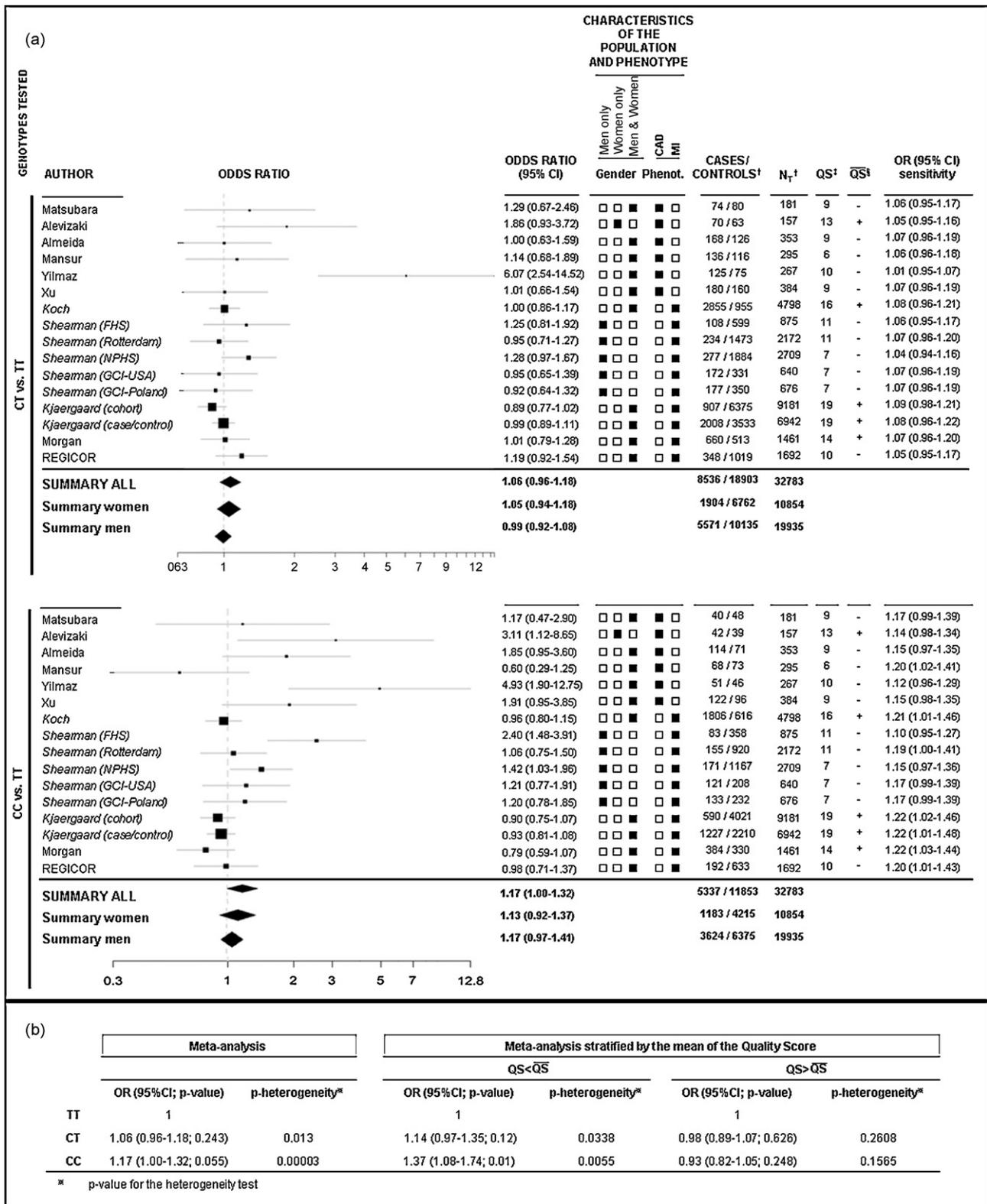


Fig. 2. Meta-analysis results for the association between rs2234693 and coronary heart disease. (a) Forest plot showing meta-analysis results. Data presented in a logarithmic scale for two genotypic tests (CC vs. TT and CT vs. TT). The pooled OR is shown as a diamond (◆), where the width of the diamond corresponds to the 95%CI. Studies in italics were included in the most recent meta-analysis [12]. The data for the gender-stratified analysis was performed using data for studies that provided gender-stratified data. OR (95%CI) sensitivity was calculated by excluding one study at a time and calculating the OR for the remaining studies. ¹N (cases/controls) for each genotypic test; ²N_T: total number of individuals in each study. ³Quality score (QS). ⁴QS less than (-) or greater than (+) the mean QS (QS; 11.06). (b) Crude meta-analysis results and stratified by QS. Using meta-regression, QS was found to account for most of the between-study heterogeneity. Stratifying by QS, the pooled odds ratio remained significant and between-study heterogeneity persisted in the meta-analysis of studies with QS < QS, while both the association and the between-study heterogeneity disappeared in the meta-analysis of studies with QS > QS.

extended two previous meta-analyses of association studies, and we observe no evidence of association between rs2234693 and CHD in >32,000 individuals. We observe significant heterogeneity between the ORs reported in previous studies and show that much of this heterogeneity can be explained by study quality. In our qualitative analysis we conclude that previous evidence in favour of this association is moderate. We observe that the reported OR is associated with both study sample size and a descriptive measure of study quality. Similar results were observed for the rs9340799 (XbaI) variant (Supplementary Data).

Neither rs2234693 nor rs9340799 was associated with MI in the REGICOR study (men/women tested separately or together), which is consistent with the results observed for higher quality studies in the meta-analyses (OR ~ 1).

The qualitative assessment showed considerable variation in quality of evidence between previous studies. While several of the criteria proposed in the guidelines used [8] might not be strictly necessary for a candidate gene association study, some studies lacked a number of important attributes, indicating that further efforts are required to standardise the design, and reporting of genetic association studies. The cumulative evidence [7] available is moderate for the rs2234693 variant and weak for the rs9340799 variant.

We have also updated previous meta-analyses regarding the putative association between rs2234693 and CHD [11,12]. As noted by Kjaergaard et al. [12], it was not possible to perform the covariate adjustment used by Shearman et al. [11] because they did not describe which covariates they adjusted for. For this reason, and since the inheritance model tested was not consistent across studies, we meta-analysed the crude results from each study. We have extended the previous meta-analyses in a number of ways: (a) expanding the phenotype definition to include both fatal MI and chronic CHD, (b) extending the meta-analysis to women, (c) adding data from 8 additional studies, including the results from the REGICOR project, increasing the total sample size from 16,706 to 32,783 samples, and most importantly (d) we show that differences in the quality of previous studies provide a convincing explanation for the lack of consistency between these studies. Using meta-regression, we found that much of the heterogeneity in our meta-analysis could be explained by study quality, and that this heterogeneity was mainly restricted to lower quality studies. Moreover, the higher quality studies showed consistently non-significant results [28]. This highlights the utility of quality assessment frameworks, such as those used in this study, for the interpretation of genetic association studies, and the motivation or design of follow-up studies. We feel that our results now allow investigators who are interested in the role of *ESR1* variation in relation to MI/CHD to confidently accept the null hypothesis for this variant.

Although we confirm the lack of association between CHD and the widely studied rs2234693 variant, this does not eliminate the possibility that other *ESR1* variation may be involved in CHD risk. A large number of variants have been reported in this gene (~2700, dbSNP Build 129, April 08), but less than 10 have been studied. The majority of studies have focused on the rs2234693 variant, which may reflect the tendency for early studies to examine the same variant as had previously been studied in relation to other complex phenotypes (e.g. cancer [29] and bone mineral density [30]).

In conclusion, this study refutes the hypothesis that the *ESR1* rs2234693 variant is involved in CHD risk. We observe significant heterogeneity between the results of previous studies, which is mainly related to their quality. Moreover, study quality is inversely associated with the magnitude of the reported OR. Further studies analyzing a broader range of variants may be able to better describe the relationship, if any exists, between variation in this gene and risk of CHD.

Acknowledgments

The REGICOR Study was partially funded by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III/FEDER (Red HERACLES RD06/0009), the CIBER Epidemiología y Salud Pública (CIBERESP), the FIS (99/0655, 99/0013-01, 99/9342, 2003/PI20471, PI06154, CP05/00290), and AGAUR Generalitat de Catalunya (SGR 2005/00577) and Comissionat per a Universitat i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (2007-BP-B1-0068 for Marta Tomás).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.05.038.

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