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No evidence for association of CTLA-4 gene polymorphisms with the risk of developing multiple sclerosis: a meta-analysis

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We conducted a meta-analysis concerning the association of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) gene polymorphisms with the risk of developing multiple sclerosis (MS). We identified 18 eligible studies summarizing information about 3375 MS cases and 2930 healthy controls. Two polymorphisms were of interest: the exon 1 +49 A/G polymorphism (in 18 studies) and the promoter −318 C/T polymorphism (in 10 studies). Using random-effects methods we found no evidence for association of the various contrasts of genotypes (or allele frequencies) with the disease. There was significant between-studies heterogeneity that could not be explained by the ethnicity of the populations studied or by other summary measures (gender, disease course, latitude). The major finding of the meta-analysis, apart from the lack of an overall association, consists of detecting a significant time trend of the OR for the contrast of GA versus GG+AA genotypes of the exon 1 +49 A/G polymorphism. In particular, using cumulative meta-analysis we found that the large number of conflicting results on the subject was triggered by the early appearance of a highly significant published result (a study that indicated a significant association of the genotype with the disease).
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Introduction

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) is a single-spanning membrane protein, a member of the immunoglobulin superfamily and it has been shown to transmit an inhibitory signal to T cells [1]. The gene encoding CTLA-4 is localized in human chromosome 2q33, contains four exons and shows significant homology to the gene encoding the functionally related protein CD28 [1,2]. The mature protein contains an extracellular V domain, a transmembrane domain and a cytoplasmic tail of unknown function. Alternate transcriptional splice variants, which encode different isoforms, have been also characterized. The membrane-anchored isoform functions as a homodimer presumably interconnected by a disulphide bond, while the soluble isoform [3] lacking exon 3 that codes for the transmembrane domain functions as a monomer.

Both CTLA-4 and CD28 bind to their native ligands, the B7 molecules (CD80 and CD86) on antigen-presenting cells. This binding completes the activation that is initiated when the antigen-specific cell-surface T-cell receptor (CD3 complex) engages the antigen bound to a major histocompatibility complex class II (MHC-II) molecule on the surface of an antigen-presenting cell [4]. CTLA-4 has a greater affinity for the B7 molecules than does CD28, and it downregulates T-cell function [5].

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Therefore, it was suggested that playing such a crucial role in T-cell-mediated autoimmunity could lead to susceptibility to various autoimmune diseases [6].

Various single nucleotide polymorphisms (SNPs) of the CTLA-4 gene have been implicated in susceptibility to various diseases of autoimmune aetiology such as insulin-dependent diabetes mellitus and Graves's disease [7], systemic lupus erythematosus [8] and multiple sclerosis (MS) [9]. Recent meta-analyses have shown that CTLA-4 is indeed implicated in rheumatoid arthritis [10], type 1 diabetes mellitus [11] and systemic lupus erythematosus [12]. Considering MS, initial evidence supporting the idea that polymorphisms of the CTLA-4 gene are associated with the disease [9,13] failed to be replicated in most of the subsequent studies [14–16], yielding, up to now, contradictory results.

In this work we conducted a meta-analysis of population-based genetic association studies, regarding the association of CTLA-4 gene polymorphisms with the risk for developing MS. Two polymorphisms were the major target of our meta-analysis: the exon 1 +A49G polymorphism, which is responsible for a substitution of threonine by alanine in the 17th position of the precursor protein (a substitution in the signal peptide), and the promoter −C318T polymorphism, which is currently of unknown function. Although the substitution of threonine by alanine is unlikely to disturb the proper function of the signal peptide, Ligers *et al*. found increased cell surface expression in individuals homozygous for the common A allele of the exon 1 polymorphism [17]. They also found increased cell surface expression of CTLA-4 in carriers of the uncommon T allele of the promoter polymorphism and that the mRNA levels were higher in the same individuals.

Materials and methods

Retrieval of published studies

We conducted a systematic computerized literature search in MEDLINE for papers published from 1 January 1966 to 31 December 2005. The electronic search was made using the following MESH headings, keywords and text words: 'Multiple Sclerosis' combined with 'CTLA-4' or 'CTLA4' or 'Cytotoxic T lymphocyte-associated antigen 4'. After an initial screening of titles and abstracts, only articles considering gene polymorphisms remained. The full text of the retrieved publications was read to decide whether information on the topic of interest was included. The reference lists of articles with information on the topic were reviewed to identify citations to other studies of the same topic. Reference lists of review articles were also screened to find further studies for inclusion. We also scrutinized special meeting issues of journals in order to identify abstracts that are usually not included in computer indices, and are shown to influence the results of a meta-analysis [18]. The investigators also contacted experts who are known to be active in the field to identify any unpublished work not provided by the MEDLINE search and consequently to maximize the pool of eligible studies under consideration.

Inclusion and exclusion criteria

Inclusion criteria for eligible studies were applied and only population-based case-control designs were considered. Articles could be included in the meta-analysis if: (i) they examined the hypothesis of CTLA-4 as a risk factor for MS using a population-based case-control design, and (ii) there were sufficient published data on the genotypes or allele frequencies for determining an estimator of relative risk (odds ratio) or a confidence interval. Manuscripts in languages other than English were also considered for review, in order to avoid the local literature bias [19]. To avoid selection bias, no study was rejected because of poor quality parameters. Furthermore, no quality scoring was attempted, as it involves subjective assignment of points, and modern approaches advocate against it [20,21]. Instead, we considered as a better choice to try investigating the sources of heterogeneity directly, incorporating various study-level covariates in a metaregression, as well as performing subgroup and sensitivity analysis (see Statistical analysis section).

Data extraction

Two reviewers (PB, AK) independently examined the retrieved articles in order to extract the information needed using a data collection form. The following data were abstracted from each study: (i) publication data, first author's last name, year of publication, ethnicity and country of the population studied; (ii) total number of subjects as well as numbers of those tested; (iii) the particular polymorphism that was under investigation; (iv) the counts of persons with different genotypes in cases and control groups; and (v) the average patient characteristics (age, sex, MS disease course, details about matching of cases and controls, MS duration in years if available) within each study. Following the extraction of data, the reviewers checked for any discordance and a consensus was reached. In all the above-mentioned procedures, the guidelines for

the meta-analyses of observational studies (MOOSE) were followed [21].

Statistical analysis

The odds ratio (OR) was used to compare distributions of alleles and genotype contrasts between cases and controls. We computed the genetic contrast of the mutant allele versus the wild-type one, and where sufficient data were available, contrasts of each genotype against the others. In secondary analyses, we calculated specific ORs according to the racial descent of subjects (separate analyses for Caucasians and Asians). We assessed the presence of between-studies heterogeneity by using the chi-square-based Cochran's Q statistic [22]. The inconsistency index I^2 (range 0–100%) was also calculated, where higher values of the index indicate the existence of heterogeneity [23].

The combined ORs were estimated using random-effects models [24]. In the presence of heterogeneity, random effects are more appropriate as it is prudent to take into account an estimate of the between-study variance (τ^2). Random-effects models assume that the true effects vary randomly between studies. Thus, in such a model, we consider the logarithm of ORs as if they were randomly sampled from a normally distributed population with mean θ and variance $\sigma_i^2 + \tau^2$, ie, the individual estimates of the log OR of each study are distributed normally with the mean centred on the true effect θ:

$$\log \mathrm{ORi} \sim \mathrm{N}(\theta,\ \sigma_i^2 + \tau^2)$$

Here, θ is the true effect (the true value of log OR), σ_i^2 the estimated variance of $\log \mathrm{OR}_i$ and τ^2 the variance between studies. If heterogeneity is absent, τ^2 equals zero and thus the random-effects and the fixed-effects models provide essentially the same results. Using the random-effects models, we could also extend the analysis by incorporating various study-level covariates (x_i) as linear predictors, in order to estimate the extent to which these covariates explain the observed heterogeneity. This approach yields thus a random-effects metaregression [25], where the component of between-studies variance is estimated by the Restricted Maximum Likelihood (REML) method.

Publication bias or other small study-related bias was evaluated initially using the rank correlation method of Begg and Mazumdar [26]. For the same purpose we also conducted a random-effects weighted regression [25] of $\log \mathrm{OR}_i$ against its estimated standard error (SE_i). This method constitutes the random-effects analogue of the Egger's regression method [27], and it was performed because the fixed-effects regression method of Egger might provide false positive evidence for publication bias in the presence of heterogeneity. We also used the non-parametric 'trim and fill' method of Duval and Tweedie [28], which adjusts the combined (random-effects) ORs, if necessary, according to the number of the estimated missing studies. With the use of the above-mentioned techniques we could be fairly sure that publication bias or any other bias arising from preferentially publishing of small studies could be detected.

In an attempt to identify potential influential studies, we calculated the random-effects estimates by removing an individual study each time and then checked whether any of these estimates can bias the results if it lies outside the confidence interval of the overall estimate. Furthermore, we undertook separate analyses excluding studies in which the genotype distribution in controls deviated from the HWE. In all the included studies, deviations from the HWE were calculated by the chi-square method. A cumulative meta-analysis was also conducted in order to identify the influence of the first published study on the subsequent publications [29].

For all analyses performed here, the statistical package Stata 8 (StataCorp) was used. In all analyses statistically significant results were declared those with a P-value <0.05.

Results

Identified studies

After the initial literature search and the subsequent screening, we came up with 18 studies concerning the association of the exon 1 +A49G polymorphism with MS and 11 studies concerning the association of promoter −C318T polymorphism with the same disease. The detailed characteristics of each study (country conducted, racial descent of subjects involved, characteristics of cases and controls, sample size, polymorphism studied and so forth) are summarized in Table 1. Some research papers contained information about distinct populations that were studied and thus they are listed twice [14,30,31]. The second group studied by Masterman *et al.* consisted of the same patients' group initially studied in the work of Ligers *et al.* and thus it was excluded from the analysis [13,30]. A subset of the patients studied by Lorentzen *et al.* was also initially studied in the work of Harbo *et al.* and consequently they were subtracted from the total counts [9,32]. However, in the later work of the Fukazawa group, some of the patients (61 in total) had also constituted a part of the sample used in the previous study of the same

Table 1 The characteristics of the eligible studies that were included in the meta-analysis

Author	Year	Country	Racial descent	Controls' characteristics	Cases' characteristics	Number of controls	Number of cases	Polymorphism studied
Harbo [9]	1999	Norway	Caucasian	Randomly selected blood donors	MS patients with clinically or laboratory-supported definite MS according to the Poser's criteria.	270	296	(+49) A/G/(−318) C/T
Ligers [13]	1999	Sweden	Caucasian	Randomly selected blood donors and cadaveric donors	Unrelated patients fulfilling Poser's criteria for clinically definite MS. All showed signs of intrathecal immunoglobulin production in analysis of the cerebrospinal fluid, as presence of oligoclonal bands and/or an increased IgG index.	237[a]	378[a]	(+49) A/G/(−318) C/T
Fukazawa [45]	1999	Japan	Asian	Healthy and unrelated members of the hospital staff undergoing yearly medical examination	Unrelated Japanese residents of Hokkaido with relapsing–remitting (RR) or secondary progressive (SP) type of MS, who were diagnosed with clinically definite MS according to the criteria of Poser	93	74	(+49) A/G
Rasmussen2 [14]	2001	China	Asian	Healthy control subjects	MS patients who fulfilled Poser's criteria of definite MS	86	42	(+49) A/G/(−318) C/T
Rasmussen1 [14]	2001	Denmark	Caucasian	Healthy control subjects	MS patients who fulfilled the Schumacher criteria of definite MS	125	84	(+49) A/G/(−318) C/T
Andreevskii [46]	2002	Russia	Caucasian	Healthy subjects	MS patients fulfilling Poser's criteria	209	168	(+49) A/G
Masterman1 [30]	2002	Sweden	Caucasian	Randomly selected blood donors and randomly selected unrelated members of a set of Swedish mono- and dizygotic twin pairs enrolled in the Swedish Twin Registry	Patients with definite MS, according to Poser's criteria	290	374	(+49) A/G/(−318) C/T
Masterman2 [30]	2002	Sweden	Caucasian	Randomly selected blood donors and cadaveric donors	Unrelated patients fulfilling Poser's criteria for clinically definite MS. All showed signs of intrathecal immunoglobulin production in analysis of the cerebrospinal fluid, as presence of oligoclonal bands and/or an increased IgG index.	237[a]	340[a]	(+49) A/G/(−318) C/T
Mauer [42]	2002	Germany	Caucasian	Age- and sex-matched healthy individuals	Adult MS patients tracked in an outpatient clinic under highly standardized follow-up conditions for several years.	152	330	(+49) A/G

Table 1 (*Continued*)

Author	Year	Country	Racial descent	Controls' characteristics	Cases' characteristics	Number of controls	Number of cases	Polymorphism studied
Kantarci1 [31]	2003	USA (Boston)	Caucasian	Unmatched healthy control subjects	Patients from the Partners Multiple Sclerosis Center of Brigham and Women's Hospital and Massachusetts General Hospital. All patients had definite MS by clinical and MRI criteria.	37	94	(+49) A/G/(− 318) C/T
Kantarci2 [31]	2003	USA (Minnesota)	Caucasian	Patients matched for ethnicity, age and gender and not having MS or another inflammatory demyelinating disease. Controls were selected from 4000 Mayo Clinic patients.	Sporadic cases with MS who constitute 73% of a prevalence cohort in Olmsted County with a broad range of disability	235	120	(+49) A/G/(−318) C/T
van Veen [15]	2003	Netherlands	Caucasian	Unrelated ethnically matched healthy volunteers	Unrelated MS patients who fulfilled the criteria for clinically definite MS as proposed by Poser *et al*.	181	514	(+49) A/G/(−318) C/T
Bocko [47]	2003	Poland	Caucasian	Age- and sex-matched healthy subjects	Unrelated, randomly selected MS patients with clinically and laboratory-supported definite MS according to Poser's criteria	101	102	(+49) A/G
Luomala [48]	2003	Finland	Caucasian	Healthy subjects of the same sex and age distribution as the patients. The control subjects include medical staff and random residents.	Randomly selected clinically definite MS patients according to the criteria of Poser	109	101	(+49) A/G
Teutsch [34]	2004	Australia	Caucasian	Randomly selected subjects from hospital staff and unrelated partners of MS subjects	Unrelated MS patients with clinically definite or laboratory-supported definite MS according to Poser's criteria	152	102	(+49) A/G
Bilinska [41]	2004	Poland	Caucasian	Sex- and age-matched healthy subjects	Unrelated MS patients with clinically definite MS according to Poser's criteria	154	152	(+49) A/G
Malferrari [49]	2005	Italy	Caucasian	Ethnically matched controls	Patients diagnosed with RRMS	104	95	(+49) A/G/(−318) C/T
Fukazawa [16]	2005	Japan	Asian	Healthy Japanese volunteers	Unrelated Japanese patients with conventional/classical MS (RR or SP course according to Poser's diagnostic criteria)	156	133	(+49) A/G/(−318) C/T
Lorentzen [32]	2005	Norway	Caucasian	Healthy blood donors recruited through the Norwegian Bone Marrow Registry	MS patients diagnosed as clinically definite or laboratory-supported definite MS according to the criteria of Poser	239[a]	216[a]	(+49) A/G

[a]Some or all of the patients used were also used by another study (see Results for more information).

group [16]; thus, we decided to include both studies in order to increase the sample size, and investigate further the influence of this choice. Some of the included studies, along with the samples they used under the population-based design, reported results on independent samples following the family-based design, using variations of the transmission disequilibrium test (TDT) [13,31]. These data were not used in the meta-analysis as well as data from other studies following solely the family-based design (data not shown). Finally, one study that was identified initially was excluded from the analysis as it did not contain all the appropriate data in order to derive an estimate for the OR and a confidence interval [33].

Exon 1 +A49G polymorphism

The 18 retrieved studies investigating the association of the exon 1 +A49G polymorphism with MS contain 3375 MS cases and 2930 control subjects (Table 2). One of the studies (the first group studied by Kantarci *et al.* [31]) contained information only about allele frequencies and thus it could not be used in the analyses concerning the various genotypes contrasts. In all the remaining studies, the control subjects were in HWE as revealed by the chi-square test. In all the contrasts studied, no significant correlation was found between the A49G polymorphism and the risk for MS (Table 3). In two out of the four contrasts (GA versus GG+AA and GG versus AA+GA) there was a statistically significant heterogeneity as indicated by the *P*-value of the corresponding test ($P < 0.02$ in both cases) and the large value of the I^2 index (48.9% and 56.7% respectively). There were no significant differences between subjects of Caucasian and Asian racial descent in any of the contrasts (Table 3, Figure 1). However, the three studies performed on Asian subjects yielded homogeneous results in all the contrasts examined, whereas the 14 studies on Caucasian subjects were responsible for the large variance between studies obtained in the two aforementioned contrasts.

There was no evidence for publication bias in the contrasts examined. The Begg's test and the random-effects regression method yielded in all cases highly insignificant results ($P > 0.3$ in all cases; data not shown). The more sensitive 'trim and fill' method imputed a missing study in the contrast of GA versus GG+AA genotypes as well as in the contrast of the G allele versus A allele. However, in both cases the adjusted OR was still not significant (1.040, 95% CI: 0.886, 1.222 and 0.994, 95% CI: 0.917, 1.078 respectively).

Even though we failed to find a significant association of the particular polymorphism with MS, we conducted a random-effects metaregression in an effort to explain the observed between-studies heterogeneity. Potential covariates for inclusion in the metaregression were considered: the proportion of primary progressive (PP) MS patients among cases, the difference in the proportion of females

Table 2 Genotype frequencies of the studies included in the meta-analysis for the association of the exon 1 +A49G polymorphism with MS

				AA genotype		AG genotype		GG genotype		G allele frequency (%)	
Author	Year	Country	Racial descent	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
Harbo	1999	Norway	Caucasian	95	89	119	169	56	38	42.8	41.4
Ligers	1999	Sweden	Caucasian	83	117	123	188	31	73	38.8	44.0
Fukazawa	1999	Japan	Asian	17	9	38	36	38	29	61.3	63.5
Rasmussen2	2001	China	Asian	7	4	34	23	45	15	55.2	56.5
Rasmussen1	2001	Denmark	Caucasian	35	28	68	39	22	17	27.9	36.9
Andreevskii	2002	Russia	Caucasian	68	51	104	85	37	32	42.6	44.3
Masterman1	2002	Sweden	Caucasian	82	120	139	188	69	66	47.8	42.8
Mauer	2002	Germany	Caucasian	66	132	69	157	17	41	33.9	36.2
Kantarci1	2003	USA	Caucasian	ND	ND	ND	ND	ND	ND	44.6	33.2
Kantarci2	2003	USA	Caucasian	35	15	111	49	89	56	38.5	37.1
van Veen	2003	Netherlands	Caucasian	72	215	85	229	24	70	36.7	35.9
Bocko	2003	Poland	Caucasian	35	31	53	57	13	14	39.1	41.7
Luomala	2003	Finland	Caucasian	36	26	46	50	27	25	46.4	49.5
Teutsch	2004	Australia	Caucasian	64	30	61	54	27	18	44.0	38.0
Bilinska	2004	Poland	Caucasian	50	47	84	80	20	25	40.3	42.8
Malferrari	2005	Italy	Caucasian	37	40	54	46	13	9	38.4	33.7
Fukazawa	2005	Japan	Asian	29	23	66	69	61	41	60.3	56.8
Lorentzen	2005	Norway	Caucasian	73	75	130	82	36	59	42.3	46.3
Total				884	1052	1384	1601	625	628	42.8	41.7

ND, non-determined.

Table 3 The results of the meta-analysis for the association of the exon 1 +A49G polymorphism with MS

Contrast	Race	Number of studies	Odds ratio (random effects)	95% Confidence interval		Between studies variance (t^2)	Cochran's Q	*P*-value for heterogeneity	Inconsistency Index (I^2)
G allele versus A allele									
	All	18	1.003	0.927	1.085	0.003	19.20	0.630	11.5%
	Caucasians	15	1.006	0.919	1.102	0.007	18.22	0.197	23.1%
	Asians	3	0.977	0.784	1.218	0.000	0.93	0.317	0.0%
GA genotype versus other (GG+AA) genotypes									
	All	17	1.060	0.902	1.246	0.063	36.98	0.002	56.7%
	Caucasians	14	1.046	0.875	1.250	0.066	32.47	0.002	60.0%
	Asians	3	1.145	0.735	1.784	0.077	4.01	0.134	50.2%
GG genotype versus other (AA+GA) genotypes									
	All	17	0.973	0.802	1.182	0.078	31.30	0.012	48.9%
	Caucasians	14	0.992	0.790	1.244	0.099	29.06	0.006	55.3%
	Asians	3	0.856	0.611	1.200	0.000	1.61	0.446	0.0%
Other (GG+GA) genotypes versus AA genotype									
	All	17	1.033	0.921	1.159	0.003	16.90	0.392	5.3%
	Caucasians	14	1.035	0.912	1.174	0.008	15.90	0.307	13.3%
	Asians	3	1.025	0.699	1.502	0.000	1.90	0.386	0.0%

We list the random effects OR with its 95% CI (confidence interval), the estimator for the between-studies variance, the *Q* statistics for heterogeneity and the associated *P*-value and the inconsistency index.

between cases and controls, and the latitude of the city where the study was conducted. None of these variables was found to significantly contribute to the regression model, and thus could not explain the between-studies heterogeneity.

The influential analysis also revealed that there was not a single study influencing the results significantly. Thus, after removing each study and calculating the overall estimate and the 95% CI interval for the remaining studies, there was no significant change; in all cases (in both four contrasts examined) the estimate for the OR was still insignificant and the confidence interval contained the value of 1. In the cumulative meta-analysis, there was strong evidence suggesting that the first published study reporting a significant association triggered the subsequent publication of other studies that failed to replicate the initial results. This is true in particular for the contrast of GA versus GG+AA genotypes as well as for the contrast of GG versus GA+AA genotypes (Figure 2). The study of Harbo *et al.* reported an OR equal to 1.689 (95% CI: 1.211, 2.355) for the contrast of GA versus GG+AA [9], however it is obvious that just after this first publication all the subsequent works failed to reproduce these findings, with the ORs being insignificant (the confidence intervals include the value of 1). Similarly, even though reverse findings hold for the contrast GG versus GA+AA, in the work of Harbo *et al.* the OR has a value of 0.563 (95% CI: 0.359, 0.883) [9], suggesting a 'protective effect' that diminishes soon after the second publication [13].

Promoter −C318T polymorphism

For the C/T polymorphism at position −318 of the promoter sequence there were 10 eligible studies, containing information about 2092 MS patients and 1721 healthy controls (Table 4). As we stated earlier, the second group studied by Masterman *et al.* consisted of the same group of patients initially studied in the work of Ligers *et al.* [13,30] However, as in the work of Masterman *et al.* detailed genotype counts were provided, we chose this study for inclusion even though it contained fewer participants. Furthermore, the first group studied by Kantarci *et al.* contained information only about allele frequencies and thus it could not be used in the analyses concerning the various genotypes contrasts [31]. In all the studies that reported genotype frequencies, the control subjects were in HWE as revealed by the chi-square test. In all the contrasts studied, no significant correlation was found between the C318T polymorphism and the risk for MS (Table 5, Figure 3). In all but one of the contrasts (TT versus CT+CC) there was statistically significant heterogeneity, as indicated by the *P*-value of the corresponding test ($P < 0.02$ in all cases) and the large value of the I^2 index (larger than 50%). There were no significant differences between subjects of Caucasian and Asian racial descent in any of the contrasts, apart from the contrast of allele T versus allele C, where a marginally significant *P*-value of 0.055 was obtained. The two studies performed on subjects of Asian origin reported a reverse association (OR > 1) compared to studies on Caucasians (OR < 1) [14,16]. However,

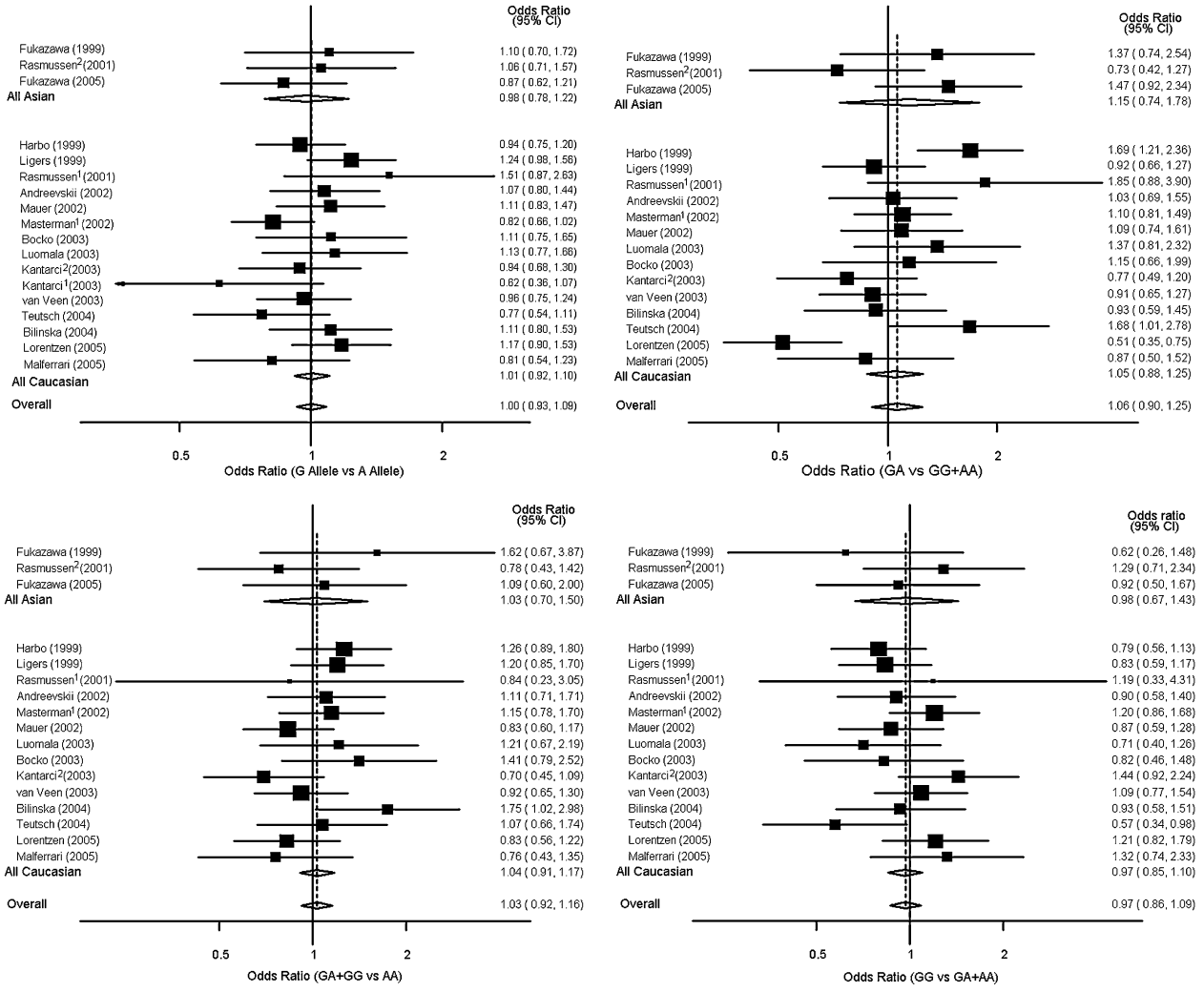

Figure 1 Forrest plots for the meta-analyses of various contrasts of the exon 1 A/G (+49) polymorphism. Studies on Asian and Caucasian subjects are analysed separately and in combination, and the random-effects results are displayed. The size of the symbol corresponding to each study's OR is shown with size proportional to the inverse of its variance.

these differences are insignificant and by performing the subanalyses the degree of heterogeneity is not affected at all.

There was no strong evidence for publication bias in the contrasts examined. In the contrast of the T allele versus C allele, the random-effects regression method yielded a marginally significant result ($P = 0.053$) that was not replicated in either the Begg's test or the 'trim and fill' test. In the TT versus TC+CC contrast, the trimming method also concluded that there were four studies missing from the meta-analysis with an adjusted estimate for the OR equal to 0.476 (95% CI: 0.240, 0.945) suggesting a 'protective' effect of the TT genotype. Even though this adjusted OR should not been taken literally, this result may indicate a preferential publication of positive findings (those suggesting that the TT genotype confers susceptibility to MS), or maybe this is just an artefact of the particular method attributable to the small number of studies included in the meta-analysis. In the remaining contrasts both methods yielded highly insignificant results ($P > 0.3$ in all cases; data not shown).

Once again, even though we failed to find a significant association with MS, we conducted a random-effects metaregression in an effort to explain the observed between-studies heterogeneity. None of the potential covariates considered, namely the proportion of PPMS patients among cases, the difference in the proportion of females between cases and controls, and the latitude of the city where the study was conducted, was found to significantly contribute to the regression model and thus could not explain the between-studies heterogeneity.

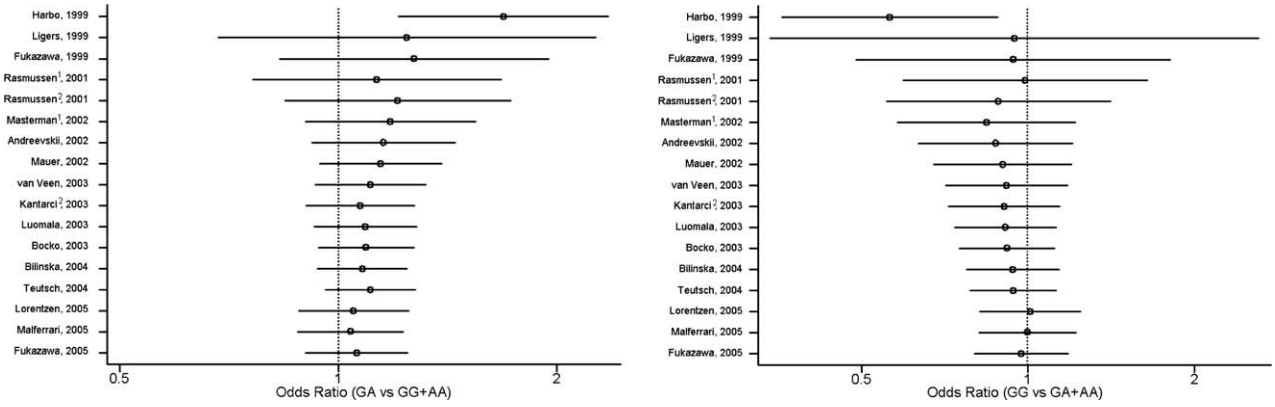

Figure 2 Cumulative meta-analysis of two of the contrasts used for the exon 1 (+49) A/G polymorphism. The studies are sorted chronologically and the random-effects results are displayed.

The influential analysis for this polymorphism also revealed that there was no single study influencing the results significantly. Finally, performing a cumulative meta-analysis we could not find any evidence suggesting a trend in the estimates of ORs during the time course from the first published study.

Discussion

To our knowledge, this is the first complete meta-analysis investigating the relation of CTLA-4 polymorphisms with MS. In one of the studies included in the meta-analysis an attempt has been made to summarize in a systematic way all the knowledge available at that time [34]. However, only nine studies were included there, concerning the association of the exon 1 polymorphisms, and no attempt has been made to summarize the results on the promoter polymorphism. The two meta-analyses performed here comprise a comparably large total number of subjects included (cases and controls). As a matter of fact, as shown in a recent evaluation of published meta-analyses [35], 33% of the evaluated meta-analyses of genetic association studies included less than 2000 subjects and 11% less than 1000, whereas only 33% of the studied meta-analyses included more than 5000 total subjects.

Our meta-analysis could not find any evidence suggesting an association of the two polymorphisms of CTLA-4 with MS. The results of the +49 A/G exon 1 polymorphism, which is the most commonly studied, indicate that there is no significant risk for developing MS attributable to this particular polymorphism. The large controversy in the literature and the large number of published articles concerning this polymorphism should be attributed to the effect of the first published study on this

Table 4 Genotype frequencies of the studies included in the meta-analysis for the association of the promoter −C318T polymorphism with MS

				CC genotype		CT genotype		TT genotype		T allele frequency (%)	
Author	Year	Country	Racial descent	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
Harbo	1999	Norway	Caucasian	226	249	39	43	6	4	9.4	8.6
Rasmussen2	2001	Denmark	Caucasian	72	29	12	12	2	1	8.2	18.6
Rasmussen1	2001	China	Asian	99	70	26	14	0	0	10.4	8.3
Masterman2	2002	Sweden	Caucasian	183	291	54	49	0	0	11.4	7.2
Masterman1	2002	Sweden	Caucasian	249	296	41	73	0	5	7.1	11.1
Kantarci1	2003	USA	Caucasian	ND	ND	ND	ND	ND	ND	1.4	8.5
Kantarci2	2003	USA	Caucasian	182	106	41	16	4	1	10.8	7.6
van Veen	2003	Netherlands	Caucasian	148	432	29	78	4	4	10.2	8.4
Malferrari	2005	Italy	Caucasian	88	78	14	16	2	1	8.7	9.5
Fukazawa	2005	Japan	Asian	27	100	16	32	0	1	11.5	12.8
Total				1274	1651	272	333	18	17	9.6	9.2

ND, non-determined.

Table 5 The results of the meta-analysis for the association of the promoter −C318T polymorphism with MS

Contrast	Race	Number of studies	Odds ratio (random effects)	95% Confidence interval		Between studies variance (t^2)	Cochran's Q	*P*-value for heterogeneity	Inconsistency index (I^2) (%)
T allele versus C allele									
	All	10	1.025	0.770	1.366	0.125	24.81	0.003	63.7
	Caucasians	8	0.926	0.684	1.254	0.107	18.09	0.012	61.3
	Asians	2	1.602	0.723	3.547	0.225	3.05	0.081	67.2
CT genotype versus other (CC+TT) genotypes									
	All	9	0.933	0.697	1.249	0.106	18.390	0.018	56.5
	Caucasians	7	0.915	0.691	1.211	0.068	11.750	0.068	48.9
	Asians	2	1.121	0.251	5.014	0.992	6.600	0.010	84.9
TT genotype versus other (CC+CT) genotypes									
	All	9	0.595	0.285	1.243	0.000	3.590	0.892	0.0
	Caucasians	7	0.545	0.249	1.191	0.000	3.100	0.796	0.0
	Asians	2	1.172	0.135	10.190	0.000	0.060	0.811	0.0
Other (TT+CT) genotypes versus CC genotype									
	All	9	0.916	0.682	1.232	0.116	17.890	0.011	59.8
	Caucasians	7	0.893	0.664	1.201	0.087	13.770	0.032	56.4
	Asians	2	1.111	0.276	4.467	0.841	6.000	0.014	83.3

We list the random effects OR with its 95% CI (confidence interval), the estimator for the between-studies variance, the Q statistics for heterogeneity and the associated *P*-value and the inconsistency index.

topic, ie, the work of Harbo *et al*. [9] The results of the cumulative meta-analysis that we conducted indicate that this is just an instance of the now well documented 'Proteus phenomenon' [29]. The Proteus phenomenon is based in recognizing the fact that controversial data are attractive to both investigators and editors, and thus the most extreme results would appear earlier as data are accumulated. This phenomenon is more frequent in molecular epidemiology, when there are plenty of possible 'risk factors' (genes and their polymorphisms) that can be studied rapidly and associated with a particular disease [36]. This phenomenon constitutes much of the so-called 'molecular bias' problem in genetic epidemiology [29,36,37], and one of the few ways, not to eliminate it but at least to control for it, is a carefully designed meta-analysis.

The results obtained from the second studied polymorphism, the −318 C/T promoter polymorphism, also indicate a non-significant association with MS. In this case, no evidence of the observed above-mentioned phenomenon was detected, but in addition to the between-studies heterogeneity, a slight indication of publication bias was present. Publication bias occurs when the most significant (favourable) results are published where the ones showing no association (null findings) are either not published at all or are extremely delayed. The fact that the researchers reporting results of the −318 C/T polymorphism were, in the majority of the cases, the same as the ones studying the +49 A/G polymorphism, and that in many published articles these results appear together, may lead one to conclude (if the case of publication bias holds) that they reported only the favourable results.

None of the factors selected to be entered as covariates in the metaregression (the proportion of PPMS patients among cases, the difference in the proportion of females between cases and controls, and the latitude of the city where the study was conducted) showed any significant correlation with the outcome. This further strengthens our belief that there is truly no significant association of these polymorphisms with MS. The lack of significant differences among subjects of Asian and Caucasian origin also points to this conclusion. We should mention here that in the three recently published meta-analyses concerning the association of CTLA-4 polymorphisms with other autoimmune diseases, all reported a positive significant association of the polymorphisms with the disease [10–12], and in two of the three cases (SLE [12], RA [10]), the magnitude of the association was different for Asians compared to Caucasians. However, there were only three eligible studies on Asian populations in this meta-analysis, and this may have inflated our results. That is, if there is a significant association of CTLA-4 with MS only among Asian subjects, our meta-analysis did not have the power to detect it and further studies are needed, particularly on Asian populations. This reminds us of another use of the meta-analytic practice: that of providing clues and directions for future research in the field.

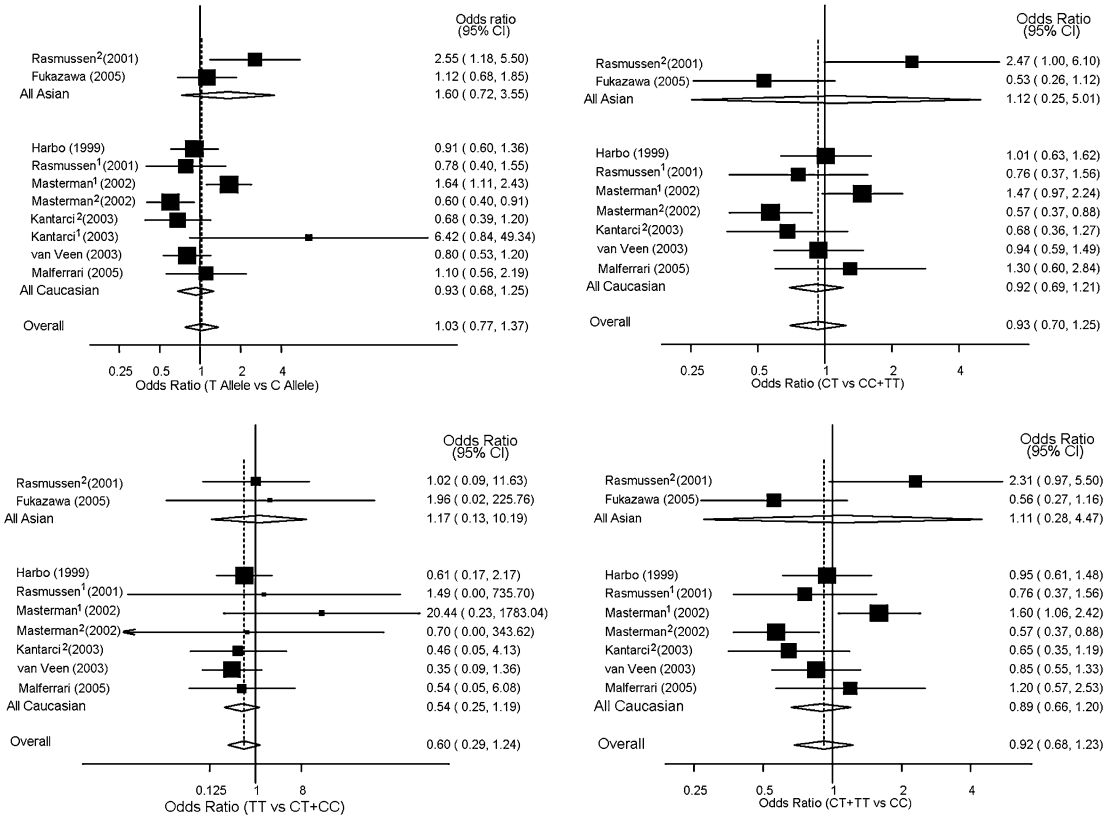

Figure 3 Forrest plots for the meta-analyses of various contrasts of the promoter (−318) C/T polymorphism. Studies on Asian and Caucasian subjects are analysed separately and in combination, and the random-effects results are displayed. The size of the symbol corresponding to each study's OR is shown with size proportional to the inverse of its variance.

Finally, based on the current meta-analysis, some limitations of the eligible studies should be pointed out; limitations that are not uncommon in genetic epidemiology studies and that are propagated in a meta-analysis. Of the 19 eligible studies, in only five were the control subjects matched for age and gender to the MS cases. This could lead to severe bias, as it is known that MS incidence and prevalence in females seems to be approximately double compared to that in men [38–40]. Thus, when randomly selected MS patients are compared to randomly selected controls, we expect the cases to be ∼67% females and the controls ∼50%. Detailed stratification according to MS course was also not available in the eligible studies. If there is a true association, for instance, of the PP course of the disease with the polymorphisms [41,42], then not reporting detailed genotypes stratified by disease course would on the one hand increase the between-studies heterogeneity, and on the other hand could seriously bias the overall results. Both these limitations, as well as the place the study was conducted, which is also a potential risk factor for MS [43], were taken into account by the metaregression without any significant result; even in this case, possible ecological confounding could not be excluded [44].

Conclusions

We have presented the first complete meta-analysis concerning the association of two SNPs of the CTLA-4 gene with MS. The results indicate no significant association, but there is strong evidence that early extreme estimates may have biased the direction of the research. The cumulative meta-analysis has shown the results of the first published study pointing to a significant association contradict the results of the full meta-analysis. Even though the overall results are insignificant, this study yields other important findings. The large variability between studies indicates the need for more stringent criteria for matching between cases and controls in genetic association studies. A more thorough investigation of the contribution of the particular polymorphisms to PPMS patients is also needed. Furthermore, more studies are needed especially on Asian subjects, as the small number of eligible studies indicates that the meta-analysis

might not have the appropriate power to detect a small but significant association, similar to what was found for the same polymorphisms and their association with other autoimmune diseases.

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