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APOE epsilon status in Hungarian patients with primary progressive multiple sclerosis

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Summary

PRINCIPLES: Apolipoprotein E (ApoE), an important glycoprotein in the transport, uptake and redistribution of cholesterol, is necessary in nerve tissue repair. The APOE gene (APOE) is involved in neurodegenerative diseases, the best-known association being that between the APOE ε4 allele and Alzheimer's disease. Multiple sclerosis (MS) is a chronic inflammatory neurological disease. The aim of this study was to assess (multicentre assessment) the possible influence of the APOE gene on the susceptibility of primary progressive MS (PPMS) in Hungary.

METHODS: Polymerase chain reaction and restriction fragment length polymorphism were carried out on DNA isolated from 135 volunteers.

RESULTS: The number of PPMS patients without the ε2 allele was found to be remarkably high, whilst the ε2 allele was overrepresented in the RRMS group. A markedly high frequency of the ε4 allele was found in the PPMS group and a very low frequency in the HC group. With regards to the clinical parameters, significant differences were observed between the RRMS and PPMS groups. Differences were also detected regarding the EDSS and MSSS scores when the patients were grouped by the presence or absence of the ε2 allele. All of the observed differences in the clinical parameters disappeared when the patients were further stratified by the type of MS.

CONCLUSIONS: Our findings suggest that the presence of the ε2 and ε4 alleles may play a role in the development of the disease. However, if any type of the disease has already developed the alleles show no association with the clinical parameters.

Key words: *Apolipoprotein E gene (APOE); Genetics; Multiple sclerosis (MS); Primary progressive (PP); Single nucleotide polymorphism (SNP)*

Introduction

Apolipoprotein E (ApoE) plays important roles in the transport, uptake and redistribution of cholesterol, which is necessary in the repair of nerve tissue. While it primarily functions as a lipid transporter, it is also linked to atherosclerosis, cognitive function, immunoregulation, neurite outgrowth, brain trauma and infectious diseases [1]. The APOE gene (APOE) is additionally involved in neurodegenerative diseases; the best-known association is that between the APOE ε4 allele and Alzheimer's disease [3]. Multiple sclerosis (MS) is a chronic inflammatory neurological disease caused by numerous factors. The heterogeneous nature of the disease leads to different clinical manifestations. In the majority of MS patients the disease begins with a relapsing course (relapsing-remitting form; RRMS), characterised by relapses and remissions, followed by a progressive phase (secondary progressive MS; SPMS). The pathological hallmark is the primary inflammation and the secondary neurodegeneration. In a smaller subset of patients, the relapsing phase is absent and the disease progresses from the very beginning (primary progressive form; PPMS). In this subtype, the neurodegeneration is the driving force [4].

Two single base polymorphisms within exon 4 of the APOE, at codons 112 and 158, result in three common alleles (ε2, ε3 and ε4); the corresponding protein variants ApoE2, E3 and E4 are distinguishable by having different

combinations of the amino acids arginine and cysteine at these positions [1, 2]. ApoE is produced in various organs and tissues, predominantly in the liver and by the astrocytes in the brain. ApoE3 seems to be the normal form, while ApoE2 and ApoE4 can each be dysfunctional. The protein is a glycoprotein that contains 299 amino acids, with a molecular weight of 34 kDa.

The literature reports on the role of APOE in MS are controversial (table 1). In addition, no Hungarian data is available regarding the APOE status of MS patients. Consequently, our aim was to evaluate the role of the APOE gene in Hungarian PPMS patients. The hypothesis of present study was that the ε4 allele is associated with progression, while the ε2 allele is rather protective.

Table 1: Summary of studies investigating the association between APOE and MS.

First author	Country	Number of MS subtype patients	Findings
Cocco	Sardinia	773 RR; 98 PP	Gender-specific association between ε4 and PP
Chapman	Israel	47 RR	ε4 allele increases rate of disease progression
Chapman	Israel	172 RR; 31 SP; 2 PP	ε4 allele associated with faster progression of disability
Fazekas	Austria	76 RR; 5 SP; 2 PP	More extensive tissue destruction or less efficient repair in carriers of the ε4 allele
Fazekas	Austria	253 RR; 97 SP; 24 PP	An association of ε4 allele with a more severe course
Høgh	Denmark	104 RR; 29 PP; 105 SP	ε4/ε4 genotype risk for developing MS and faster disease progression
Schmidt	USA	379 RR; 30 PP; 182 SP; 19 PR	Association between ε4 and a more severe form and between ε2 and mild disease
Pinholt	Denmark	249 RR; 94 SP; 42 PP	ε4 allele associated with faster progression
Evangelou	UK	52 RR; 32 SP; 11 PP	ε4 allele associated with more rapid progression
Al-Shammri	Kuwait	33 RR; 5 RP; 1benign MS	No association (only trend toward) between disease severity and ε4 allele
Ferri	Italy	161 RR	No association with occurrence of MS
Niino	Japan	95 RR; 40 SP	No association with disease progression
Weatherby	UK	162 RR; 188 SP; 20 PP	No association with occurrence or clinical course
Zwemmer	The Netherlands	159 RR; 159 SP; 90 PP	Disease severity not associated to carriership of ε4 or ε2
Masterman	Sweden	124 benign; 140 severe	No significant differences between the benign and severe MS
Santos	Portugal	34 PP; 184 other form	ε4 not associated to disease progression only in a subset of patients with <10 yrs disease duration
Portaccio	Italy	75 RR; 40 BMS; 49 SP; 9 PP	No association with disease course and severity
Bonetti	Finland	459 MS trio families	No association
Savettieri	Italy	319 RR; 90 SP; 19 PP	Association between ε2 allele and longer disease duration
Ballerini	Italy	32 CP; 34 stable MS	ε2 allele has protective role against the onset of the progression form
Kantarci	Turkey	221 MS	Gender-specific association between APOE ε2 and lesser disease severity

Abbreviations: APOE= apolipoprotein E gene; BMS = benign multiple sclerosis; CP = chronic progressive; MS = multiple sclerosis; PP = primary progressive; RR = relapsing remitting; SP = secondary progressive;

Patients and methods

Patients

All patients gave their informed consent for participation in accordance with the Declaration of Helsinki (1964) and the study was approved by the local ethics committee (protocol no. 16/2006). Over a period of one year (between 2006 June and 2007 June), participation in the study was offered to PPMS patients who were consecutively referred to one of the 5 involved Hungarian MS Centres at the regular check-up every 5 months. The APOE genotype was determined from the blood of 45 PPMS patients, 45 age- and sex-matched RRMS patients and 45 healthy controls (HC). The two latter groups served as control groups for the PPMS group. A total of 13 of the 45 PPMS patients were followed up at the MS Outpatient Unit of the Department of Neurology at the University of Szeged, 12 patients at the Department of Neurology at the University of Pécs, 7 patients at the Department of Neurology at Ferenc Jahn Hospital in Budapest, 7 patients at the Department of Neurology at the County Hospital in Kecskemét, and 6 patients were followed up at the Department of Neurology at the University of Debrecen. The RRMS patients and HC subjects were selected according to age and sex from Szeged. Clinical data (the Expanded Disability Status Scale (EDSS) score [5], date of birth, year of diagnosis and onset of the disease) were collected by using the up-to-date MS register. The Multiple Sclerosis Severity Score (MSSS) [6] and the progression index (PI; the ratio between the EDSS score and the disease duration in years) were also determined. The EDSS scores the disability by evaluating the degree of neurological impairment and it has steps from 0 (normal) to 10 (death due to MS). The MSSS gives a hint as to the severity of the disease, expressed numerically via the extent of disability and the disease duration. The RRMS patients met the McDonald diagnostic criteria [7]. The PPMS patients had undergone at least one year of disease progression and had had a positive brain or spinal cord MRI or

positive cerebrospinal fluid [8]. The patients were relapse-free and were not taking steroids for at least 1 month before the assessment. In Hungary, a central database of healthy controls for genetic research is not available. We therefore offered study participation to age and sex-matched attendants and relatives of MS patients and to healthy staff at the clinic. If the health inclusion criteria were satisfied (no current acute or chronic physical or mental illness), the participant signed an informed consent form.

Methods

DNA analysis and genotyping

Genomic DNA was extracted from 1 ml EDTA-treated peripheral blood. For extraction, the Genisol^{TM} Maxi-Prep Kit (ABgene House, Epsom, UK) was used.

To determine the genotype, we applied a polymerase chain reaction (PCR) on a 2720 Thermal Cycler (produced by PE Applied Biosystems) by using the primers 5'-TCCAAGGAGCTGCAGGCG-3' and 5'-CCGGCCTGGTACACTGCC-3'. The oligonucleotides were from the Biological Research Centre, Hungarian Academy of Sciences, Szeged.

The primers created a restriction site for the Hin6I enzyme (Fermentas) (5'-G^CGC-3'). The digestion products were resolved on polyacrylamide gel and detected under ultraviolet light after staining with ethidium bromide. The ε2 allele gave visible fragments of 91 and 83 bp, the ε3 allele gave fragments of 91 and 48 bp, and the ε4 allele gave fragments of 72 and 48 bp.

Statistical analysis

T-test and variance analysis were used to detect the differences among the three groups with regards to the demographic and clinical parameters. We grouped the subjects by the presence or absence of the ε2, ε3 or ε4 alleles because the sample size did not allow groups to be made for all possible genotypes. The Pearson chi squared test was performed to study the distribution of the alleles by the investigated groups. The *p* values were calculated on the basis of adjusted residual values. These values revealed the extent of the discrepancy between the observed and the expected frequencies in the cell (residual) in the examined group and standardised it. The combined effect of the MS course and the alleles on the clinical parameters was analysed with a two-way analysis of variance. Due to the multiple comparisons, the level of significance was chosen to be $p < 0.005$. For all statistical analyses, the SPSS 15.0 statistical package was used.

Results

The population analysed was in Hardy-Weinberg equilibrium, because the value of the goodness-of-fit test was $p = 0.082$ ($Chi^2 = 9.78$, d.f = 5). The demographic data on the three groups are displayed in table 2. As the groups were age- and sex-matched, there were no differences by gender or mean age ($p = 0.998$). The age at onset in the PPMS patients was higher and the disease duration was shorter than in the RRMS patients but not significantly ($p = 0.404$ and $p = 0.316$, respectively). In the PPMS form, the EDSS score, the PI and the MSSS values were higher compared with those for the RRMS group ($p < 0.001$).

Due to the low number of subjects with absence of the ε3 allele (5 RRMS and 1PPMS patients) grouping patients by this allele was not possible (table 3). Neither ε2/ε2 nor ε4/ε4 homozygote genotype was detected.

Table 4a and 4b show the occurrence of ε2 and ε4 alleles in the investigated groups. The number of the PPMS patients without the ε2 allele was found to be remarkably high ($p < 0.001$), whilst the ε2 allele was overrepresented in the RRMS group ($p < 0.003$) (table 4a). In addition, the pairwise comparisons indicated that the difference between the RRMS and HC groups was also significant ($p < 0.001$).

The presence of the ε4 allele was typical in the PPMS and the RRMS groups (table 4b). A markedly high frequency of this allele was found in the PPMS group ($p < 0.001$) and a very low frequency in the HC group ($p < 0.001$). The pairwise comparisons revealed that the frequency of the ε4 allele was higher also in the RRMS group than that in the HC group ($p < 0.05$) and was twice as frequent as in the PPMS group ($p < 0.005$).

With regards to the clinical parameters (EDSS, PI and MSSS), significant differences were observed between the RRMS and PPMS groups ($p < 0.001$ for all parameters; table 2). Differences were also detected regarding the EDSS and MSSS scores when the patients were grouped by the presence or absence of the ε2 allele ($p < 0.004$ and $p < 0.001$, respectively; table 5). As for the ε4 allele, there were no differences found in any of the clinical parameters on the $p = 0.005$ decision level but on the $p = 0.05$ level the MSSS value differed significantly ($p = 0.045$). All of the observed differences in the clinical parameters disappeared when the patients were further stratified by the type of MS.

Two-way analysis of the combined effect of the two variables (presence or absence of the alleles and type of MS) revealed that the difference in the clinical parameters could only be attributed to the type of MS (table 6). Interaction between these variables could not be detected.

Table 2: Demographic and clinical data of the three groups.

Group	Patients number Male/Female	Age (years)	Age at onset (years)	Disease duration (years)	EDSS	PI	MSSS
PPMS	23/22	49.1 ± 10.5	38.0 ± 9.4	11.1 ± 8.9	5.7 ±1.8	0.96 ± 1.13	7.2 ± 2.0
RRMS	23/22	49.2 ± 9.8	36.3 ± 9.5	12.9 ±8.0	2.8 ± 1.9	0.32 ± 0.35	3.2 ± 2.5
HC	23/22	49.0 ± 10.6	–	–	–	–	–
p	–	0.998	0.404	0.316	<0.001	<0.001	<0.001

Values are given as mean ± SD

Abbreviations: EDSS = Expanded Disability Status Scale; HC = healthy control; MSSS = Multiple Sclerosis Severity Score; PI = progression index; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis

Table 3: Distribution of all APOE genotypes by the investigated groups.

APOE gene	MS patients			Total
	PPMS (*n* = 45)	RRMS (*n* = 45)	HC (*n* = 45)	
Genotype				
ε2/ε2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ε2/ε3	3 (6.7%)	17 (37.8%)	17 (37.8%)	37 (27.4)
ε2/ε4	1 (2.2%)	5 (11.1%)	0 (0.0%)	6 (4.4%)
ε3/ε3	18 (40.0%)	17 (37.8%)	24 (53.3%)	59 (43.7%)
ε3/ε4	23 (51.1%)	6 (13.3%)	4 (8.9%)	33 (24.4%)
ε4/ε4	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	45 (100.0%)	45 (100.0%)	45 (100.0%)	135 (100.0%)

Abbreviations: APOE = apolipoprotein E gene; HC = healthy control; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis

Table 4a: The occurrence of the ε2 allele in the investigated groups.

		HC	PPMS	RRMS	Total
non ε2	**Count**	28	41	23	92
	% within group	62.2	91.1	51.1	68.1
	Adjusted residual (*p*)	–1.0 (0.317)	4.0 (<0.001)	–3.0 (0.003)	
ε2	**Count**	17	4	22	43
	% within group	37.8	8.9	48.9	31.9
	Adjusted residual (*p*)	1.0 (0.317)	–4.0 (<0.001)	3.0 (0.003)	
Total	**Count**	45	45	45	135
	% within group	100.0	100.0	100.0	100.0

Pearson Chi-Square p <0.001

Table 4b: The occurrence of the ε4 allele in the investigated groups.

		HC	PPMS	RRMS	Total
non ε4	**Count**	41	21	34	96
	% within group	91.1	46.7	75.6	71.1
	Adjusted residual (*p*)	3.6 (<0.001)	–4.4 (<0.001)	0.8 (0.424)	
ε4	**Count**	4	24	11	39
	% within group	8.9	53.3	24.4	28.9
	Adjusted residual (*p*)	–3.6 (<0.001)	4.4 (<0.001)	–0.8 (0.424)	
Total	**Count**	45	45	45	135
	% within group	100.0	100.0	100.0	100.0
	Pearson Chi-Square p <0.001				

Abbreviations: HC = healthy control; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis

Table 5: Mean values of the measured clinical parameters of MS groups dependant on having or not having the ε2 or ε4 alleles.

	PPMS				RRMS				PPMS and RRMS			
	N = 41	N = 4	N = 45	*p*	N = 23	N = 22	N = 45	*p*	N = 64	N = 26	N = 90	*p*
	non ε2	ε2	Total		non ε2	ε2	Total		non ε2	ε2	Total	
Disease duration (year)	11.2 ± 8.9	10.3 ± 10.2	11.1 ± 8.9	0.872	12.7 ± 6.9	13.1 ± 9.2	12.9 ± 8.0	0.842	11.7 ± 8.2	12.7±9.2	12.0 ± 8.5	0.618
EDSS	5.6 ± 1.8	6.3 ± 2.1	5.7 ± 1.8	0.586	3.0 ± 2.1	2.6 ± 1.7	2.8 ± 1.9	0.429	4.7 ± 2.2	3.2±2.2	4.2 ± 2.3	**0.004**
PI	1.0 ± 1.2	0.6 ± 0.6	1.0 ± 1.1	0.475	0.3 ± 0.3	0.4 ± 0.4	0.3 ± 0.4	0.456	0.7 ± 1.0	0.4±0.5	0.6 ± 0.9	0.093
MSSS	7.2 ± 2.1	7.0 ± 0.8	7.2 ± 2.0	0.858	3.5 ± 2.6	3.0 ± 2.4	3.2 ± 2.5	0.534	5.9 ± 2.9	3.6±2.6	5.2 ± 3.0	**0.001**
	PPMS				**RRMS**				**PPMS and RRMS**			
	N = 21	**N = 24**	**N = 45**	***p***	**N = 34**	**N = 11**	**N = 45**	***p***	**N = 55**	**N = 35**	**N = 90**	***P***
	non ε4	**ε4**	**Total**		**non ε4**	**ε4**	**Total**		**non ε4**	**ε4**	**Total**	
Disease duration (year)	10.7 ± 8.4	11.5 ± 9.5	11.1 ± 8.9	0.768	14.2 ± 8.0	8.8 ± 6.9	12.9 ± 8.0	0.052	12.9 ± 8.3	10.6 ± 8.7	12.0 ± 8.5	0.226
EDSS	5.6 ± 1.9	5.7 ± 1.7	5.7 ± 1.8	0.934	3.0 ± 1.9	2.1 ± 1.8	2.8 ± 1.9	0.169	4.0 ± 2.3	4.6 ± 2.4	4.2 ± 2.3	0.287
PI	0.9 ± 0.9	1.0 ± 1.3	1.0 ± 1.1	0.819	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.4	0.851	0.6 ± 0.7	0.8 ± 1.2	0.6 ± 0.9	0.242
MSSS	7.0 ± 2.2	7.4 ± 1.8	7.2 ± 2.0	0.530	3.3 ± 2.5	3.0 ± 2.7	3.2 ± 2.5	0.759	4.7 ± 3.0	6.0 ± 2.9	5.2 ± 3.0	0.045

Values are given as mean ± SD

Abbreviations: EDSS = Expanded Disability Status Scale; MSSS = Multiple Sclerosis Severity Score; PI = progression index; PPMS = primary progressive multiple sclerosis; RRMS= relapsing-remitting multiple sclerosis

Table 6: Results of the two-way analysis of variance.

		p
Dependent variable: EDSS		**–**
Grouping variables	**Type of MS**	<0.001
	ε2	0.866
	Type of MS* ε2	0.327
Dependent variable: MSSS		**–**
Grouping variables	**Type of MS**	<0.001
	ε2	0.631
	Type of MS* ε2	0.838

Abbreviations: EDSS = Expanded Disability Status Scale; MSSS = Multiple Sclerosis Severity Score; MS = multiple sclerosis

Discussion

The literature reports on the role of APOE in MS are controversial, with claims that the presence [9–18] or absence [19–28] of the APOE ε4 allele is connected with the susceptibility to the disease or its severity. The role of the APOE gene in MS has been extensively studied in recent years, but the debate remains open (table 1). Studies on the APOE status in the Hungarian MS population have not been published so far, though the role of chromosome 19 was raised by Rajda et al. [29].

In the literature, the information relating to the genetic background of PPMS patients is incomplete because of the low number of such patients [10, 12–15, 17, 18, 24, 25]. Only two studies on APOE analysis (from Sardinia and the Netherlands) involved a larger PPMS group than that in the present study (table 1) [11, 26]. The prevalence of MS in the Netherlands is 76 per 100,000 inhabitants [30], while in Sardinia it is approximately 2.3-fold higher than in Hungary [31, 32]. Accordingly, our group (approximately 7% of the Hungarian PPMS population) comprises of a comparatively large sample, considering that the Hungarian prevalence is 62 per 100,000, the population of Hungary is 10 million and 11% of Hungarian MS patients have the PPMS form [31]. The study from Sardinia revealed that the ε4 allele increases the risk of PPMS, but only in women [11] whereas the study from the Netherlands did not confirm any association.

A recent meta-analysis by Burwick et al. of the results from a pooled analysis (353 PPMS cases) did not provide any evidence of an association between the ε2 or ε4 carrier status in PPMS [20]. The pooled analysis was performed on the results of 11 published (from 10 different countries) and one unpublished article, which is an average of 32 ± 26 PPMS subjects per article. However, this meta-analysis did not include the results from Sardinia with 98 PPMS patients [11] or from Denmark with 42 PPMS patients [16]. Cocco et al. detected a gender-specific association between the ε4 allele and the PPMS course whilst Pinholt et al. found that the ε4 allele is associated with faster progression (table 1). A study from a country geographically adjacent to Hungary (Austria) detected an association between the ε4 allele and rapid progression (24 PPMS subjects) [14].

The present study did not consider the genotype-phenotype relationship, however, in the series in the study by Masterman et al. the APOE ε3/ε4 genotype was more common in severe MS than in benign MS [21]. Fazekas et al. found that patients carrying the ε3/ε4 genotype exhibited a significantly higher black hole ratio, demonstrating the disabling

effect of the ϵ4 allele [13]. The black hole ratio ((T1-LL/T2-LL)*100), calculated by the total lesion load on proton density weighted (T2-LL) and T1 weighted scans (T1-LL), indicates the proportion of more severe tissue destruction among MS lesions. A study from Denmark indicated that the ϵ4/ϵ4 homozygote genotype is a risk factor for MS and determines the clinical progression [15].

Similarly as in other studies, we could not identify patients homozygous for the ϵ4 allele [10, 11, 22, 34], most probably because of the low number in the overall examined population. In contrast, 2.1–7.7% of the patients were genotyped as ϵ4/ϵ4 in studies from Denmark [15, 16], Sweden [21] and Kuwait [19].

The frequencies of the APOE alleles vary worldwide. The predominant allele for most populations is the ε3 allele (70–80%), which is often considered to be the ancestral allele. We found this to be the most frequent allele in our population (68.9% in the PPMS group, 63.3% in the RRMS group and 76.7% in the HC group). While the proportion of ε4 carriers increases steadily from 10–15% in southern Europe to 40-50% in the north, the proportion of ε2 allele carriers is slightly higher in central Europe than in the south or the north [1]. The distributions of the ϵ4 allele in this study exhibited a wide range, from 4.4% to 26.7%, and its frequency was outstandingly high in the PPMS group. The ϵ2 allele frequency was underrepresented in the PPMS group (only 4.4%).

Concerning the relationship between the age at onset and the APOE polymorphisms, a previous study concluded that the ϵ4 allele is associated with an earlier age of onset in MS patients [10]. We did not observe a significant association between either the ϵ4 or the ϵ2 allele and the disease duration.

Fewer studies have investigated the association of the ε2 allele in MS. Some authors reported evidence of a protective effect of the ε2 allele [18, 33], but the meta-analysis by Burwick et al. disputed this [20]. Our results provide support for an association between carrying the ϵ2 allele and more favourable disease parameters. Furthermore, this is in line with the findings of Savettieri et al. [24], Kantarci et al. [35] and a study which demonstrated that patients with the ϵ3/ϵ2 genotype had a significantly reduced and delayed risk of chronic progressive MS [33]. The presence of the ϵ2 allele may possibly exert a protective effect against progression.

The strength of the present study is that it was based on a comparatively large number of a subpopulation with a rare disease and examined a wide range of clinical parameters.

The limitations of this study are the low number of subjects in the control groups; consequently increases in the number of RRMS patients and healthy controls are clearly needed. In addition, further SNP assessments (HLA) related to disease progression or association, and longitudinal follow-ups completed with magnetic resonance findings are suggested for a reliable result.

The present study is the first that has examined the possible association involving the APOE status in the population of Hungarian PPMS patients. Based on the observed differential occurrence of the ε2 allele in the PPMS and the RRMS groups, we suspect that the presence of this allele makes the patients susceptible to the RRMS course. The observed distribution of the ε4 allele across the groups indicated that this allele is linked with both forms of the disease but with a higher propensity to the PPMS course. Our findings suggest that the presence of the ε2 and ε4 alleles may play a role in the development of the disease. However, if any type of the disease has already developed the alleles show no association with the clinical parameters.

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