

Gene variants of adhesion molecules act as modifiers of disease severity in MS

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ABSTRACT

Objective: To assess the potential effect of variants in genes encoding molecules that are implicated in leukocyte trafficking into the CNS on the clinical phenotype of multiple sclerosis (MS).

Methods: A total of 389 Greek MS cases and 336 controls were recruited in 3 MS centers from Cyprus and Greece. We genotyped 147 tagging single nucleotide polymorphisms (SNPs) in 9 genes encoding for P-selectin (*SELP*), integrins (*ITGA4*, *ITGB1*, and *ITGB7*), adhesion molecules (*ICAM1*, *VCAM1*, and *MADCAM1*), fibronectin 1 (*FN1*), and osteopontin (*SPP1*) involved in lymphocyte adhesion and trafficking into the CNS. Clinical end points of the study were age at MS onset and MS severity as measured by the Multiple Sclerosis Severity Score. Permutation testing was applied to all analyses.

Results: SNPs rs6721763 of the *ITGA4* and rs6532040 of the *SPP1* were found to significantly influence disease severity (permutation *p* values: 3.00e-06 and 0.009884, respectively). SNP rs1250249 of the *FN1* had a dose-dependent effect on age at disease onset (permutation *p* value: 0.0002).

Conclusions: This study provides evidence implicating variants encoding adhesion molecules, responsible for lymphocyte adhesion and trafficking within the CNS, as modifiers of MS disease severity. These genetic biomarkers, which can be available at the time of diagnosis, may be used to assess the biological aggressiveness of the disease and thus guide decisions on treatment.

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GLOSSARY

DMT = disease-modifying therapy; **GWAS** = genome-wide association study; **MAF** = mean allele frequency; **PI** = progression index; **SNP** = single nucleotide polymorphism.

Multiple sclerosis (MS) is considered to result from an interplay between environmental exposures and genetic background.^{1–3} The majority of genetic studies have focused on susceptibility variants, and this effort has yielded more than 110 genetic risk factors of MS.⁴ In addition, there is evidence that genetic factors may influence disease phenotype such as age at onset, disease severity, and the clinical course.^{5–7} However, 3 genome-wide association studies (GWASs) investigating MS severity have failed to reach a genome-wide significance threshold.^{8–10} A recent meta-analysis of 10 cohorts in 7,125 patients with MS also failed to detect any significant signal.¹¹

Disability in MS, either resulting from relapses or accumulating during the progressive phase of the disease, is partly a result of immune attack from adaptive and innate immunity. A number of molecules are important in leukocyte adhesion and diapedesis through the blood-brain barrier, as well as their migration through the extracellular matrix to the target tissue. The use of natalizumab, a monoclonal antibody against $\alpha 4$ integrin, was found to markedly reduce relapses,

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new lesion formation, and disability progression^{12,13} by blocking lymphocyte adhesion and migration through the vascular endothelial cells and by blocking interaction with extracellular matrix proteins. The impressive effectiveness of natalizumab argues strongly in favor of the significance of this pathogenic pathway.

This study was designed to test the effect of genetic variants of molecules, that are implicated in leukocyte trafficking through blood vessel walls into the CNS, on disease severity as measured by the Multiple Sclerosis Severity Score (MSSS).

METHODS Study population. Greek patients with MS and controls were recruited from 3 MS centers: The Cyprus Institute of Neurology and Genetics in Cyprus, the University Hospital of Larissa, Greece, and the AHEPA Hospital of Aristotle University in Thessaloniki, Greece.^{14,15} In total, 389 MS cases and 336 controls were recruited for the study. Patients were included in the study if they had clinically definite MS according to the 2005 revised McDonald criteria, were aged ≥ 18 years, and had disease duration of ≥ 5 years. Cases with primary progressive MS were excluded.

Clinical and demographic data were collected using the same criteria in each center. Neurologists specialized in MS collected data such as sex, current age, and age at disease onset, as age at the first neurologic symptom suggestive of MS. Use of disease-modifying therapies (DMTs) and treatment duration were also recorded. Patients were receiving one of the following DMTs: interferon- β , glatiramer acetate, or natalizumab. Disability status was assessed in the absence of relapse for at least 6 months, using the Kurtzke Expanded Disability Status Scale (EDSS). Disease duration was defined as the time (in years) from the first symptom to the last EDSS assessment. The rate of disease progression was expressed by the progression index, which is the ratio of EDSS to MS duration. Finally, disease severity in each individual was assessed by means of MSSS which is a useful algorithm for disability distribution in MS groups evaluated cross-sectionally.¹⁶ The control group consisted of healthy volunteers from the same ethnic regions and was matched with cases for age and sex.

Standard protocol approvals, registrations, and patient consents. The study protocol was approved by the local ethics committees, and written informed consent was obtained from all patients.

Isolation of DNA, SNP selection, and genotyping. DNA was isolated from peripheral blood samples using the Puregene DNA purification kit (Qiagen, Valencia, CA). Based on the study rationale, we chose to genotype genes that encode for the following adhesion and extracellular matrix molecules: (1) P-selectin (OMIM #173610) encoded by the *SELP* gene located on chromosome 1q23-q25, (2) integrin α -4 (OMIM #1929750, *ITGA4*, 2q31-q32), (3) integrin β -1 (OMIM #135630; *ITGB1*, 10p11.2), (4) integrin β -7 (OMIM #147559, *ITGB7*, 12q13.13), (5) intercellular adhesion molecule 1 (OMIM #147840, *ICAMI*, 19p13.3-p13.2), (6) vascular cell adhesion molecule 1 (OMIM #192225, *VCAMI*, 1p32-p31), (7) mucosal vascular addressin cell adhesion molecule 1 (OMIM #102670, *MADCAMI*, 19p13.3), (8) fibronectin 1 (OMIM #135600,

FNI, 2q34), and (9) osteopontin (OMIM #166490, *SPP1*, 4q21-q25).

To cover most of the polymorphic variability along with the above-mentioned genes, we used the linkage disequilibrium tagger algorithm of the HapMap project for selection of tag-SNPs. Selection of tagging single nucleotide polymorphisms (SNPs) was based on the HapMap Release 27 database (Phase II + III, Feb09, on NCBI B36 assembly, dbSNP b126) for CEU population, using the criteria of pairwise r^2 values ≥ 0.8 and minor allele frequency > 0.05 . This approach retrieved 32 tagging SNPs in *SELP* gene, 36 in *ITGA4* gene, 16 in *ITGB1*, 3 in *ITGB7*, 5 in *ICAMI*, 15 in *VCAMI*, 3 in *MADCAMI*, 38 in *FNI*, and 8 in *SPP1* which sum to 156 SNPs. Chromosome position of each SNP, the minor allele frequency for CEU population and TSI population (Tuscan in Italy), which is considered closer to the Greek population, and the minor allele frequency (MAF) of our control group are presented in table e-1 at Neurology.org/nn.

TaqMan 5'-nuclease assays were used to genotype SNPs by acquiring specific assays for each SNP and then performing allelic discrimination assays on the ABI 7900HT Real-Time System (Applied Biosystems, Foster City, CA). Genotype success rates were above 96%. However, 9 SNPs as shown in table e-1 failed to be genotyped and therefore were not included in the analysis.

Statistical analysis. Quality control was performed by evaluating the genotype distributions of all SNPs in the control samples for the Hardy-Weinberg equilibrium using an exact test¹⁷ with a threshold of $p \leq 0.05$. MAFs were estimated and used for quality control (threshold of MAF $< 0.05\%$). Linear regression analysis for the age at disease onset and MSSS were performed for association testing. The cutoff threshold for significance was a priori set to a permutation-derived p value of at most 0.01. Analyses for age at onset were adjusted for sex and site. Analyses with the MSSS, as a dependent variable, were additionally adjusted for age at onset and treatment status. For treatment status, the duration of treatment with DMT in months was used as a covariate to ensure that any detected effect was not driven by treatment duration. Permutation testing was applied to adjust for multiple testing for known or unknown factors that may affect the balance of the distribution of the data. A total of 10^6 permutations were performed by using a high-performance computing platform.

Label-swapping permutations were used with 2 sets of empirical significance p values to estimate individual SNPs significance. The minimum possible p value depends on the number of tests performed; in our case, it was 10^{-6} . Because the permutation scheme followed preserves the correlational structure between SNPs, there is no assumption of independence unlike Bonferroni, which assumes all tests are independent. As this is a candidate gene study with relatively dense coverage, there is a likelihood of dependence between neighboring SNPs due to linkage disequilibrium; therefore, permutation offers a better alternative for adjusting for multiple testing.

RESULTS Clinical and demographic data of controls and patients as a group and in each MS center are presented in table 1. More than two-thirds of the patients were women. The mean disease duration was 12.9 years, and most patients (70%) were under DMT. The mean EDSS was 3.4, whereas half of the patients had EDSS less than 3. Severe accumulation of disability (MSSS: 7–10) was observed in approximately 20% of the patients. Patients recruited from

Table 1 Demographic and clinical characteristics of study participants

	Total patients	Controls	Cyprus patients	Cyprus controls	Larissa patients	Larissa controls	Thessaloniki patients
No.	389	336	104	127	206	209	79
Female, n (%)	251 (64.5)	234 (69.6)	74 (71.2)	88 (69.3)	126 (61.2)	146 (69.9)	51 (64.6)
Male, n (%)	138 (35.5)	102 (30.4)	30 (28.8)	39 (30.7)	80 (38.8)	63 (30.1)	28 (35.4)
Female:male ratio	1.8:1	2.3:1	2.5:1	2.3:1	1.6:1	2.3:1	1.8:1
Age at disease onset, y, mean (range)	30.4 (18-63)		35.3 (18-58)		29.0 (18-63)		27.9 (18-50)
Age at the time of analysis, y, mean (range)	43.3 (23-74)	36.3 (23-78)	43.8 (25-66)	47.4 (20-78)	43.0 (23-74)	29.6 (23-58)	41.9 (23-63)
Disease duration, y, mean	12.9		12.44		12.77		13.90
Median (range)	11 (5-52)		11 (5-35)		10 (5-52)		12 (5-38)
DMT, n (%)	271 (69.7)		71 (68.3)		131 (63.6)		69 (87.3)
DMT time, mo, mean (SD)	72.1 (44.2)		73.6 (49.6)		70.4 (43.3)		73.9 (40.2)
EDSS, n (%)							
<3	188 (47.8)		69 (66.3)		84 (40.8)		33 (41.8)
3 to <6	114 (29.3)		27 (26.0)		54 (26.2)		33 (41.8)
6-6.5	40 (10.3)		6 (5.8)		25 (21.1)		9 (11.4)
≥7	35 (9.0)		2 (1.9)		31 (15.0)		2 (2.5)
Mean (SD)	3.40 (2.16)		2.44 (1.54)		3.83 (2.46)		3.61 (1.59)
Progression index, mean (SD)	0.31 (0.24)		0.22 (0.15)		0.34 (0.24)		0.35 (0.31)
MSSS							
Median	4.00		3.00		5.00		5.00
Mean (SD)	4.39 (2.61)		3.18 (1.98)		4.92 (2.89)		4.71 (2.00)
Benign MS (MSSS = [1-2]), n (%)	109 (28.0)		49 (47.1)		51 (24.8)		9 (11.4)
Severe MS (MSSS = [7-10]), n (%)	86 (22.1)		9 (8.7)		61 (29.6)		16 (20.3)

Abbreviations: DMT = disease-modifying therapy; EDSS = Kurtzke Expanded Disability Status Scale; MSSS = Multiple Sclerosis Severity Score.

the Larissa and Thessaloniki sites presented a more severe phenotype regarding EDSS, progression index, and MSSS scores compared with the patients recruited from Cypriot ($p < 0.05$).

The distribution of SNP alleles in patients, their respective scores in MSSS, and the results of linear regression analysis are presented in table e-2. Significant associations with $p < 0.05$ were found with 4 *ITGA4* polymorphisms (rs10930969, rs2124440, rs3770136, and rs6721763), in rs3780873 *ITGB1*, in 2 *FNI* polymorphisms (rs1250258 and rs13432972), and in rs9138 of *SPP1*. Permutation analysis was performed to adjust for multiple testing (table 2). Significant associations were found with rs6721763 of *ITGA4* (permutation p value: 3.00e-06) and rs6532040 of *SPP1* (permutation p value: 0.009884). A dose-dependent effect of minor allele on MSSS was noted with worse scores in minor allele homozygotes for both SNPs. In particular, for rs6721763 of *ITGA4*, the mean MSSS increased from 3.191 in AA homozygotes to 3.94 in Ab heterozygotes and finally 5.012 in bb homozygotes. The same trend was observed for rs6532040 of *SPP1* with an

increase from a mean MSSS of 3.538 to 4.034 and 4.176 in AA, Ab, and bb genotypes, respectively.

Permutation analyses of the effects of SNP alleles on age at onset are shown in table e-3. The permutation p value significance threshold of 0.01 was exceeded by rs1250249 of *FN1* ($p = 0.0002$). A dose-dependent effect of this polymorphism on age at onset was observed: age at onset dropped from 32.3 years in AA homozygotes to 28.1 years in Ab heterozygotes and to 26.3 years in bb homozygotes.

DISCUSSION Despite the considerable progress in identifying genetic susceptibility variants,⁴ there is limited evidence in identifying genetic factors that may contribute to MS disease severity. Genetic association studies have focused on candidate genes such as *APOE*, genes related to immune function, autoimmunity, neurobiology, myelin structure, and the human leukocyte antigen (*HLA*), which resulted in conflicting data.^{18,19} In addition, 3 GWAS investigating MS severity failed to surpass a genome-wide significance threshold.⁸⁻¹⁰ However, analysis of SNPs in genes involved in glycosylation and calcium and

Table 2 Permutation analysis results in MSSS

	SNPs	Permutation p value	SNPs	Permutation p value	SNPs	Permutation p value	SNPs	Permutation p value	SNPs	Permutation p value	SNPs	Permutation p value
	SELP		27. rs6128	0.1919	23. rs4667310	0.1279	1. rs3825084	0.6863	2. rs6510810	0.2457	25. rs13652	0.6824
1.	rs732314	0.9147	28. rs3917843	0.3343	24. rs7607758	0.1746	2. rs2272300	0.2438	3. rs3745925	0.2051	26. rs13432972	0.06204
2.	rs3917862	0.6987	29. rs3917853	0.1037	25. rs11689738	0.6311	3. rs11170465	0.5258	FN1		27. rs1250203	0.7171
3.	rs3917683	0.9941	30. rs3917854	0.1719	26. rs12469449	0.5248	ICAM1		1. rs1250258	0.0634	28. rs7608342	0.6461
4.	rs3917686	0.5239	ITGA4		27. rs6721763	3.00e-06 ^a	1. rs5030390	0.6959	2. rs2577290	0.7659	29. rs6707530	0.348
5.	rs2244529	0.4432	1. rs12988934	0.3794	28. rs6728886	0.4004	2. rs1799969	0.6981	3. rs1250246	0.209	30. rs1250211	0.9535
6.	rs2244526	0.9039	2. rs3770138	0.7438	29. rs6707704	0.2942	3. rs5498	0.5103	4. rs1898536	0.9657	31. rs2289200	0.8463
7.	rs3917709	0.6725	3. rs10930969	0.01169	30. rs1449259	0.3535	4. rs3093032	0.2641	5. rs1250250	0.885	32. rs7568287	0.6808
8.	rs2236868	0.6458	4. rs2124440	0.01847	31. rs1047307	0.2376	5. rs281437	0.1168	6. rs11693652	0.9635	33. rs33996776	0.7985
9.	rs3917714	0.7683	5. rs3770136	0.01521	ITGB1		VCAM1		7. rs16854041	0.5775	34. rs10498037	0.7494
10.	rs6125	0.7683	6. rs155149	0.3515	1. rs11009157	0.2657	1. rs3176860	0.3093	8. rs1250249	0.5565	35. rs7579867	0.8239
11.	rs3917727	0.2448	7. rs3770132	0.3451	2. rs1187070	0.5029	2. rs3176861	0.3179	9. rs1250248	0.1671	36. rs11651	0.8754
12.	rs6131	0.3165	8. rs711801	0.8058	3. rs1009002	0.696	3. rs3917009	0.7551	10. rs17517509	0.9489	SPP1	
13.	rs3917731	0.4606	9. rs1449264	0.2445	4. rs2488336	0.3562	4. rs3917010	0.2939	11. rs1437799	0.9584	1. rs2853749	0.7142
14.	rs3917739	0.8051	10. rs1449265	0.8729	5. rs10827164	0.7011	5. rs3765685	0.2097	12. rs724617	0.9754	2. rs11728697	0.1396
15.	rs3917740	0.8371	11. rs3770126	0.06531	6. rs16933819	0.2905	6. rs3176867	0.2108	13. rs13021679	0.6185	3. rs6840362	0.4792
16.	rs2076074	0.9684	12. rs11694175	0.9604	7. rs9417094	0.2937	7. rs3917012	0.1209	14. rs2372545	0.4479	4. rs6532040	0.009884 ^a
17.	rs3917744	0.6447	13. rs17224277	0.3591	8. rs2504001	0.6565	8. rs3176869	0.539	15. rs7575234	0.6593	5. rs6811536	0.4773
18.	rs2142760	0.4416	14. rs155087	0.04326	9. rs2488331	0.4218	9. rs3181088	0.1947	16. rs7572169	0.6593	6. rs4754	0.6128
19.	rs3917768	0.6865	15. rs2305588	0.1206	10. rs10763902	0.7189	10. rs2209627	0.288	17. rs10172425	0.6431	7. rs1126772	0.6849
20.	rs2205895	0.6823	16. rs155099	0.07616	11. rs3780873	0.1267	11. rs3176876	0.5796	18. rs34255697	0.5046	8. rs9138	0.03449
21.	rs3917779	0.9829	17. rs3755021	0.1005	12. rs4587680	0.3719	12. rs3917016	0.533	19. rs1525351	0.7776		
22.	rs2420378	0.9013	18. rs155103	0.3347	13. rs2488320	0.8601	13. rs3917018	0.3098	20. rs2692228	0.917		
23.	rs3917786	0.7186	19. rs155106	0.9674	14. rs1316757	0.853	14. rs3917019	0.4335	21. rs11883812	0.5302		
24.	rs3917788	0.5852	20. rs3770120	0.1899	15. rs2488318	0.8561	15. rs3176878	0.857	22. rs10804242	0.9766		
25.	rs3917792	0.6765	21. rs17225354	0.6191	16. rs2153875	0.8525	MADCAM1		23. rs6709607	0.9355		
26.	rs1569471	0.743	22. rs2305586	0.1351	INTEGRIN, β-7		1. rs12982646	0.5785	24. rs7588661	0.7678		

Abbreviations: MSSS = Multiple Sclerosis Severity Score; SNP = single nucleotide polymorphism.

^aSignificant.

glutamate signaling that may represent disease modifiers reached levels close to genome-wide significance threshold. The effect of HLA on disease onset was relatively consistent among studies.^{20,21} Carriers of the DRB1*15:01 allele had an earlier onset of MS, with each copy accounting for a decrease in age at onset by about 10.6 months.²²

This study detected a statistically significant association between rs6721763 of *ITGA4* gene and disease severity as measured with the MSSS in a dose-dependent manner. *ITGA4* encodes for the $\alpha 4$ subunit of $\alpha 4\beta 1$ integrin, which is involved in leukocyte adhesion and migration through the blood-brain barrier.²³ The monoclonal antibody, natalizumab, which is approved for the treatment of relapsing-remitting MS, exerts its favorable effects on relapses and disability progression by targeting $\alpha 4$ integrin.²⁴ Polymorphisms in *ITGA4* have been linked so far only to MS susceptibility.^{25–27} In particular rs1143676, a nonsynonymous polymorphism (R878Q) located in exon 24 of *ITGA4* has been reported to be associated with disease susceptibility in Slovaks²⁵ but not in other populations.^{26,27} This polymorphism is located 9.3 kb distant from our polymorphism. The second *ITGA4* polymorphism that was linked so far to disease susceptibility in a Basque group is the intronic SNP rs1449263,²⁶ which however is 66.5 kb away from our polymorphism. Finally, rs155100 (36 kb far from rs6721763) has been reported to influence the risk of autism.²⁸ SNP rs6721763 is an intronic polymorphism (intron 17–18) located only just 929 bp from the splicing site of exon 18 of *ITGA4*.

The second genetic modifier of disease course identified in our study is rs6532040 of *SPP1*. The presence of the minor allele worsens the disease disability course. Osteopontin is a secreted matrix protein with pleiotropic, but mainly, proinflammatory functions involved in T-cell activation, proliferation, and migration. A part of its actions is mediated by binding to integrins. In addition, intracellular osteopontin activates intracellular signaling pathways.²⁹ Osteopontin transcripts are upregulated in MS brain lesions and in normal-appearing white matter. In the mouse model of experimental autoimmune encephalomyelitis, osteopontin was implicated in disease severity and activity.³⁰ Genetic polymorphisms altered osteopontin levels.³¹ A genetic association study showed that a *G/A* polymorphism located in the 3' untranslated region of exon 7 (position 9583) had a significant effect on the age at onset.³² SNP rs6532040, which emerged as significant in our study, is located in intron 5–6, which is only 1.5 kb distant from the start of exon 7. In another study, rs9138 of the osteopontin gene, located at exon 7 and 2.14 kb distant from our polymorphism, showed

a modest trend for association. In particular, patients with the wild-type genotype were less likely to have a mild course ($p = 0.08$) and had increased risk of having a secondary-progressive course of the disease ($p = 0.05$).³³ In addition, a haplotype study of 4 SNPs (rs4754, rs1126616, rs1126772, and rs9138; 0.49 kb, 1.65 kb, 1.98 kb, and 2.14 kb away from our polymorphism, respectively) showed that homozygotes combining the wild-type alleles of all 4 SNPs displayed a milder disease course, slower accumulation of disability, and delayed conversion from relapsing-remitting to secondary-progressive disease course.³¹ The same research group replicated these findings in an independent group and also detected similar effects on a polymorphism located in the promoter region of the osteopontin gene (rs7687316, 5.5 kb distant from our polymorphism).³⁴ However, a study of 8 SNPs located in exons 6 and 7 failed to detect any significant association with MS severity.³⁵

Finally, our study found that rs1250249 of *FNI* exerts a dose-dependent effect on disease onset. The presence of the minor allele predisposes to an earlier age at onset. Fibronectin 1 is an extracellular matrix glycoprotein that interacts with a number of integrins mediating a variety of functions including cytoskeleton organization, cell adhesion, and migration.³⁶ Polymorphisms of *FNI* have been associated so far among others with schizophrenia³⁷ and the development of intracranial aneurysms.³⁸

The difficulty in identifying genetic modifiers of MS course and severity may reflect either the complexity or diversity of inflammatory and neurodegenerative mechanisms that contribute to the clinical expression or some inherent flaws in measuring relevant clinical endpoints. In addition, the advent of immunomodulatory treatments has proved to alter the course of the disease.^{39,40} Early initiation and timely escalation of disease-modifying drugs are known to prevent the accumulation of disability. Therefore, the inclusion in a genetic association study of patients with MS without any treatment along with those who may or may not receive the best medical treatment is an important confounding factor that may obscure the detection of significant genetic effects.

Our study included a clinically well-characterized and ethnically homogenous group. Disease severity in each individual was assessed by means of MSSS, a robust and validated measure, that shows stability over time. In addition, patients were only included if they had at least 5 years duration of the disease, as it is known that the first 5 years of the disease represent a significant outcome milestone of MS. Also, a number of meaningful clinical predictors that may have an effect on long-term severity were collected and included as covariates in the analysis. Moreover, genetic variability across the tested genes was covered

by a large number of tagging SNPs. Finally, permutation analysis, a powerful unbiased test, was implemented to adjust for multiple testing of known and unknown factors.

In brief, our study provides evidence implicating variants in genes that encode adhesion molecules, responsible for lymphocyte trafficking and activation, in the severity of MS. Replication of these associations in other independent populations will render our findings more robust. Such biomarkers, if validated, may become valuable prognostic tools at an individual level at the time of diagnosis. Furthermore, therapies targeting adhesion molecules may be worth exploring in the future.

AUTHOR CONTRIBUTIONS

Efthimios Dardiotis, Georgios M. Hadjigeorgiou, and Theodoros Kyriakides: original idea, study design, clinical management and cohort recruitment, interpretation of results, and drafting of the manuscript. Elena Panayiotou, Kyproula Christodoulou, and Andreas Hadjisavvas: experimental work. Antonios Provas, Athanasios Loubopoulos, Marios Pantzaris, and Nikolaos Grigoriadis: clinical management and cohort recruitment. Athos Antoniadis: statistical analysis.

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