

Serum antibodies to phosphatidylcholine in MS

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Abstract

Objective

To evaluate the value of serum immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies reactive with phosphatidylcholine (PC) and lactosylceramide (LC) as biomarkers in MS.

Methods

We developed an ultrasensitive ELISA technique to analyze serum IgG and IgM antibodies to LC and PC, which we used to analyze samples from 362 patients with MS, 10 patients with non-MS myelin diseases (Non-MSMYDs), 11 patients with nonmyelin neurologic diseases (Non-MYNDs), and 80 controls. MS serum samples included clinically isolated syndrome (CIS, n = 17), relapsing-remitting MS (RRMS, n = 62), secondary progressive MS (SPMS, n = 50), primary progressive MS (PPMS, n = 37), and benign MS (BENMS, n = 36).

Results

We detected higher levels of serum IgM antibodies to PC (IgM-PC) in MS than control samples; patients with CIS and RRMS showed higher IgM-PC levels than patients with SPMS, PPMS, and BENMS and controls. MS and control samples did not differ in serum levels of IgM antibodies reactive with LC, nor in IgG antibodies reactive with LC or PC.

Conclusions

Serum IgM-PC antibodies are elevated in patients with MS, particularly during the CIS and RRMS phases of the disease. Thus, serum IgM-PC is a candidate biomarker for early inflammatory stages of MS.

Classification of evidence

This study provides Class III evidence that serum antibodies to PC are elevated in patients with MS. The study is rated Class III because of the case control design and the risk of spectrum bias: antibody levels in patients with MS were compared with healthy controls.

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→ Class of Evidence

Criteria for rating therapeutic and diagnostic studies

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Glossary

BCR = B-cell receptor; **BENMS** = benign MS; **CIS** = clinically isolated syndrome; **IgG** = immunoglobulin G; **IgG-PC** = IgG to PC; **IgG-LC** = IgG to LC; **IgM** = immunoglobulin M; **IgM-LC** = IgM to LC; **IgM-PC** = IgM to PC; **LC** = lactosylceramide; **Non-MSMYD** = non-MS myelin disease; **Non-MYND** = nonmyelin neurologic disease; **PC** = phosphatidylcholine; **PPMS** = primary progressive MS; **RRMS** = relapsing-remitting MS; **SPMS** = secondary progressive MS.

The high prevalence of immunoglobulin G (IgG) and immunoglobulin M (IgM) oligoclonal bands in the CSF has guided multiple studies focused on the identification of antibody targets in MS.^{1,2} Lipids are a major component of myelin, the main target of the autoimmune response in MS.³ New antigen microarray techniques have allowed the detection of lipid-reactive antibodies in the CSF of patients with MS.^{4,5} Indeed, IgG antibodies to sulfatide, ganglioside GM4, and galactocerebroside^{6–9} worsen disease pathogenesis in MS experimental models.⁴ In addition, patients with MS harboring CSF IgM reactive with phosphatidylcholine (PC) develop a more severe disease course.^{6,7,10–12} Moreover, serum IgG antibodies to lactosylceramide (LC) are associated with cerebral tissue damage in patients with MS.¹³ These data suggest that lipid-reactive antibodies may contribute to disease pathogenesis and constitute useful as biomarkers in MS.

In this study, we evaluated the potential of lipid-reactive antibodies as biomarkers in MS. We detected increased levels of serum IgM antibodies to PC (IgM-PC) in patients with MS. In contrast, we did not detect differences in serum IgG antibodies to PC (IgG-PC), nor IgM or IgG to LC (IgM-LC and IgG-LC, respectively) between MS and controls. Thus, serum IgM-PC is a candidate biomarker for MS.

Methods

Study design

We developed an ELISA technique to study IgG and IgM antibodies reactive with LC and PC in patients with MS. This Class III retrospective cohort control study included individuals from different cohorts (United States and Spain). The study included a narrow spectrum of persons with MS, non-MS myelin diseases (Non-MSMYDs) and nonmyelin neurologic diseases (Non-MYNDs) with a retrospective longitudinal follow-up, and without neurologic disease (controls). The MS patient and control samples analyzed are described in table 1. The samples were analyzed by ELISA by an investigator blinded to disease status; the diagnostic test results and disease status were determined by different team members. Clinical information was provided from a clinical database of the different patients with associated biobanks (blood and CSF samples).

Patients

Samples were obtained from the Comprehensive Longitudinal Investigation of Multiple Sclerosis (CLIMB, Boston,

MA) and EPIC (San Francisco, CA), as part of the SUMMIT consortium^{14,15}; from the Servicio de Neurología Hospital Virgen del Rocío (Sevilla, Spain); from the Servicio de Inmunología del Hospital Clínico San Carlos (Madrid, Spain); and from the Servicio de Neurología del Hospital Universitario Quirónsalud (Madrid, Spain). The classification and clinical data of all samples analyzed are summarized in table 1.

Peripheral blood samples from patients with MS (362), patients with Non-MSMYD (10), patients with Non-MYND (11), and from 80 individuals without neurologic diseases (control group) were collected. Patients were free of relapses at the time of blood sample collection. Sera samples were aliquoted and stored at -80°C until analyzed.

ELISA assay

To detect IgG and IgM antibodies reactive with LC (Matreya, State College, PA) and PC (Sigma-Aldrich, St. Louis, MO), 96-well plates were first coated with LC diluted in dimethyl sulfoxide and PC diluted in methanol at $10\ \mu\text{g}/\text{mL}$. After washing 3 times with phosphate-buffered saline, the wells were treated with blocking solution. Serum samples were diluted (1/50, 1/100, 1/200) in blocking solution and pipetted into the wells because we obtained the higher signal and a lower background. IgG or IgM antibodies were detected with anti-human IgG biotin and anti-human IgM biotin, respectively (Jackson ImmunoResearch, West Grove, PA), followed by avidin–horse peroxidase (Sigma-Aldrich). Finally, we used TMB-one (Kementec, Taastrup, Denmark) as substrate. Plates were read at 450 nm using an Infinite 200 PRO spectrophotometer (Tecan instrument, Zürich, Switzerland). We classified a serum as positive when the optic density was higher than $\text{mean} \pm 2 \times \text{SEM}$ of the control group.

Statistics

Statistical analyzes were performed with GraphPad Prism (version 6.0) and IBM SPSS 24 statistical packages. *p* Values <0.05 were considered statistically significant. We used the Mann-Whitney *U* test for comparisons between 2 groups and the Kruskal-Wallis test for multiple group comparisons. To analyze the fraction of individuals harboring lipid-reactive antibodies, we used the Fisher test for comparisons between MS and control group and the Pearson χ^2 test for comparisons among different MS types. OR and 95% CI were used to estimate the association between the presence of antibodies and the disease.

Table 1 Demographic and clinical data from patients with MS, Non-MSMYD, and Non-MYND and control group

Figure	Cohorts	Females	Age	Disease duration	EDSS score	Treatment
1, 2	Controls (80)					0 (0%)
	United States (60)	47 (78.3%)	44 (25–63)			
	Spanish (20)	17 (85%)	58 (28–88)			
	MS (362)	246 (70.7%)	48 (19–92)	13 (0–57)	3.0 (0–9)	120 (33%)
	United States (235)	173 (73.6%)	47 (19–92)	13 (0–51)	2.2 (0–9)	113 (48%)
	Spanish (127)	73 (57.5%)	49 (19–77)	15 (2–57)	4.4 (0–9)	7 (6%)
3, 4	CIS (17)	10 (58.8%)	44 (19–76)	10 (2–36)	1.9 (0–6)	0 (0%)
	RR (63)	43 (70.5%)	49 (23–92)	15 (2–56)	2.0 (0–7)	4 (6%)
	SP (50)	31 (62.0%)	59 (37–79)	22 (8–57)	6.4 (3–9)	7 (14%)
	PP (37)	18 (48.6%)	54 (31–75)	12 (4–27)	6.0 (2–9)	0 (0%)
	Benign (36)	20 (55.6%)	48 (34–61)	22 (4–42)	2.7 (0–8)	6 (17%)
	Control (80)	64 (80%)	48 (25–88)			0 (0%)
	5	Non-MSMYD (10)	4 (40%)	41 (25–59)		
	Myelitis (4)					
	Polyneuropathy (2)					
	Optic neuritis (2)					
	Autoimmune myelopathy (1)					
	Secondary myelopathy to tumor (1)					
5	Non-MYND (11)	8 (72.7%)	38 (2–78)			
	Intracranial hypertension (2)					
	Lymphocytic meningitis (1)					
	Schizophrenia (1)					
	Cerebellar syndrome (1)					
	Non-Hodgkin lymphoma (1)					
	Ataxia telangiectasia (1)					
	Cephalea (2)					
	ELA (1)					
	Meningoencephalitis (1)					

Abbreviations: CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale; IFN = interferon; Non-MSMYD = non-MS myelin disease; Non-MNYD = nonmyelin neurologic disease; PP = primary progressive; RR = relapsing-remitting; SP = secondary progressive. Females: number of cases analyzed and the percentage in the group. Age, disease duration, and EDSS score: mean (minimum and maximum). Treatment: number and percentage of treated patients. Patients in figure 1 were treated with IFN-beta (84), glatiramer acetate (24), and natalizumab (6). Treatment used for the RR patients was IFN (5); SP patients were treated with IFN (1), glatiramer acetate (3), and natalizumab (3), and benign patients were treated with IFN (3) and natalizumab (3). PP patients were not treated.

Standard protocol approvals, registrations, and patient consents

The study was approved by the Bioethics Committees of the participant hospitals. All the participants gave verbal and written informed consent for sample collection.

Data availability

The corresponding authors will provide anonymized data of this study on reasonable request from any

qualified investigator, following relevant data protection regulations.

Results

Increased serum IgM antibodies to PC in MS

We first analyzed serum IgG and IgM antibodies to PC and LC in patients with MS and controls. We detected higher IgM

reactivity against PC (IgM-PC) in MS serum samples (95% CI 0.03–0.15; 95% CI 0.04–0.17; United States and Spain cohorts, respectively) (figure 1A). Serum IgM-PC levels were not related to sex, age, or disease duration (table e-1, links. lww.com/NXI/A265). In a preliminary study, we detected lower serum IgM-PC levels in patients with MS treated with natalizumab than in those untreated (data not shown). We did not detect significant differences in serum IgM to LC (IgM-LC) nor in IgG reactivity to PC (IgG-PC) or LC (IgG-LC) between patients with MS and controls (figure 1B and not shown).

IgM-PC antibodies are associated with disease course and subtype

We then analyzed serum IgM-PC levels at different MS phases. We detected higher serum IgM-PC levels in clinically isolated syndrome (CIS) (95% CI 0.17–0.31; 95% CI 0.12–0.29), relapsing-remitting MS (RRMS) (95% CI 0.16–0.25; 95% CI 0.10–0.23), secondary progressive MS (SPMS) (95% CI 0.09–0.22; 95% CI 0.03–0.20), and primary progressive MS (PPMS) (95% CI 0.00–0.06; 95% CI 0.04–0.14) samples compared with benign MS (BENMS) or controls, respectively (figure 2A). Moreover, we detected higher serum IgM-PC antibody levels in CIS (95% CI –0.02 to 0.19; 95% CI 0.04–0.19) and RRMS (95% CI –0.01 to 0.10; 95% CI 0.02–0.13) samples than in SPMS or PPMS samples; controls harbored higher serum IgM-PC levels than BENMS (95% CI 0.00–0.07). We did not observe differences in IgM-PC serum antibodies between CIS and RRMS samples, nor between SPMS and PPMS samples. We detected increased IgM-PC levels (above percentile 50) in 23% of control, 89% of CIS, 87% of RRMS, 48% of SPMS, 59% of PPMS, and 8% of BENMS samples. We did not detect differences in IgM-LC levels when

we analyzed samples taken from different MS subtypes (figure 2B).

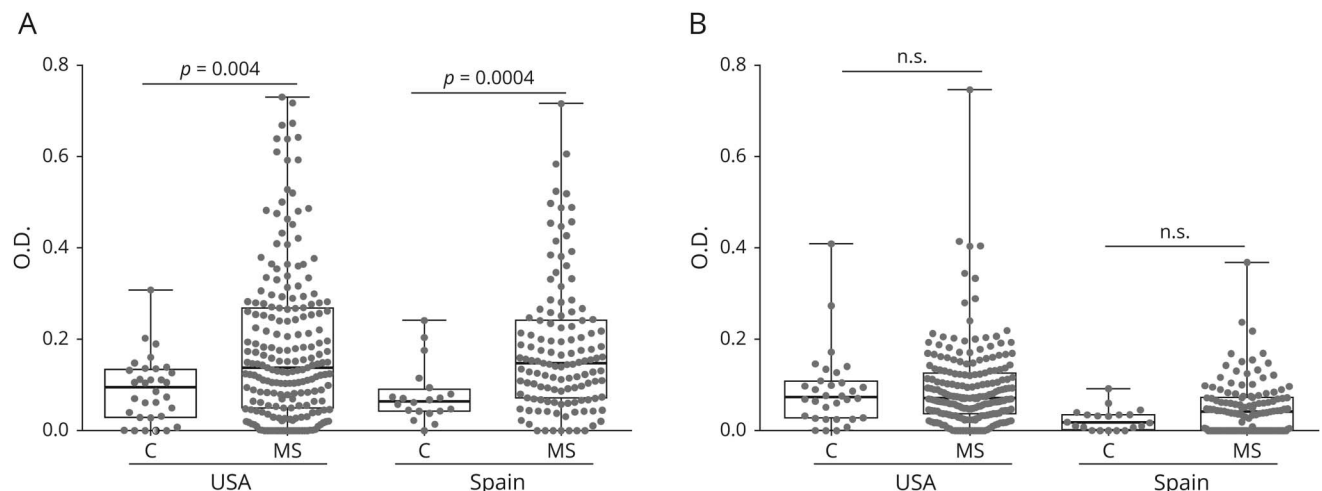
To further investigate the value of IgM-PC antibodies as biomarkers, we analyzed the fraction of IgM-PC positive samples, which was higher in the MS group than in controls (OR 4.66; 95% CI 2.40–9.07) (table 2). The fraction of IgM-PC positive samples was higher in the CIS (OR 22.50; 95% CI 4.53–111.90), RRMS (OR 24.00; 95% CI 8.78–65.63), SPMS (OR 4.14; 95% CI 1.78–9.62), and PPMS (OR 4.40; 95% CI 1.77–10.91) groups than in controls. We also observed significant differences between CIS (OR 60.00; 95% CI 9.87–364.90), RRMS (OR 64.00; 95% CI 17.38–235.60), SPMS (OR 11.05; 95% CI 1.39–36.02), or PPMS (OR 11.73; 95% CI 3.43–40.13) and BENMS. CIS (OR 5.43; 95% CI 1.12–26.34) and RRMS (OR 5.79; 95% CI 2.20–15.22) showed the highest fraction of IgM-PC compared with SPMS. We also detected significant differences in the frequency of IgM-PC positive serums between CIS (OR 5.11; 95% CI 1.02–25.72) or RRMS (OR 5.45; 95% CI 1.95–15.19) and PPMS (figure 3).

IgM-PC antibodies in other neurologic diseases

Finally, we analyzed serum IgM-PC levels in samples from patients affected by other neurologic diseases. Patients with MS exhibited higher IgM-PC levels compared with the Non-MSMYD group (95% CI 0.02–0.23); these differences were larger compared with CIS samples (95% CI 0.09–0.34); similar IgM-PC levels were detected between MS or CIS samples and Non-MNYD (figure 4A).

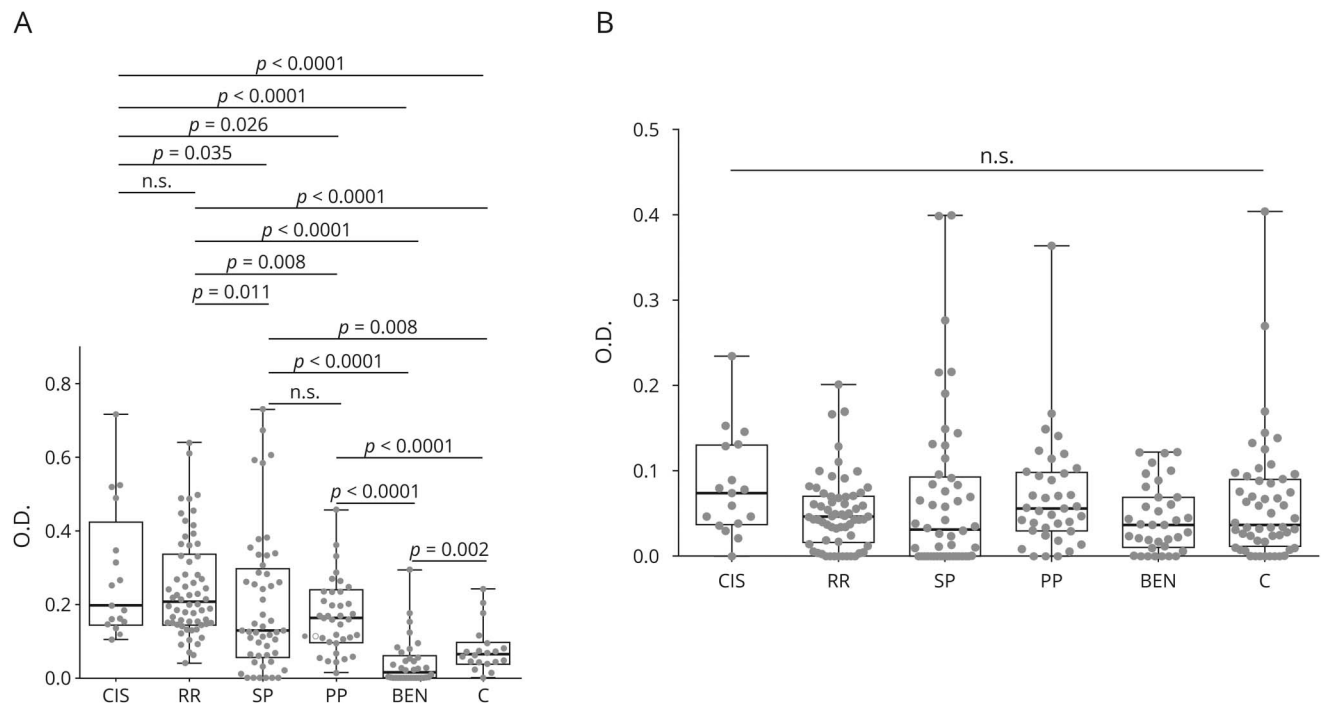
We detected IgM-PC (above percentile 50) in 30% of Non-MSMYD and 63.7% Non-MNYD samples. In addition, we

Figure 1 Serum IgM-PC and IgM-LC in patients with MS and controls



(A) Serum IgM-PC controls. Boxes represent the median of OD ± percentiles 25–75, and whiskers include 100% of the patients. Percentile 25 = 0.0559. Percentile 75 = 0.2416. (B) Serum IgM-LC. Boxes represent the median of OD ± percentiles 25–75, and whiskers include 100% of the patients. Percentile 25 = 0.0559. Percentile 75 = 0.2416. The Mann-Whitney *U* test was used to compare different groups. C = control group; IgM-LC = IgM to lactosylceramide; IgM-PC = IgM to phosphatidylcholine; NS = nonsignificant; OD = optic density.

Figure 2 IgM-PC and IgM-LC in different MS phases



(A) Serum IgM-PC. Boxes represent the median of OD \pm percentiles 25–75, and whiskers include 100% of the patients. Percentile 25 = 0.0559. Percentile 75 = 0.2416. (B) Serum IgM-LC. Boxes represent the median of OD \pm percentiles 25–75, and whiskers include 100% of the patients. Percentile 25 = 0.0559. Percentile 75 = 0.2416. The Kruskal-Wallis test was used for multiple group comparisons, and when significances were detected, Mann-Whitney *U* test to compare 2 groups. BEN = benign; C = control group; CIS = clinically isolated syndrome; IgM-LC = IgM to lactosylceramide; IgM-PC = IgM to phosphatidylcholine; NS = nonsignificant; PP = primary progressive; RR = relapsing-remitting; SP = secondary progressive.

also detected significant differences in the fraction of IgM-PC-positive MS (OR 5.179; 95% CI 1.40–19.18) or CIS (OR 25.00; 95% CI 3.52–177.60) and Non-MSMYD samples (figure 4B).

Table 2 IgM-PC and IgM-LC in patients with MS, Non-MSMYD, and Non-MYND and control group

	IgM-PC	IgM-LC	IgM-LIP
CIS	15 (88.2%)	5 (29.4%)	16 (94.1%)
RR	55 (88.7%)	4 (6.5%)	55 (88.7%)
SP	29 (58.0%)	10 (20.0%)	29 (58.0%)
PP	22 (59.5%)	6 (16.2%)	23 (62.2%)
BEN	4 (11.1%)	3 (8.3%)	7 (19.4%)
Control	13 (25.0%)	7 (13.5%)	16 (30.8%)
Non-MSMYD	3 (30.0%)		
Non-MYND	7 (63.6%)		

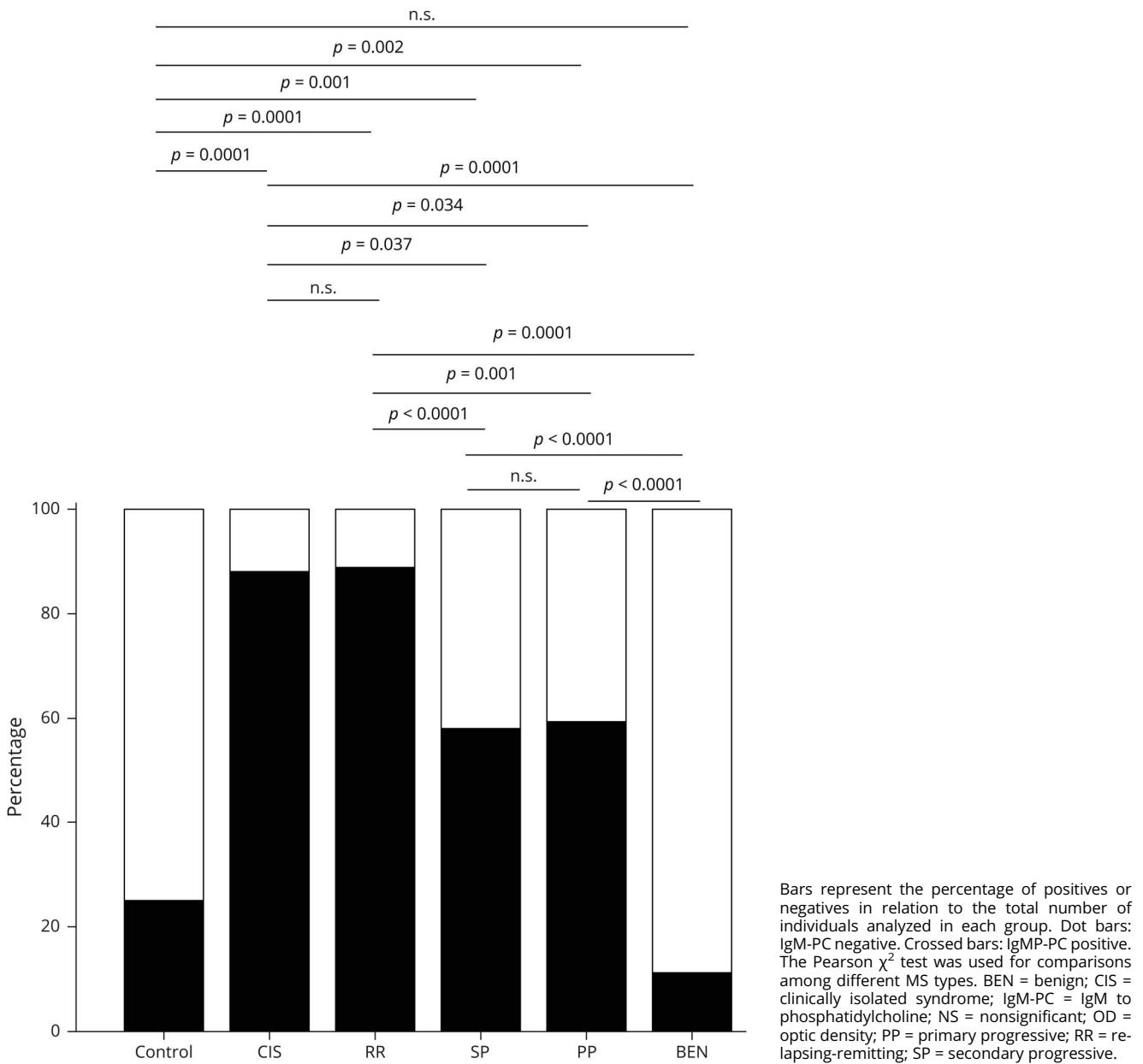
Abbreviations: BEN = benign; CIS = clinically isolated syndrome; IgM-LC = sera positive for IgM-LC; IgM-LIP = sera positive for IgM-PC or IgM-LC; IgM-PC = sera positive for IgM-PC; LC = lactosylceramide; LIP = lipids; Non-MSMYD = non-MS myelin disease; Non-MYND = nonmyelin neurologic disease; PC = phosphatidylcholine; PP = primary progressive; RR = relapsing-remitting; SP = secondary progressive.

Discussion

In this work, we report the detection by ELISA of IgM serum antibodies reactive with PC in patients with MS. Microarray-based assays detected lipid-reactive serum antibodies in patients with MS, potentially associated with different disease processes.¹³ Serum antibodies to gangliosides have been described in 13–50% of patients with MS and about 40% of patients affected by other autoimmune diseases.¹⁶ IgM antibodies reactive with cholesterol have also been detected in patients with MS, but their roles as biomarkers were not investigated.¹⁷ Our study differs from these reports in that we analyzed serum antibody reactivity directed against antigens different from those previously investigated; in particular, we focused on IgM serum antibodies reactive with PC detected by ELISA. In addition, we analyzed different disease stages and found that most of the patients in the first relapse or during the relapsing-remitting phase present IgM-PC.

Technical problems related to antigen solubility, accessibility of reactive groups, and the requirement of auxiliary lipids are linked to the detection of lipid-reactive antibodies.^{17,18} Moreover, the detection of IgG oligoclonal bands is cumbersome, among other reasons because it requires CSF collection, and the sensitivity achieved varies between laboratories, ranging from 91% to 69%.¹⁹ We detected increased serum IgM-PC in most MS patient samples, with a sensitivity comparable to that

Figure 3 Percentage of positive IgM-PC serums in MS and control group



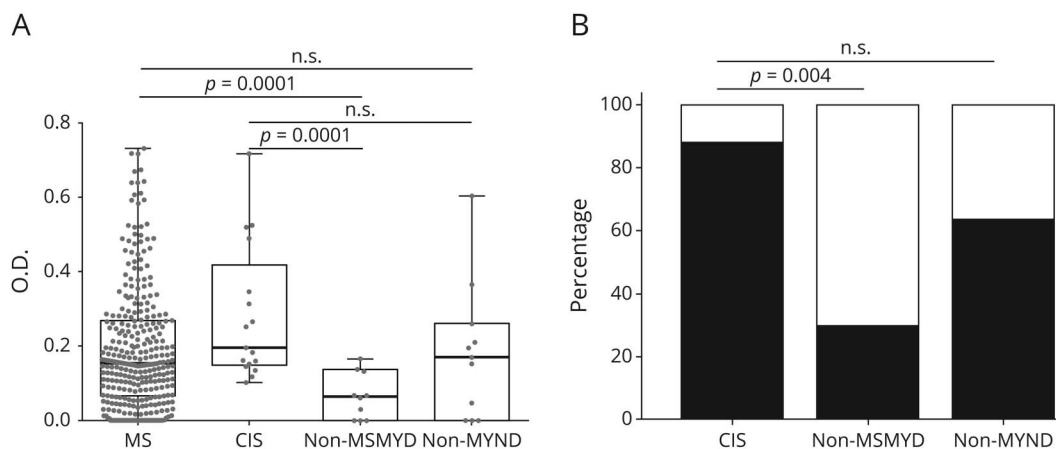
of IgG oligoclonal bands. The ELISA assay described in our article showed a sensitivity of 87.5% and a specificity of 77.2%. Although we detected IgM-PC in some control group non-myelin neurologic disease samples, these pathologies are not usually mistaken for MS during the differential diagnosis. Thus, the analysis of serum IgM-PC may complement other prognostic markers such as CSF antibodies.¹² Future studies should analyze the relevance of IgM-PC for MS pathology and diagnosis.

It should be kept in mind that other lipids may also be relevant antibody targets in MS. Moreover, as we already mentioned, we also detected serum IgM-PC in some control and Non-MSMYD and Non-MYND samples. The detection

of IgM-PC in patients with MS and also in Non-MYND samples suggests that IgM-PC antibodies are generated in the context of CNS damage. Indeed, we did not detect a high prevalence of IgM-LC antibodies, probably reflecting the fact that PC, and not LC, is one of the main components of myelin. These findings are in line with the generation of IgM reactive with PC in response to its release from damaged myelin. However, it is still unclear whether IgM-PC contributes to disease pathology.

The presence of B cells in blood reactive with brain antigens has been linked to disease relapses in MS.²⁰ Conversely, B cell-targeting therapies are efficacious in the management of MS.²¹ We detected higher serum IgM-PC

Figure 4 IgM-PC in patients with MS, Non-MSMYD, and Non-MYND and control group



(A) Serum levels of IgM-PC in MS, clinically isolated syndrome, Non-MSMYDs, and Non-MYNDs. Boxes represent the median of OD \pm percentiles 25–75, and whiskers include 100% of the patients. Percentile 25 = 0.0559. Percentile 75 = 0.2416. (B) Percentage of positive IgM-PC serums in patients with MS, Non-MSMYD, and Non-MYND. Bars represent the percentage of positives or negatives in relation to the total number of individuals analyzed in each group. Dot bars: IgM-PC negative. Crossed bars: IgM-PC positive. The Fisher test was used for comparisons between MS or CIS groups and patients with other neurologic diseases. CIS = clinically isolated syndrome; IgM-PC = IgM to phosphatidylcholine; Non-MSMYD = non-MS myelin disease; Non-MYND = nonmyelin neurologic disease; NS = nonsignificant; OD = optic density.

levels in patients with CIS and RRMS than patients with SPMS and PPMS, suggesting that serum IgM-PC reflects ongoing inflammation and myelin destruction driven by peripheral immune cells. These data are in agreement with a central role for CNS intrinsic mechanisms during the progressive phase of MS.^{22,23} Indeed, we previously reported that lipid-reactive IgM OCBs in CSF persist during disease course,¹⁰ and we also detected IgM and IgG antibodies deposited on axons and oligodendrocytes in MS CNS samples,²⁴ suggesting that these antibodies are produced locally within the CNS, probably in meningeal follicle-like structures.^{25–28} Although pathogenic activities have been assigned to some lipid-reactive antibodies,⁴ the role of IgM-PC antibodies in MS pathogenesis is still unclear, and they may simply reflect the release of target lipids as a result of myelin destruction.^{4,29–31}

PC-reactive IgMs are part of the natural antibody repertoire, which provides protection against multiple pathogens.^{32–34} Natural autoantibodies are produced in a T cell-independent manner by B-1 cells^{35,36} and usually do not undergo isotype switching to IgG.³⁷ One mechanism thought to drive the generation of natural antibodies is the ability of their target antigens to trigger signaling via the B-cell receptor (BCR) as well as additional stimulatory pathways in B cells.³⁸ Because PC is reported to modulate multiple aspects of B-cell biology,^{39,40} it is possible that PC released from myelin in the context of inflammation simultaneously activates PC-reactive B-1 cells via the BCR and additional pathways to expand preexisting natural antibody responses. Indeed, increased serum levels of the myelin components GM1 and GD1a are detected in patients with RRMS.⁴¹ Moreover, our finding of increased serum IgM-PC suggests that B-1 cell hyperactivity may also contribute to MS pathology,⁴² as

reported in other autoimmune diseases such as rheumatoid arthritis and idiopathic thrombocytopenic purpura.⁴³ Indeed, the number of B-1 cells in the CSF is linked to poor prognosis in patients with MS.¹²

In conclusion, our results identify IgM-PC antibodies as potential biomarkers for MS. Future studies should extend these observations to additional patient cohorts, analyze the effect of disease-modifying therapies on serum concentration of IgM-PC antibodies, and investigate the role of these immunoglobulins in MS pathogenesis.

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Disclosure

The authors declare no conflict of interest. Go to Neurology.org/NN for full disclosures.

Publication history

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Veit Rothhammer, MD, PhD	Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA	Performed experiments and wrote the manuscript
Úrsula Muñoz, PhD	Facultad de Medicina, Instituto de Medicina Molecular Aplicada (INMA), Universidad San Pablo-CEU, CEU Universities, Madrid, Spain	Provided unique reagents and discussed and interpreted findings
Cristina Sebal, PhD	Facultad de Medicina, Instituto de Medicina Molecular Aplicada (INMA), Universidad San Pablo-CEU, CEU Universities, Madrid, Spain	Performed experiments
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Guillermo Izquierdo, MD, PhD	Molecular Biology Service and MS Unit, University of Sevilla, Sevilla, Spain	Provided unique reagents and discussed and interpreted findings
Stephen L. Hauser, MD	Department of Neurology, University of California, San Francisco	Provided unique reagents and discussed and interpreted findings
Sergio E. Baranzini, PhD	Department of Neurology, University of California, San Francisco	Provided unique reagents and discussed and interpreted findings
Jorge R. Oksenberg, PhD	Department of Neurology, University of California, San Francisco	Provided unique reagents and discussed and interpreted findings

Appendix (continued)

Name	Location	Contribution
Roberto Álvarez-Lafuente, PhD	Instituto de Investigación Sanitaria San Carlos (IdISSC), Hospital Clínico San Carlos, Madrid, Spain	Provided unique reagents and discussed and interpreted findings
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Francisco J. Quintana, PhD	Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; The Broad Institute of Harvard and MIT, Cambridge, MA	Provided unique reagents, discussed and interpreted findings, and wrote the manuscript

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