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Association of circulating anti-CD64 IgM levels with favourable long-term clinical outcomes in multiple sclerosis patients

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ABSTRACT

Circulating levels of IgM anti-CD64, an immunosuppressive antibody recently identified in long-term stable multiple sclerosis (MS) patients, were found to fluctuate over time in MS patients. Antibody-positive patients showed a significantly lower annualized relapse rate value as well as reached sustained disability worsening and had a relapse in a significantly longer median time than those without antibody. Disease-modifying therapies (DMTs) only were the covariate influencing both the relapse occurrence and the disability accrual. Serum IgM anti-CD64 levels are associated with maintenance of clinical stability in MS and may be tested as a candidate biomarker predictive of benign course and favourable long-term response to DMTs treatment.

1. Introduction

There is wide evidence that subgroups of patients with multiple sclerosis (MS) may have a benign course (Lublin et al., 2014; Lublin and Reingold, 1996) or at least be clinically stable in a frequency ranging from 5 to 40% (Hutchinson, 1986; Pittock et al., 2004; Poser and Wikstrom, 1979). However, this favourable outcome exhausts over time in 50% of these patients after 10 years from disease onset (Hawkins and McDonnell, 1999). However, as patients showing clinical stability prior to the advent of the DMTs era as well as long-term stable patients delaying the start of DMTs exist, it is reasonable to hypothesize that any immunological factors underlying the maintenance of clinical stability status in MS might concur with this outcome and have therefore to be searched for. Recently, a monoclonal antibody (mAb) IgM to the high affinity Ig Fc-receptor I (CD64) with immunosuppressive properties has been isolated from Epstein-Barr virus$^+$ B cell clones of long-term stable relapsing-remitting (RR) and secondary progressive MS patients (Annunziata et al., 2013). Moreover, these patients also showed high levels of circulating polyclonal IgM anti-CD64 displaying the same in vitro immunosuppressive activity as the mAb. These immunosuppressive properties include inhibition of myelin antigen-driven T cell lines and non-antigen dependent T cell proliferation. Moreover, this antibody induced in monocytes up-regulation and release of interleukin-10 (IL-10), an anti-inflammatory cytokine, and down-regulation of IL-12, a powerful pro-inflammatory cytokine, and was found to bind to CD64 transfected cells showing capability to bind native CD64 (Annunziata et al., 2013). However, data on possible fluctuation of these antibodies in relation to disease activity and their capability of maintaining the clinical stability status over time are lacking. To address these issues, we searched for circulating IgM anti-CD64 over disease course in a cohort of MS patients undergoing periodical clinical and magnetic resonance imaging (MRI) examinations over a long time of observation. In this cohort, we tested whether circulating anti-CD64 IgM levels might change over time and play a role in influencing the disease activity and disability progression in association or less with a number of clinical parameters thought to be associated with benign course such as disease duration (Zivadinov et al., 2016), age at disease onset (Poser et al., 1986), prior relapse rate (Weinshenker et al., 1991), considering also the role of MRI lesion load accumulation as well as of immunomodulatory treatments.

2. Methods

2.1. Patients and clinical features

Ninety-two patients with definite RRMS according to the established criteria of McDonald et al. (2001) were consecutively recruited for this study. Baseline demographic and clinical characteristics are shown in supplementary table 1. These patients had regular periodical neurological evaluations (at least three-months) at the Clinical Neuroimmunology Unit of the Medical School of the University of Siena and underwent multiple brain and spinal cord MRI examinations at 1.5 T at

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the Neuroradiology Unit of the Medical School of the University of Siena in a time period ranging from 1998 to 2015. The MRI scans were obtained at the time of periodical radiological monitoring according to routine clinical practice (at a median time among the different scans of 18 months, interquartile range [IQR] = 12–27). All clinical features including relapses and disability assessed with EDSS (Kurtzke, 1983) were prospectively recorded and retrospectively analyzed. Brain and spinal cord MRI findings were recorded and new or enlarging T2-weighted and T1 gadolinium-enhancing lesions were counted. Clinical relapses were defined as occurrence of new neurological symptoms or signs not associated with infections or fever and lasting > 24 h. Sustained disability progression was defined as occurrence of disability worsening by at least 1 point on the EDSS assessed after the first blood sample collection and definitively confirmed in the subsequent examinations and not ameliorating during the entire clinical follow-up up to the last visit recorded within December 2015. The median follow-up time was 166 months (IQR = 96–204). All patients underwent two different blood sample collections for monitoring blood chemistry at the time of any clinical relapse prior to any treatment with high dose intravenous methylprednisolone or of clinical stability during a median follow-up time of 54 months (IQR = 31–88). Clinical stability was defined as absence of any clinically documented relapse or disability worsening by at least one year and with no new lesions at the last MRI performed within the last 3 months. As control group, 47 sex- and age-matched normal healthy subjects (NHS) blood donors at the Blood Transfusion Centre of the Siena University Medical School were included and their demographic characteristics are reported in supplementary Table 1. All serum samples were stored frozen in aliquots at −80 °C until the analysis. All patients and normal subjects gave written informed consent. The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of the Siena University Medical School.

2.2. Serum anti-CD64 antibody assay

Anti-CD64 IgG and IgM were measured by ELISA using microplates coated with CD64 222–228 peptide (synthesized by PRIMM, Italy) as previously described (Annunziata et al., 2013). In particular, serum samples were assayed at 1:500 dilution chosen on the basis of findings of a titration curve (Supplementary Fig. 1A) and in order to minimize any eventual role of different serum total IgM levels among samples. The assay specificity was established with a competitive ELISA assessing inhibition of binding of serum anti-CD64 IgM in the presence of different concentrations of CD64 222–228 peptide or scrambled CD64 peptide (Annunziata et al., 2013) (Supplementary Fig. 1B). For all patients, both serum samples were simultaneously assayed in triplicate in the same microplate and all assays were performed blindly to the clinical characteristics. Inter-assay coefficient variability (CV) (n = 10) was 8.6%, and intra-assay CV (n = 10) was 8.4%.

2.3. Statistical analysis

To establish a cut-off value distinguishing patients with anti-CD64 IgM from those without, a receiver-operator curve (ROC) analysis of absorbance values of MS patients at the first blood collection and NHS was performed. Comparison of the antibody levels and clinical variables was made with the non-parametric Mann-Whitney test for unpaired values and Wilcoxon matched-pairs signed rank test for paired values. Analysis of the frequency of clinical variables was made with Fisher's exact test. The sustained disability progression and the time to have the first clinical relapse from the first serum sample collection with respect to presence or absence of serum anti-CD64 IgM were analyzed with survival analysis using Kaplan-Meier method. To analyze the role of other covariates continuous such as age at disease onset, disease duration defined as the time from disease onset to the first blood sample collection, annualized relapse rate (ARR) prior to the first blood sampling, number of new MRI lesions/year (calculated dividing the total new lesion number by the years of the time interval from the first blood sample collection to the last recorded visit in order to minimize the possible bias effect of different follow-up times among patients) and categorical such as current immunomodulatory treatment with DMTs with respect to the antibody status, the Cox proportional hazard survival regression was used. For all statistical analyses a significance of $P < .05$ was chosen. All statistics was performed with GraphPad PRISM 7.02 software, SanDiego, CA, USA except for Cox proportional hazard regression performed with Javastat (http://statpages.org/prophaz.html).

3. Results

3.1. Serum anti-CD64 antibody assay

Based on the results of ROC analysis, a cut-off absorbance value 0.074 (sensitivity 70.6%, specificity 100%, area under the curve [AUC] 0.86, $p < .0001$) was chosen for distinguishing patients with or without serum anti-CD64 IgM (supplementary fig. 2). Sixty-four of 92 patients (69.5%) had serum anti-CD64 IgM but not IgG. In the antibody-positive patients, two subgroups showing high and low levels, respectively, (above and below median: 0.183, respectively) were identified (Fig. 1A). Of 64 antibody-positive patients, at the second blood sampling, 47 (73%) had a very significant level decline (mainly above the cut-off value) ($P < .0001$) whereas 17 (26.5%), showing lower absorbance values, remained unchanged ($P = .75$). However, in 2 of 64 antibody-positive patients (3.1%), at the second blood sampling, antibody declined below cut-off value (Fig. 1B).

3.2. Clinical characteristics and IgM anti-CD64 at the first blood sampling

Forty-seven of 92 (51%) patients underwent first blood sample collection at the time of a clinical relapse in a median time of 13 days (IQR = 6–21) and 45 of 92 (49%) at the time of clinical stability. The clinical characteristics of the MS patients with respect to the anti-CD64 IgM status at the first blood sampling, are shown in Table 1. There was no significant difference in terms of number of patients on relapse ($P = .36$) or clinical stability with respect to the antibody status ($P = .17$). There was also no significant difference between antibody levels of patients with respect to the presence or absence of relapse at the first blood sampling time: (O.D. mean ± SD) 0.294 ± 0.13 and 0.256 ± 0.12, respectively; ($P = .34$). Patients with anti-CD64 IgM had a significantly higher age at disease onset compared with those without ($P = .014$). However, there was no correlation between CD64 IgM levels at the first blood sampling, and age at disease onset ($r = 0.16$; $P = .12$) indicating no role of age in determining antibody levels. There were no significant differences with regard to the disease duration, annualized relapse rate as well as EDSS score and the number of patients on or without DMT treatment at the time of first blood sampling. With regard to DMTs, of 37 patients with anti-CD64 IgM at the first blood sampling, 17 were on interferon beta-1a (2 shifted to glatiramer acetate during the follow-up), 20 on interferon beta-1b (6 shifted to fingolimod, 3 at and 3 after the second blood sampling, 1 to dimethyl fumarate at the second sampling). However, of 16 patients without anti-CD64 IgM, 6 were on interferon beta-1a (1 shifted to glatiramer acetate at the second blood sampling), 10 on interferon beta-1b (1 shifted to glatiramer acetate).

3.3. Follow-up disease activity and IgM anti-CD64

At the end of follow-up, antibody-positive patients showed a significantly lower ARR value compared with antibody-negative subjects ($P = .0086$). A statistical trend towards a lower EDSS value in patients with anti-CD64 antibody was observed ($P = .06$). Conversely, there were no significant differences both in total new T2-weighted/ T1

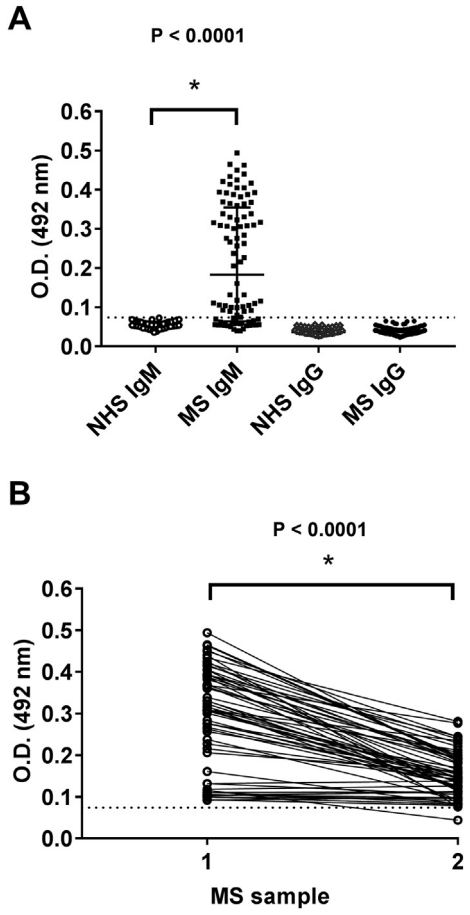

Fig. 1. Serum IgM and IgG anti-CD64 assay. (A) Serum levels of IgM and IgG anti-CD64 in multiple sclerosis (MS) patients and normal healthy subjects (NHS). The levels are expressed as optical density (O.D.) values of serum samples (1:500). Horizontal and vertical line indicate median and 75% percentile, respectively. The dotted line indicates the cut-off normal value established as described in methods.(B) Changes of anti-CD64 IgM levels in antibody-positive MS patients (n = 64) at the time of two different blood sample collections (sample 1 and 2) during clinical follow-up at a median time of 54 months. The dotted line indicates the cut-off normal value established as described in methods. * = statistical significance established with the Mann-Whitney test (A) and Wilcoxon matched-pairs signed rank test (B), respectively.

Table 1
Clinical characteristics and serum IgM anti-CD64 at the first blood sampling.

	Anti-CD64$^+$	Anti-CD64$^-$	P value
	(n = 64)	(n = 28)	
Relapse$^+$, n, (percentage)	36 (56)	11 (39)	0.36[a]
Clinical stability$^+$, n, (percentage)	28 (44)	17 (61)	0.17[a]
Age at onset, mean (SD), y	29 (8)	25 (9)	0.014[b]
Disease duration, mean (SD), y	8.2 (9)	7.8 (5)	0.35[b]
ARR, mean (SD)	0.47 (0.6)	0.39 (0.3)	0.64[b]
EDSS, mean (SD)	1.9 (0.9)	1.9 (1)	0.87[b]
DMTs treatment$^+$, n, (percentage)	39 (61)	16 (57)	0.82[a]

Abbreviations: anti-CD64$^+$ = patients with anti-CD64 IgM; anti-CD64$^-$ = patients without anti-CD64 IgM; n = number of subjects; relapse$^+$ = patients on relapse; clinical stability$^+$ = patients on clinical stability; SD = standard deviation; y = years; ARR = annualized relapse rate; EDSS = expanded disability status scale; DMTs treatment$^+$ = patients on treatment with disease-modifying therapies.
[a] Fisher's exact test.
[b] Mann-Whitney test.

Table 2
Follow-up disease activity and serum IgM anti-CD64.

	Anti-CD64$^+$	Anti-CD64$^-$	P value
	(n = 64)	(n = 28)	
ARR[a], mean (SD)	0.15 (0.18)	0.23 (0.17)	0.0086[b]
EDSS[a], mean (SD)	2.6 (2.2)	3.2 (2.4)	0.06[b]
Total new MRI lesions, mean (SD)	5.0 (6.7)	3.5 (3.4)	0.67[b]
New MRI lesions/yr, mean (SD)	0.50 (0.68)	0.45 (0.39)	0.44[b]

Abbreviations: anti-CD64$^+$ = patients with anti-CD64 IgM; anti-CD64$^-$ = patients without anti-CD64 IgM; n = number of subjects; SD = standard deviation; yr = year; ARR = annualized relapse rate; EDSS = expanded disability status scale; MRI = magnetic resonance imaging.
[a] Calculated from the time of the first blood sample collection to the end of follow-up.
[b] Mann-Whitney test.

gadolinium enhancing (GAD$^+$) lesions and new T2/T1 GAD$^+$ / year with respect to the antibody status (Table 2). However, in 2 antibody-positive patients showing antibody decline below cut-off value at the second blood sampling, a dramatic increase in disability reaching an EDSS score 6.0 and 7.0, respectively, was observed.

3.4. Time to disability progression and clinical relapse

The antibody-positive patients reached sustained disability worsening from the first blood sampling in a significantly longer median time than that of antibody-negative subjects (239 and 96 months, respectively, chi-square = 10.4, P = .0012; hazard ratio [HR] = 0.36, 95% CI, 0.17–0.79) (Fig. 2A). The antibody-positive patients also experienced a clinical relapse from the first blood sampling in a significantly longer median time compared to that of antibody-negative group (39 and 32 months, respectively, chi-square = 4.6, P = .03; HR = 0.6; 95% CI, 0.36–1.02) (Fig. 2B). The statistical significance of this difference was however weaker than that of disability and became a statistical trend (Chi-square 3.618, P = .057; HR = 0.59; 95% CI, 0.35–1.01) when performing the analysis up to 100 months of observation. There was no significant difference in the time to reach disability progression (chi-square = 0.69; P = .40) or to experience a relapse (chi-square = 1.49; P = .22) between patients with high and low antibody levels, respectively. When analyzing the role of any covariates thought to be associated with disease outcome, we observed a significant involvement of DMTs in influencing the relapse occurrence (OR = 1.03; 95% CI 1.01–1.06; P = .0007) as well as the disability progression (OR = 1.03; 95% CI 1.01–1.05; P = .0008). A statistical trend towards a significant role of disease duration in influencing the disability progression was observed (OR = 1.04; 95% CI 0.99–1.09; P = .07) (Table 3).

4. Discussion

In this study, we demonstrated that serum anti-CD64 IgM was present in the majority of a cohort of MS patients with a bi-modal expression identifying two subgroups characterized by high and low antibody levels, respectively, and independent of relapse or clinical stability. However, this antibody was unchanged in low-level patients, while in those with high levels, decreased over time reaching, even though rarely, cut-off values. This antibody recognizing the 222–228 sequence of the extracellular domain of the high affinity immunoglobulin gamma Fc-receptor (CD64), was previously isolated from a cohort of long-term stable MS patients and found to exert important in vitro immunosuppressive effects (Annunziata et al., 2013).

A number of clinical and demographic factors have been identified in the MS natural history both in the pre- and DMTs era such as gender, age at onset and disease onset symptoms (Runmarker and Andersen,

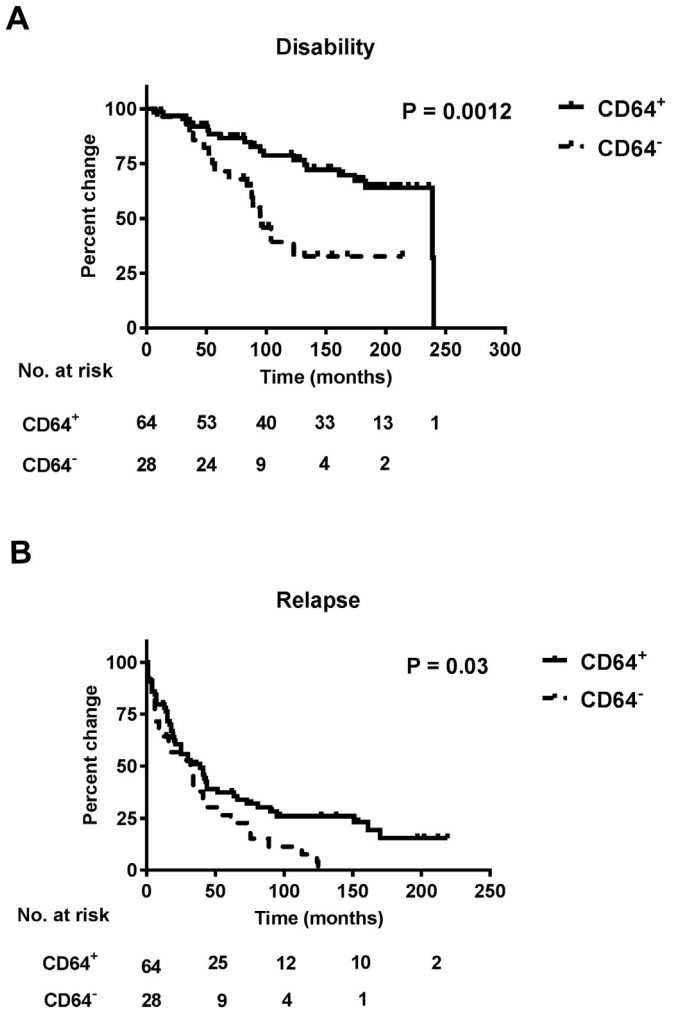

Fig. 2. Time to disability progression and clinical relapse. (A) Time to sustained disability progression from the first blood sample collection calculated with Kaplan-Meier analysis in the total cohort with respect to presence or absence of anti-CD64 IgM. (B) Time to the first clinical relapse from the first blood sample collection in the total cohort with respect to presence or absence of anti-CD64 IgM. $CD64^+$ = patients with anti-CD64 IgM; $CD64^-$ = patients without anti-CD64 IgM.

Table 3
Analysis of covariates on the time to relapse occurrence and disability progression.

Covariate	OR	95% CI	P
Relapse occurrence			
Age at onset	1.00	0.97–1.04	0.63
Disease duration	1.01	0.98–1.05	0.34
ARR	0.78	0.42–1.44	0.43
New MRI lesions/y	0.89	0.51–1.55	0.67
DMTs treatment	1.035	1.01–1.06	0.0007
Disability progression			
Age at onset	1.02	0.98–1.06	0.34
Disease duration	1.04	1.00–1.09	0.07
ARR	0.94	0.44–2.02	0.89
New MRI lesions/y	1.19	0.64–2.20	0.58
DMTs treatment	1.03	1.01–1.05	0.0008

Abbreviations: OR = odds ratio; CI = confidence intervals; ARR = annualized relapse rate prior to the time of the first blood sample collection; MRI = magnetic resonance imaging; DMTs = disease-modifying therapies; y = years.

1993; Scalfari et al., 2011) and long-term prognostic impact of DMTs as well as of vitamin D levels has also been analyzed (Cree et al., 2016; Jokubaitis et al., 2016) but, to date, evidence for the involvement of any immunological factors is lacking although some immunological prognostic indicators may derive from the mechanisms of action of the DMTs (reviewed by Damal et al., 2013). In our study, we demonstrated, for the first time, that serum levels of an immunosuppressive antibody mainly fluctuate over the disease course raising the hypothesis of a synthesizing mechanism exhausting over time with eventual loss of protective activity. The demonstration of protective activity of this antibody, even at low circulating levels, could be explained by the pentameric structure of IgM antibodies displaying high avidity to the related antigen (reviewed by Lutz, 2007). We provided evidence for a role of IgM anti-CD64 on the capability of influencing the MS disease activity in terms of reduction of relapse rate, supporting its role in the maintenance of clinical stability independent of the MRI activity in terms of number of new lesions. This apparently contradictory finding is consistent with the previous demonstration that clinical benign course in MS may be maintained despite a large T2 lesion load accumulation and presence of brain tissue loss as assessed by quantitative MRI techniques such as magnetization transfer as well as magnetic resonance spectroscopy (Strasser-Fuchs et al., 2008). However, we can't rule out that this antibody could influence other MRI parameters such as lesion volume or cortical lesions. This possibility is further suggested by the presence of monocytes/macrophages (the cell target of IgM anti-CD64) in the early active demyelinating lesions (Bruck et al., 1995; Henderson et al., 2009) contributing to the process of MS lesion formation and progression.

The relationship between relapse and disability in MS has been subject of several investigations in the last two decades with heterogeneous findings. The number of relapses in the first 5 years was found to significantly predict the disability progression over time (Confavreux et al., 2003) as well as severity of relapses was of value (Lublin et al., 2003). Moreover, other studies failed to assign to relapses a significant role in the natural disease course (Confavreux et al., 2000; Scalfari et al., 2010; Tremlett et al., 2009). Although at the early stages of MS, disability depends on inflammatory demyelination, it is known that disability accumulation over time correlates with axonal loss pathologically documented (Bjartmar et al., 2000) and is due to an early neurodegenerative process (Trapp et al., 1999). Our study has demonstrated a significant role of IgM anti-CD64 in delaying the disability progression as well as in reducing the relapse rate rather than the time to relapse occurrence over a long time of observation supporting any relationship between relapses, more related to inflammation, and disability accumulation mainly related to neurodegeneration. Of interest is the observation that in some patients of our cohort, the exhaustion of the circulating antibody was associated with a dramatic increase and irreversible acceleration of disability progression. The evidence for a role of DMTs as the main covariate in influencing the delaying of relapses as well as of disability progression is consistent with previous extension studies of pivotal trials on first-line DMTs demonstrating a long-term decrease of relapses as well as disability accrual (Ford et al., 2006); Kappos et al., 2015) and suggests a possible synergism of anti-CD64 IgM with DMTs in maintaining a favourable outcome over long time. This hypothesis is supported by the immunological activity of this antibody able to induce in vitro up-regulation of interleukin-10 (IL-10), a powerful anti-inflammatory cytokine, in monocytes and to inhibit activated T cell proliferation resembling the anti-inflammatory effects induced by first-line and oral DMTs such as interferon beta (Rudick et al., 1998; Weber et al., 1999), glatiramer acetate (Gran et al., 2000), dimethyl fumarate (Albrecht et al., 2012) and fingolimod (Blumenfeld et al., 2016). The retrospective nature of our study may be a limitation partially mitigated by the fact that the data were prospectively collected and all patients were diagnosed and constantly monitored in a single center by the same neurologists. The use of other quantitative MRI measures such as brain volume (Sanfilipo et al., 2005), atrophy

(Bermel and Bakshi, 2006) or detection of cortical lesions (Van Munster et al., 2015) and cognitive assessment (Penner, 2016) thought to better assess the clinical outcome in MS could be taken into account. In our cohort, the absence of differences in baseline clinical characteristics including relapse rate and disability except for higher age at disease onset in antibody-positive patients, that however is considered a bad prognostic factor (Poser et al., 1986), excludes an eventual original bias in the patient selection. Moreover, the presence of 40% of DMT-free patients in this cohort limits the impact of different immunomodulatory therapies in assessing the long-term clinical outcome.

We demonstrated a primary role of the circulating immunosuppressive IgM anti-CD64, in synergism with DMTs, in maintaining the clinical stability over time. However, a larger multicenter study is warranted to draw definitive conclusions on IgM anti-CD64 as a candidate biomarker predictive of benign course and favourable long-term response to treatment with DMTs in MS.

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Disclosures

P.A. reports personal fees for speaking and participation in scientific boards from Novartis, Biogen and Almirall, outside the submitted work; C.C., and G.M.declare no competing interests.

Contributorship statement

P.A designed the study, recruited and monitored the patients, contributed to the analysis of data and drafted the manuscript. G.M. monitored the patients and contributed to the analysis of data. C.C., performed the assays and contributed to the analysis of data. All authors reviewed and approved the final version of the manuscript.

Data sharing

There are no additional unpublished data related to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.03.005.

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