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Decrease of blood anti- α 1,3 Galactose Abs levels in multiple sclerosis (MS) and clinically isolated syndrome (CIS) patients[☆]



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ABSTRACT

The etiology of multiple sclerosis (MS) remains elusive. Among the possible causes, the increase of anti-Neu5Gc antibodies during EBV primo-infection of Infectious mononucleosis (IMN) may damage the integrity of the blood-brain barrier facilitating the transfer of EBV-infected B cells and anti-EBV T cell clones in the brain.

We investigated the change in titers of anti-Neu5Gc and anti- α 1,3 Galactose antibodies in 49 IMN, in 76 MS, and 73 clinically isolated syndrome (CIS) patients, as well as age/gender-matched healthy individuals.

Anti-Gal and anti-Neu5Gc are significantly increased during IMN ($p = 0.02$ and $p < 1.10^{-4}$ respectively), but not in acute CMV primo-infection. We show that, whereas there was no change in anti-Neu5Gc in MS/CIS, the two populations exhibit a significant decrease in anti-Gal (combined $p = 2.7.10^{-3}$), in contrast with patients with non-MS/CIS central nervous system pathologies.

Since anti-Gal result from an immunization against α 1,3 Gal, lacking in humans but produced in the gut, our data suggest that CIS and MS patients have an altered microbiota or an altered response to this microbiotic epitope.

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1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system of unknown etiology. Despite the fact that an elusive primary “degenerative” abnormality cannot be ruled out [1], the disease displays a clear autoimmune component at its overt clinical state [2] reinforced by evidence for genetic linkages to genes

affecting antigen presenting cells, including B cells, T cell homing or cytokine receptors [3,4]. Several viruses have been linked to MS etiology [5], particularly, the Epstein-Barr virus (EBV) shows a strong association with disease occurrence [6,7]. The potential role of EBV in initiating the autoimmune disease has been based on the presence of lymphocytes committed to EBV (or to cross reacting antigens [8]) in the CNS, EBV in leptomeningitis lesions and possible effects of EBV infection on blood-brain barrier (BBB) endothelial cells [9] and on the requirement for a past EBV infection for the disease to occur [10]. High titers of anti-EBNA1 antibodies prior to MS onset [11] or at the time of a clinically isolated syndrome [12] as well as a previous history of infectious mononucleosis (IMN) [13] or high anti-EBV titers [14] have also been shown to increase the risk of developing MS.

The relationship between MS and response to EBV has led to speculation in the context of a “Hygiene theory” of autoimmunity [15,16]. Recently, we have elaborated a working hypothesis on a new etiological

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link between an overt clinical presentation of an EBV primary infection and the occurrence of MS [17]. We suggested that the vigorous production of anti-Neu5Gc (a major component of the Paul-Bunnell-Davidsohn reaction [18]), which occurs in IMN [19,20], may represent a key factor in altering the BBB selectivity, consistent with the high frequency of circulating EBV committed T cells [21] and of EBV infected memory B cells [22,23] of the EBV primary infection. Indeed, diet-derived Neu5Gc epitope has been observed on endothelial cells (EC) from healthy individuals [24,25]. We suggested that the anti-myelin process is a secondary event, resulting from an anti-EBV immune response imported in the central nervous system (CNS) and initiating local autoimmunity.

Establishing evidence for a causal role for a peculiar immune response to IMN in MS etiology faces various difficulties. Indeed, the “remnant immunological traces” of such an initial biological signal are only assessed when clinical symptoms of the disease are identified, years after the initiating event.

We first revisited the change of anti-Neu5Gc and anti-α1,3 Galactose antibodies resulting from the lack of synthesis of these two sugars following the lost-mutation of the CMAH and GGT1 enzymes respectively [26], during IMN and show a significant increase in anti-Neu5Gc as well as in anti-Gal during this disease. In addition, we tested the levels of anti-Neu5Gc and anti-Gal in patients who developed MS or who presented with CIS (clinically isolated syndrome) or non-MS central nervous system diseases compared to age and sex matched healthy individuals. We observed no “remnant” high levels of anti-Neu5Gc in MS or CIS patients. However, compared to age- and sex-matched healthy individuals, we report for the first time that both MS and CIS patients exhibit a significant decreased level of anti-Gal IgG and discuss the potential interest of this new observation with respect to MS etiology.

2. Patients

2.1. Patients with infectious mononucleosis (IMN)

Sera from 49 patients with IMN were collected at disease onset from the University Hospital of Grenoble and Nantes between 2007 and 2014. The gender ratio was 22F/27M. The average age was 24.1 years (range: 7 to 65 years) (Table 1). IMN was serologically confirmed by the detection of VCA IgM without anti-EBNA1 IgG. EBV serostatus was determined in plasma with a DiaSorin LIAISON XL automat, using EBNA1 IgG, EBV-VCA IgG and EBV VCA IgM kits (DiaSorin, Saluggia, Italy). Four patients had multiple blood samples harvested from 7 to 820 days after IMN diagnosis. Patients and samples were coded for anonymity. Sera were taken for routine clinical diagnosis and used in conformity with legal regulations. In addition the sera of 37 patients with clinically overt CMV primo infection (gender ratio: 19 M/18F; age average: 37 years, from 16 to 68 years) were studied and compared to age- and gender-matched healthy individuals. Virological diagnosis of CMV primary infection was assessed by the detection of specific anti-CMV IgM associated or not with specific IgG (DiaSorin LIAISON XL). In the case of the detection of IgG, antibody levels and avidity were very low (avidity < 5%).

For each cohort of this study, samples from normal individuals, matched for age (± 3 years) and gender for each patient, were obtained from the regional blood bank (Rennes) and from Nantes University Hospital (in conformity with regulatory and ethical requirements). When indicated, the sera of EBV negative individuals, with 3 negative tests, were tested for anti-Glycan antibodies.

2.2. Patients with multiple sclerosis (MS) or clinically isolated syndrome (CIS)

A first cohort of 55 patients with MS (including 9 from twins) diagnosed at the Nantes and Toulouse University Hospitals was included in the study (Table 1). For the 46 non-twin patients, the gender ratio was 30F/16M and the average age of 47.6 years (range: 22 to 73). Thirty

Table 1 Summary of all cohorts of patients and of corresponding control individuals tested in the study. Table summarizes the different cohorts of patients tested for anti-Neu5Gc, anti-Gal and anti-EBNA1 in this study. IMN (infectious mononucleosis) patients (n = 49) were compared to healthy age/sex matched controls (n = 49). The global MS cohort includes 76 patients. The MS patient cohort was composed of a “classical” MS population (n = 46), MS twins (n = 9), pregnant MS patients in pre- (n = 21) and post-partum (n = 21), including 7 post-partum relapses. The CIS cohort included 73 patients also compared to 73 age/sex matched healthy controls. The mean age at blood harvesting time (in years), age range of cohort (minimal age/maximal age in years), sex ratio (female/male), MS status (RR for relapsing remitting, PP for primary progressive, SP for secondary progressive), MS relapses and treatments were noted.

Groups	IMN		MS		Twins		Twins		Pregnant/Post-partum		Pregnant		Non pregnant		Global		CIS		
	IMN	Matched controls	MS	MS	MS	MS	Non-MS	MS	MS	MS	Matched controls	Matched controls	Matched controls	Matched controls	MS	MS	Matched controls	Matched controls	
N	49	49	46	46	9	9	9	21 (x2)	42	21	42	21	76	73	76	73	73	73	
Age	24.1	24.1	47.6	47.5	49.7	49.7	49.7	32.1	31	32.1	31	32.1	43.2	42	43.2	42	41.5	41.5	
Age range	Jul-65	Jul-65	22/73	22/77	22/69	22/69	22/69	26/37	25/40	26/38	25/40	26/38	22/73	22/78	22/73	22/78	22/77	22/77	
Sexratio (F/M)	22/27	21/28	30/16	29/17	9F	9F	9F	21F	42F	21F	42F	21F	60/16	56/17	60/16	56/17	57/16	57/16	
MS status: RR			30																
PP/SP			16																
MS relapses			4																
Treatments			19 w/o TTT 11 IFNs 1 Glatiramer Ac 7 Natalizumab/Fingolimod 7 Mycophenolate Mofetil 1 Prenisone				4 w/o TTT 3 IFNs 1 Cortancyl 1 Rivotril												No TTT

Abbreviations: IMN: infectious mononucleosis; MS: Multiple sclerosis; CIS: clinically isolated syndrome; TTT: treatments; RR: relapsing remitting; PP: primary progressive; SP: secondary progressive; IFN: interferons; CS: corticosteroids.

patients presented a remitting relapsing (RR) form (63.04%) and 16 presented a secondary or primary progressive (SP/PP) form (34.78%) of the disease. Patients received either no therapy ($n = 19$, 41.3%) or IFNs ($n = 11$, 23.9%), Glatiramer Acetate ($n = 1$, 2.17%), Natalizumab or Fingolimod ($n = 7$, 15.21%), Mycophenolate Mofetil ($n = 7$, 15.21%) or Prednisone ($n = 1$, 2.17%). The 9 patients from the twin pairs, with one patient of the pair having MS, were diagnosed at the University Hospital of Toulouse, the average age was 49.7 years (from 22 to 69), and all were monozygotic females (Table 1).

A second cohort consisted of 21 pregnant patients with MS obtained from the Lyon University Hospital. The average age was of 31.4 years (range 25–37) (Table 1). A total of 42 blood samples, stored at the Neurobiotec bank (biological resource center of the Lyon University Hospital) was obtained from these women, with one sample obtained during pregnancy and another one post-partum. Seven patients presented a post-partum relapse (from 3 weeks to 7 months post-partum). Pregnant women had taken no drugs, and post-partum medications are given in Table 1. Sera from women with MS were compared to samples from a specific control group of healthy pregnant women, matched for age and also for the trimester of pregnancy or post-partum at harvesting ($n = 42$, ratio 1/1). In addition, the samples of healthy pregnant women were compared to samples of healthy non pregnant women ($n = 21$) obtained from the Pediatric Department of Nantes University Hospital and blood bank of Rennes, respectively. Altogether, the MS cohort was composed of 76 patients (age mean: 43.2 years, from 22 to 73 years).

The last cohort consisted of 73 patients presenting with a clinically isolated syndrome (CIS) from the Université Catholique de Louvain (UCL). CIS was defined as a first mono- or multifocal neurological deficit lasting for >24 h, not associated with fever or infection, and compatible with a first presentation of MS [27,28]. The gender ratio was 56F/17M, and the average age was 42 ± 1.2 years (range: 22 to 78). None of the CIS patients were under treatment at the time of blood harvesting.

Finally, anti-Gal levels were also tested in a last cohort of 34 patients with non-MS/CIS pathologies of the central nervous system (CNS) (gender ratio: 11 M/23F; age average: 50, from 23 to 86 years) according to the classification of Teunissen et al. [29] and compared to age and sex-matched normal individuals (see Supplementary Fig.4 and Supplementary Table 1).

2.3. Age and sex paired controls, sampling and ethics

To decrease experimental variables as much as possible, anonymous test tubes were coded and dispatched by a third party who was unaware of the study to allow an appropriate division of controls and experimental samples for serum testing. All ELISAs were thus carried out blind. Each sample from MS/CIS, IMN, CMV primo-infection or non-MS/CIS central nervous system diseases was compared to a sample from a normal individual paired for age and gender. All patients and controls signed an informed consent for the experiments.

3. Materials and methods

3.1. Anti-Neu5Gc antibodies

Anti-Neu5Gc was quantified by an ELISA using mouse serum proteins as coating antigens according to [30]. Briefly, the plates were coated overnight at 4 °C with wild-type mouse serum at a concentration of 1 µg/well diluted in 50 µl of coating buffer (50 mM sodium carbonate-bicarbonate buffer (pH 9)). Diluted human sera were pre-incubated for two hours at 4 °C with CMAH-KO mouse serum diluted 1:4000 in PBSTO (PBS 1 × - 0.05% Tween 20–1% Ovalbumin; Sigma Aldrich). After washing and blocking, pre-treated human sera were added to the plates for two hours at room temperature (RT). IgG/Ms. were detected by HRP-goat anti-human IgGs or PO-rabbit anti-Human IgMs (Dako). Then, TMB (Sigma Aldrich) was added, and the reaction was stopped by H₂SO₄. Optical density was read at 450 nm (630 nm as reference) using an MRX

spectrophotometer (Dynatech laboratories, El Paso, US). Serial dilutions of IVIg (Privigen) or purified human IgM (Sigma) at concentrations starting at 800 or 400 ng/ml, respectively, served as standards.

3.2. Anti-α1,3 Gal antibodies

The ELISA was adapted from Buonomano et al. [31]. Plates were coated with Gal1–3Gal-polyacrylamide conjugate (5 µg/ml, PAA-Bdi; Lectinya, Moscow, Russia) overnight at 4 °C, and then blocked with PBS 0.5% fish gelatin (Sigma-Aldrich) for two hours at 37 °C. Human sera (at 1:1000, 1:2000 and 1:4000 in PBST) were incubated for two hours at 37 °C. According to the quantity of sera available, a rabbit anti-human IgG/M followed by a HRP-goat anti-rabbit antibody (1:2000 and 1:2000, respectively; Jackson ImmunoResearch) or a HRP-donkey anti-human IgG (1:2000; Jackson ImmunoResearch) were used as secondary antibodies. A titration curve used serial dilutions of human IVIg (Privigen) at a first concentration of 200 ng/ml. For anti-Gal IgM ELISA, extensive washing with PBS - 0.1% Tween20 was performed between each step, and we developed the assay using HRP-F(ab')₂-anti-human IgM (1:1000; Jackson ImmunoResearch). Serial dilutions of purified IgM at concentrations starting at 800 ng/ml served as standards.

3.3. Statistical analysis

3.3.1. Metrology

Each ELISA was tested for reproducibility in duplicates in a panel of 90 samples. To assess the accuracy of the measurements, we computed an intra-class correlation coefficient (ICC) and tested its statistical significance (H₀: ICC = 0.5; H₁: ICC > 0.5) using an F-test. Both anti-Neu5Gc IgG and anti-Gal IgG or IgG/M tests were highly accurate (ICC = 0.69 $p = 0.014$ and ICC = 0.83 $p < 0.001$, respectively). In contrast, the anti-Neu5Gc IgM test had low reproducibility and was further excluded from the analysis (ICC = 0.32 $p = 0.93$).

3.3.2. Statistics

For each antibody, the statistical significance of the comparisons were assessed using non-parametric matched pairs-Wilcoxon tests to account for any possible effect of age or gender on the observed traits (IMN and MS/CIS are age-dependent traits and gender is a well-established dimorphism in MS). Neu5gc sensitization may be associated with age as observed in the controls ($r = 0.14$, $p = 0.048$). Statistical analyses were performed using Graphpad, Stata 13 SE and R statistical software.

4. Results

4.1. Anti-Neu5Gc and anti-Gal antibodies in a normal population

Since MS has a strong bias for gender and age, our initial design predicted a possible similar bias in the anti-Neu5Gc IgG, without any published reference values in the literature and with a trend for an effect of age in normal individuals. The option of comparing patients with age/gender matched normal individuals was thus retained. However, although the analyses were performed using age/gender matching values, the various patient cohorts (including IMN, MS and CIS) tested did not show a correlation with age/gender. Only healthy individuals showed a trend for a decrease of antibodies with age ($p = 0.048$). Fig. 1 shows the distribution values of the antibodies in the healthy individuals ($n = 120$). The age average was 40.65 (range from 20 to 80 years). Means \pm SEM of anti-Neu5Gc IgG were 2.7 ± 0.3 ng/ul and 44.8 ± 8.1 ng/ul for anti-Gal IgG. All normal individuals were found to be positive for anti-Neu5Gc IgG, consistent with a previous report [25], and only 8% were negative for anti-Gal IgG.

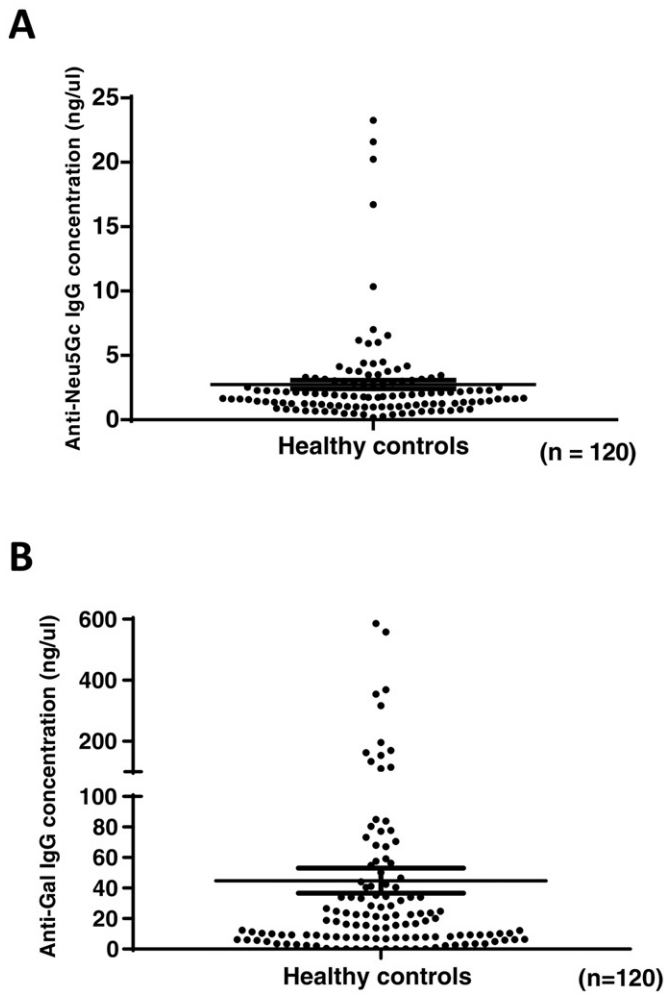


Fig. 1. Anti-Neu5Gc and anti-Gal IgGs levels in normal individuals. The results are expressed in ng/ul as individual plots with median and interquartile range for anti-Neu5Gc IgG (a) and anti-Gal IgG (b) (n = 120).

4.2. Elevated anti-Neu5Gc and anti-Gal in infectious mononucleosis

Anti-Neu5Gc and anti-Gal were first tested in a population of 49 subjects with IMN. Fig. 2A shows that there was a significant increase in anti-Neu5Gc IgG in IMN patients (3.3 ± 0.4 vs 1.8 ± 0.2 ng/ul for controls, a 1.8-fold increase, $p = 0.02$), consistent with studies suggesting that the “anti-heterophile” antibodies of the Paul-Bunnell-Davidsohn reaction were mainly anti-Neu5Gc Abs [19,20]. However, we also measured the levels of anti-Gal, initially chosen as a specificity control using another type of natural xenoantibody also directed against a sugar not synthesized by normal individuals [32]. A highly significant increase in anti-Gal titers was however also observed during IMN (Fig. 2B: 10.4 ± 0.7 vs 4.2 ± 0.7 ng/ul for controls, a 2.4-fold increase, $p < 0.0001$) ruling out a specific response against Neu5Gc in IMN disease and suggesting a non-specific response. Supplementary Fig. 1 shows examples of the length of exposure to abnormal anti-Neu5Gc and anti-Gal titers after the first blood sample following IMN diagnostic, suggesting variable time intervals before reverting to normal values for the two types of antibodies. However, this time interval was usually restricted to a month, with the exception of one patient exposed to high levels of anti-Neu5Gc for approximately 25 months after the disease. In contrast, there was no significant change for anti-Gal and anti-Neu5Gc in patients with clinically overt CMV primo-infection (n = 34) (Supplementary Fig. 3).

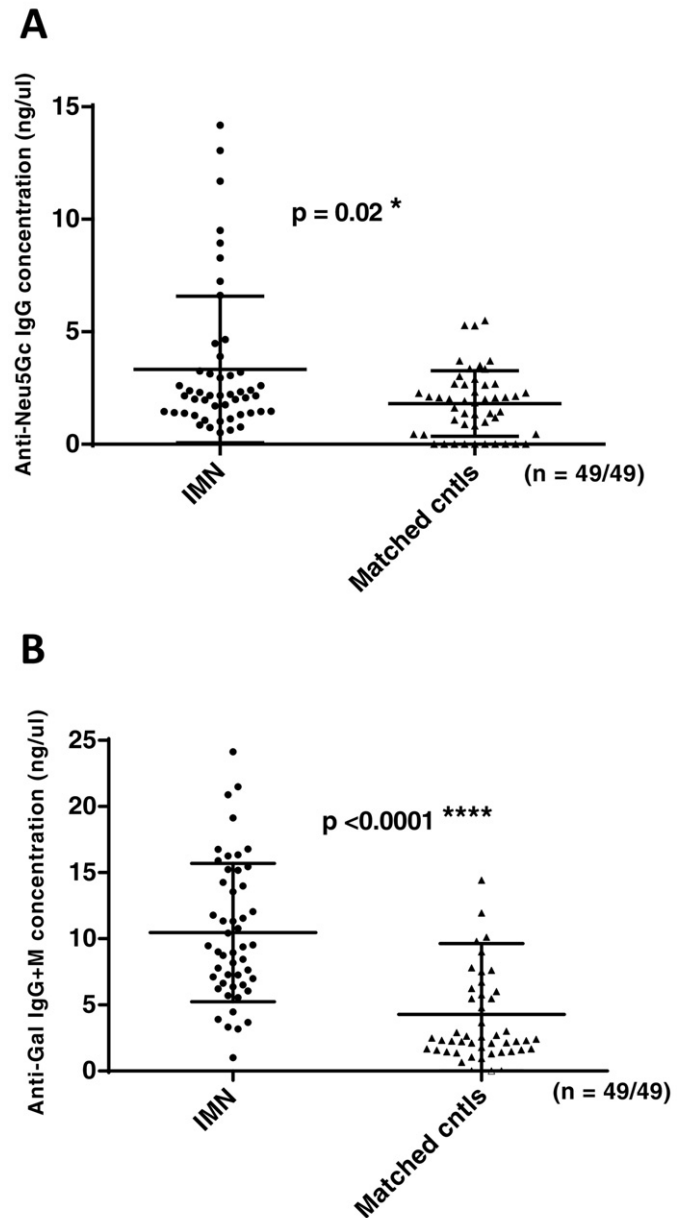


Fig. 2. Anti-Neu5Gc and anti-Gal levels in IMN patients. The results are expressed in ng/ul as individual plots with median and interquartile range for each group, for anti-Neu5Gc IgG (a) and anti-Gal IgG/M (b) (n = 49). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ using a Wilcoxon non-parametric paired test.

4.3. Lower anti-Gal, with unmodified anti-Neu5Gc in patients with MS

Since IMN has been documented as a risk factor for MS [11] and characterized by an overproduction of anti-Neu5Gc [17,24,25], anti-Neu5Gc were also measured in patients with MS and several MS patient sub-groups: a small cohort of twin patients who did or did not present with the disease (n = 9), a group of RR (n = 30), of SP/PP form of MS (n = 16), and a group of women (n = 21) during gestation and in the post-partum period who did (n = 7) or did not present with a disease relapse in the months following delivery. No significant difference between the levels of anti-Neu5Gc of the MS patients compared to matched healthy controls was noted when all the MS patients (Fig. 3A: 2.9 ± 0.5 vs 2.3 ± 0.2 ng/ul; $p = 0.71$) or the sub-groups was observed (data not shown).

However, we observed a significant drop in the mean anti-Gal IgG levels (29.2%) in the MS patients compared to their matched controls (Fig. 3B: 9.2 ± 1.2 vs 13 ± 1.6 ng/ul for controls; $p = 0.03$). No significant

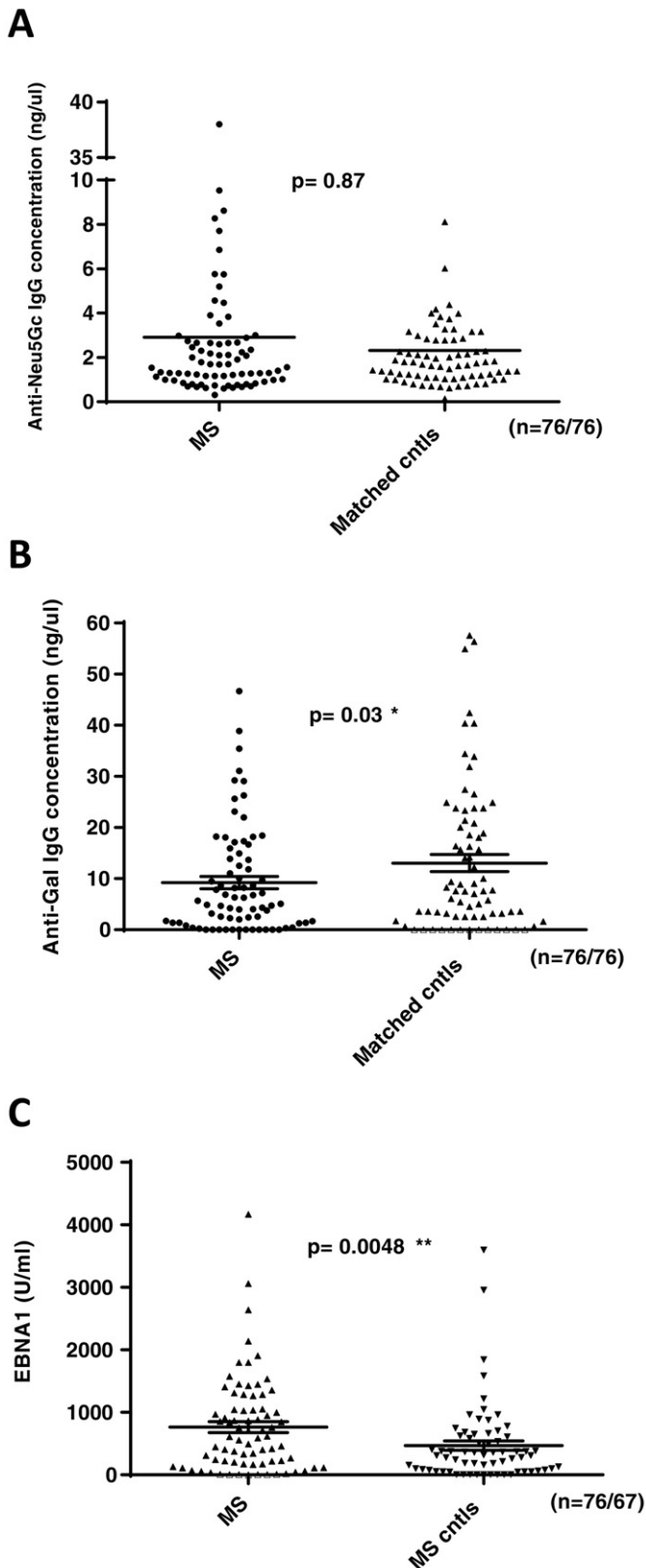


Fig. 3. Anti-Neu5Gc and anti-Gal IgGs levels in a global MS cohort. The results are expressed in ng/ul as individual plots with median and interquartile range for each group, for anti-Neu5Gc IgG (a) and anti-Gal IgG (b) ($n = 76/76$) and in U/ml for anti-EBNA1 (c) ($n = 76/67$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using a Wilcoxon non-parametric paired test for anti-Neu5Gc and -Gal IgGs and a Mann Whitney non-parametric unpaired test for anti-EBNA1.

results were found when the MS population was divided into the different subgroups, including comparisons between MS patients undergoing treatment or without treatment (Supplementary Fig. 2). This significant decrease in anti-Gal was not observed in patients with non-MS central nervous system pathologies (Supplementary Fig. 4).

4.4. Lower anti-Gal IgG with unmodified anti-Neu5Gc in clinically isolated syndrome

Anti-Neu5Gc and anti-Gal levels were also tested in a cohort of 73 patients with CIS tested independently after the MS cohort. As for overt MS disease, we found no difference in the anti-Neu5Gc IgG levels between CIS and the matched normal individuals (Fig. 4A; $p = 0.87$). However, we also observed a significant decrease in the levels of the anti-Gal IgG (Fig. 4B: 44.8 ± 7.1 vs 64.7 ± 13.4 ng/ul; a 30.7% decrease; $p = 0.02$). Taken together, our data on CIS provides additional support for the new observation that patients with MS have a decrease in their anti-Gal IgG levels (combination of the two cohorts vs. matched controls: $p = 0.0027$).

4.5. Increased anti-EBNA1 titers in MS and CIS patients

Finally, we also observed a highly significant increase in anti-EBNA1 titers both in MS (Fig. 3C: 763.3 ± 87.6 vs 468.7 ± 76.8 ; $p = 0.0048$) and CIS (Fig. 4C: 667.4 ± 60 vs 333.5 ± 51 ; $p = 0.0003$) compared to matched normal individual controls. No significant correlation between the anti-EBNA1 levels and levels of the various antibodies tested was observed.

5. Discussion

Elevated titers of so called “heterophile” antibodies against bovine or horse red blood cells during IMN have been reported several decades ago, an observation that has been the basis of the Paul-Bunnell-Davisohn test in IMN [20]. Biochemical analyses have also suggested that a substantial fraction of these “heterophile” antibodies was directed against the Neu5Gc [19,33]. To our knowledge, our paper is the first to revisit this assumption in a cohort of IMN compared to normal gender/age matched individuals using modern tools for dosing anti-Neu5Gc, here based on their binding to wild mouse sera after their pre-absorption on CMHA KO ones [30]. Our study shows that the anti-Neu5Gc IgG titers were significantly different from gender/age control individuals. However, the reasons for the elevated production of anti-Neu5Gc antibodies in IMN remain speculative. As EVB does not contain the CMAH sequence and does not produce Neu5Gc, the EBV infection of endothelial and epithelial cells loaded with Neu5Gc from a dietary origin [34] may favor the immune presentation of Neu5Gc positive self molecules to B cells, inducing a more vigorous response to the diet absorbed Neu5Gc epitopes than under basal conditions. Patients with influenza vaccination or influenza can increase their anti-HLA titles [35–37].

However, our data show that there is no significant increase of anti-Gal and anti-Neu5Gc in the acute CMV disease. These data are in agreement with reports indicating that the rise in “heterophile” antibodies (anti-horse and anti-bovine red blood cell antibodies) is mostly associated with IMN [19]. They also suggest that the rise of anti-Gal and anti-Neu5Gc cannot be explained by inflammation only. As EBV also infects B cells during IMN [23], we suggest that the very high proportion of B-cells infected by EBV during IMN provides a better rationale to explain the rise of anti-Neu5Gc and anti-Gal in this disease, by comparison with acute CMV primo-infection. Indeed, it is likely that the B cells progenitors with a BCR committed to the recognition of these two antigens (about 1% of IgG and several % of IgM in human blood are directed against Gal and Neu5Gc) are concerned by the acute EBV infection and produce more of the corresponding antibodies.

Altogether, the elevation of anti-Gal IgG and anti-Neu5Gc during IMN are thus likely due to the same factor, because of the loss-mutation of the

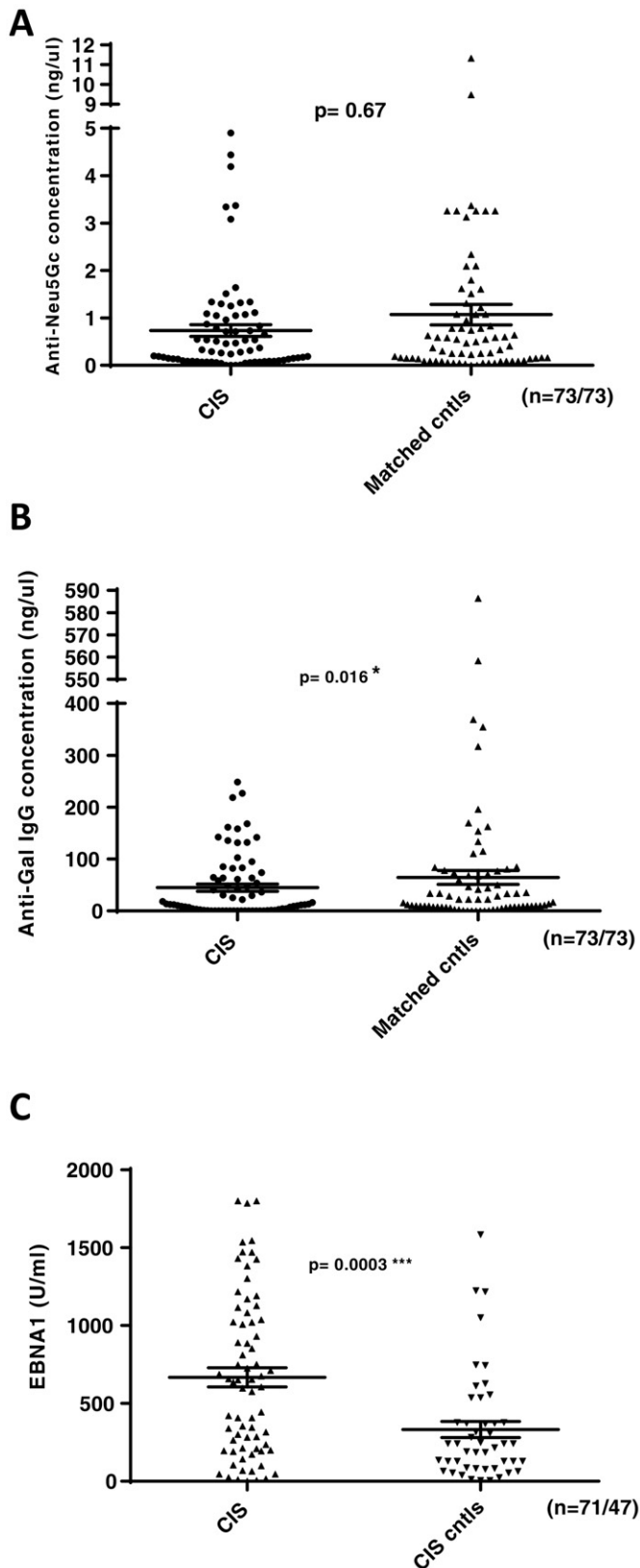


Fig. 4. Anti-Neu5Gc and anti-Gal IgGs levels in the CIS cohort. The results are expressed in ng/ul as individual plots with median and interquartile range for each group, for anti-Neu5Gc IgG (a) and anti-Gal IgG (b) ($n = 73/73$) and in U/ml for anti-EBNA1 (c) ($n = 71/47$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using a Wilcoxon non-parametric paired test for IgGs and a Mann Whitney non-parametric unpaired test for anti-EBNA1.

gene controlling their synthesis [34,38,39]. Since 1,3 Gal antigen, in contrast to Neu5Gc, is not derived from the diet and does not accumulate in human ECs, we favor again that the elevated antibodies titers against both epitopes are the result of an increase of the antibody pool in the context of a strong percentage of EBV-infected B cells affecting both anti-Gal and anti-Neu5Gc progenitors.

Nevertheless, this first set of data suggests that anti-Neu5Gc increase during the IMN disease may enhance the transfer of anti-EBV committed T cells and EBV infected B cells in the brain following endothelial cell activation during IMN [24,25] possibly favoring an autoimmunity process [17].

The second aim of the study was to explore whether patients having developed a clinically definite MS exhibit elevated anti-Neu5Gc compared to age and gender matched normal individuals after having experienced an EBV infection (as it occurs for anti-EBNA antibodies for instances). There are obvious limitations associated with trying to speculate on a contribution of IMN to MS etiology by analyzing patients at the time of diagnosis of an overt autoimmune disease. Indeed, subclinical disease activity may have begun long time before. However, a correlation between clinical presentation of the EBV primo-infection with IMN and an elevated response against EBV antigens has been correlated to the occurrence of MS [11], and anti-EBNA1 antibodies were also significantly elevated in MS patients compared to age/gender matched controls in our study. The seminal data of Ascherio et al. [11] have launched the hypothesis that an acute EBV infection may contribute to triggering the disease in genetically susceptible individuals and in the context of “favorable” geographic and social ecology [17]. Unfortunately, we could only study an anecdotal number of patients for whom several sera post IMN were available, and a limitation of our study is the absence of long term survey of anti-Neu5Gc titers and possible exposure to these antibodies. Large data has fueled several reviews with pro or con rationale for considering the role of an “abnormal” immune response against EBV in MS [40]. The present work failed to show that anti-Neu5Gc was, as for anti-EBNA1, still significantly elevated at the time of MS disease diagnosis, that is, years after the IMN. This was also not observed when an additional cohort of 73 patients with CIS was tested. Nevertheless, if we consider that the early increase of the antibodies tested is due to EBV acute infection of progenitors of anti-Gal and anti-Neu5Gc, a slow decrease of the antibodies against Gal and Neu5Gc can be expected when the acute disease terminates. In contrast, antibodies which occur in the context of *persisting viral antigens* (anti-EBNA1) are expected to remain elevated.

An important, but unexpected, finding of this study was however the significant decrease of anti-Gal titers, which were significantly lower in the MS patients compared to the age/gender matched controls. Furthermore, this decrease was also observed in the cohort of CIS patients but absent in the serum of patients presenting non-MS pathologies of the central nervous system (Supplementary Fig.4) tested independently, suggesting a potential link between low anti-Gal and MS etiology. Anti-Gal antibodies appear in almost all individuals within the first months of life following immunization by $\alpha 1,3$ -Gal produced by the microbiota and can represent several percent of circulating Ig [38]. In addition to being involved in hyper-acute xenograft rejection, IgE anti-Gal antibodies have been responsible for anaphylaxis [41]. Tick-induced Th2 bias of the immune response has also been shown to induce a red meat food allergy related to anti-Gal IgE [42]. Elevated anti-Gal has been reported in falciparum disease due to Gal expression by the parasite [43] as well as in other chronic infections such as Leishmania [44,45] and Yersinia [46]. Anti-Gal may be beneficial for the protection against some infectious agents, either Gal⁺ [43] or embedded in Gal-coated viral buds [47], owing that the evolution pressure would have censured that all humans exhibit the loss-mutation GT1 phenotype. Abnormal levels of the anti-gal antibody have been reported in autoimmune diseases [48] [49]. Decrease levels of anti-Gal antibodies is a much rare event [50]. Auto-antibodies that bind to cytokine receptors and inhibit the binding of the cytokine to its receptor in vitro have been also

shown belong to anti-Gal antibodies which can cross-react with an α -galactosyl like moiety of these receptors [51]. The biological effects of this cross-reactivity for sugar determinants of these receptors to modulate inflammation have remained elusive. In contrast to anti-Gal, the unmodified anti-Neu5Gc does not evoke an unspecific altered immune response against highly immunogenic sugar epitopes [26], which is not synthesized by the gut microbiota [39], and a non-specific down-regulation of the antibody responses on the long range in the disease. Rather, our observation suggests specific mechanisms for an anti-Gal decrease, such as a modified gut microbiota in MS patients, more specifically affecting microorganisms producing α 1,3-Gal. So, low α 1,3-Gal-producing gut microorganisms can modify the global inflammation homeostasis of individuals who are genetically predisposed to develop MS. Drastic modification of microbiota affects the susceptibility to autoimmunity, including EAE, in experimental animal models [52–55]. Recent reports have shown abnormal bacterial microbiota in the stools of patients with MS [56,57]. Although no specific information on the amount of GGT1 positive bacteria is available, one of these studies reports a global decrease of the global gut microbiota magnitude in MS patients compared to matched controls (48). Another study reports a striking depletion of 19 species among the 21 studied in MS samples [58]. These changes that may be a basis for a new etiological factor of the disease. We hope that our observation, which may offer a concrete support to speculation linking gut microbiota and MS, will spur further work on anti-Gal in MS and CIS patients, as well as in other autoimmune diseases.

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Conflict of interest

None of the authors has any potential financial conflict of interest related to the manuscript.

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