The association between IgG and IgM antibodies against cardiolipin, β2-glycoprotein I and Domain I of β2-glycoprotein I with disease profile in patients with multiple sclerosis

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Abstract

Antiphospholipid antibodies (aPL) occur in patients with multiple sclerosis (MS) with a number of studies reporting elevated levels; their exact prevalence and pathogenic role remain unclear. Epidemiological studies associate MS with an increased risk of deep venous thromboembolism and stroke; overlapping clinical features with APS. Antibodies against the first domain - Domain I (DI) - of β2glycoprotein I (β2GPI), show the most clinical significance and evidence for pathogenicity in the antiphospholipid syndrome (APS), but have not yet been investigated in MS. Serum from a well-defined cohort of 127 MS patients and 92 healthy controls were tested for IgM and IgG antibodies against cardiolipin (CL), β2GPI and DI. Higher frequency of IgM and IgG anti-CL were found in MS patients (18.1% and 21.3%), compared to controls (1.1% in both cases, $p<0.0001$). We report that anti-DI antibodies were associated with MS patients, with 6.3% and 7.1% positive for IgM and IgG, respectively, compared to controls, 1.1% ($p<0.05$). IgM anti-CL antibodies were elevated in secondary progressive MS and primary progressive MS compared to relapse-remitting MS, ($p<0.005$). This study enrolled the largest number of patients with definite MS for studying the association with aPL. Although we confirmed IgM and IgG anti-CL antibodies occur in patients with MS, this is the first study that identified anti-DI antibodies in MS patients. This new finding may prove valuable and future studies are required to evaluate its role as a potential risk factor of thromboembolic phenomena in MS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of unknown etiology, in which chronic inflammation drives multifocal demyelination of axons in the central nervous system (CNS) [1]. The pathological course of the disease is heterogeneous and involves an early, predominantly inflammatory demyelinating disease phase of relapsing-remitting MS (RRMS), which over a variable period of time, develops into a progressively degenerative stage associated with axonal destruction and scar formation, causing physical and cognitive disability [2].

Autoantibodies play an important role in MS pathogenesis [3-5]. During the last decade, much effort has been made to characterize the autoantibodies present in MS patients in order to find early predictors for diagnosis and disease progression [6]. Despite intense research, due to the overlap in autoantibody profiles in autoimmune diseases and to the complexity of MS, there is no known MS-associated antibody specificity that can individually discriminate between MS patients and controls [7, 8]. Antiphospholipid antibodies (aPL) are amongst the most commonly investigated autoreactive antibodies in MS [4, 9]. Autoimmune disorders such as the antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) are associated with persistently positive aPL and recurrent venous or arterial thrombosis and/or fetal loss. aPL are known to react not only with phospholipid alone but with phospholipid binding proteins, β2glycoprotein I (β2GPI) being the most important target autoantigen. In addition, the IgG isotype appears to be more closely associated with clinical manifestations than either the IgM or IgA isotype. Anti-β2GPI antibodies form a rather heterogeneous group of antibodies with some patients

positive for these antibodies suffering from thrombosis whereas others do not [10]. All domains of β2GPI have been described to be targeted by aPL, although most studies regarding clinical significance point to antibodies to domain I (DI) as being important [11-13]. Extensive epitope mapping studies were then undertaken to characterize the immunodominant epitope within DI. It was discovered that patient-derived anti-β2GPI antibodies targeting DI of β2GPI bind a conformational epitope that includes residues Arg39–Arg43; Asp8–Asp9 and the interlinker region connecting Domains I and II [14]. The role of DI as the immunodominant epitope of β2GPI is supported by various epidemiological studies, which indicate the diagnostic/predictive value of anti-DI antibodies [15, 16].

Most often, clinical findings cannot clearly distinguish between MS and APS [4]. An acute isolated neurological syndrome poses the biggest diagnostic problem, since it is the most common symptom in MS but can also be the only feature or first manifestation in APS/SLE, before other features of these diseases, such as thrombosis and/or miscarriages for APS and systemic manifestations for SLE, appear [17]. It has been reported that MS patients with atypical features had much higher prevalence of aPL than patients with classic MS and slower progression of the disease [18].

Noteworthy, defining neuromyelitis optica as a distinct disease entity from MS came from the identification of a highly specific serum antibody, which is absent in patients with conventional MS [19]. Whereas IgG specific autoantibodies for aquaporin 4 is pathognomonic for neuromyelitis optica, the presence of T cells, complement, and inflammation is required for the development of parenchymal tissue damage. Activated $CD4^+$ T helper 1 (T_H1) and T helper 17 (T_H17) cells differentiate and

secrete IFN-γ, IL-2, or IL-17A, IL-17F and IL-21 respectively [20, 21]. These effects can largely promote inflammation per se, as well as recruiting other inflammatory cells such as macrophages and monocytes [22, 23]. CD8$^+$ T cells are found more abundant than CD4+ T cells and their clonal expansion is also encountered more commonly [24], whilst there is evidence that they may differentially contribute to disease activity and plaque formation [25].

In MS, the reported frequencies of positive aPL have ranged between 2% and 88%, although these are predominantly of IgM over IgG isotype, possibly due to patients not having undergone antibody class switch yet [9, 26-29]. Notably, the implications of aPL on the clinical presentation of MS and their role in its pathogenesis are still not fully understood [30, 31]. Many potential pathophysiological mechanisms have been identified by which aPL could lead to neurological dysfunction. A proposed role in the pathogenesis is by targeting antigens on the blood brain barrier (BBB) and compromising its integrity [27] or the antibodies could be related to modification of structure and function of proteins involved in the inflammatory–thrombotic processes during disease re-activation [32]. Alternatively, some studies reported that anti-β2GPI stimulates the expression of adhesion molecules, endothelial cell activation and adherence to CNS cells. In particular, IgG anti-β2GPI affinity purified from the serum of a patient with SLE/APS bound to CNS cells such as astrocytes, neurons and cerebral vascular endothelium [33]. It has also been suggested that molecular mimicry of aPL-target antigens with myelin, myelin-related proteins and brain phospholipids may lead to cross-reactivity and predispose to a prothrombotic state [4, 34]. Although a role of aPL on MS disease pathogenesis cannot be ruled out, the findings of other studies suggest that the presence of aPL may represent a by-product of disease

progression since it has been shown that more patients with MS had elevated titres during a disease flare with aPL positivity having a higher prevalence in secondary progressive MS (SPMS) compared to RRMS [9, 35-37].

Consequently, the aim of the present investigation is to use standardized ELISA protocols for the detection of aPL in the serum of a large well-defined cohort of patients with MS and compare these with controls, to assess their frequency and to attempt to better explain the possible association of these antibodies in the pathogenesis of MS. Our study, has enrolled the largest number of patients that are well characterized in terms of their clinical history to date. Interestingly, to our knowledge this is the first study conducted that explores the potential relevance of antibodies against DI of β2GPI in patients with MS.

2. Patients & Methods

2.1 Patient population

Blood samples from 127 patients diagnosed with MS, as defined by the McDonald criteria [38] and followed at The Cyprus Institute of Neurology and Genetics clinic, were collected prospectively for the purposes of this study. None of the MS patients participating in the study had secondary APS or any clinical manifestation such as thrombotic events or pregnancy complications that are associated with APS or any underlying autoimmune disease. In parallel, sera were also obtained from 92 controls with no long-term illness or history of immune-mediated disorders. All subjects had provided written informed consent. MS patients consisted of 88 RRMS patients, 11 primary-progressive MS patients (PPMS), 28 SPMS (5 with relapse) diagnosed using

the McDonald criteria [38], aged between 22 and 79 years, of whom 62 patients were receiving treatment. A relapse was defined as an episode of neurological disturbance attributable to MS, lasting at least 24 h and confirmed by objective observation [38].

2.1.1. Blood processing

Blood samples were allowed to clot, centrifuged within 3 h of collection for 10 min (1500 g at 25 °C) and the sera were frozen in aliquots at -20° C. The frozen aliquots were freeze-thawed once.

2.2. aPL detection

ELISA for detecting IgG and IgM anti-CL

Anti-CL were measured as previously described [39], using commercially sourced calibrators (Louisville APL Diagnostics, USA). A set of polyclonal calibrators (7 IgG calibrators; 7 IgM calibrators) was used to construct IgG and IgM calibration curves from which IgG and IgM anti-CL values, respectively, of unknown samples were determined. These calibrators were used according to the manufacturer's instructions. Briefly, 96-well plates were coated with 50 μg/ml cardiolipin (Sigma,UK) in ethanol on the test half and ethanol alone on the control half. Plates were incubated overnight at 4°C and then blocked with 10% FBS/PBS for one hour at room temperature. Sera were tested in triplicate at a dilution 1:50 with 10% FCS/PBS, incubated at room temperature for 90 minutes. Bound IgG and IgM was detected by addition of anti-human IgG or IgM horseradish peroxidase conjugate (Sigma, UK) in 10% FCS/PBS for one hour followed by addition of substrate, and absorbance was read at 450 nm. The net OD reading for each sample was calculated by subtracting the readings obtained using buffer alone from the corresponding antigen-coated readings in order

to exclude non-specific background binding. Activity was defined in IgG and IgM phospholipid units, GPLU and MPLU units respectively. The IgG and IgM calibrator activity ranges were 16-96 GPLU and 16-96 MPLU respectively. Inter and intra plate variations were assessed using a control on each ELISA plate.

2.2.1. ELISA for detecting IgG and IgM anti-β2GPI

Anti-β2GPI binding was measured as previously described [39]. The test half of the plate was coated with 4 μg/ml human β2GPI (Louisville Diagnostics, USA) in PBS; PBS alone was used on the control half. The plates were blocked using 0.5% porcine gelatin/PBS. Sera were diluted in PBS/1% BSA and incubated for one hour at room temperature. Commercially sourced positive control sera (Louisville APL Diagnostics, USA) for IgG and IgM anti-β2GPI antibodies were used to define activity in SGU and SMU units respectively. Positive controls were serially diluted to obtain a standard curve and the positivity of the first point was assigned as 40 SGU and 40 SMU units according to the manufacturer's instructions.

2.2.2. ELISA for detecting IgG and IgM anti-DI

The anti-DI antibodies were measured [40] in the same manner as human β2GPI, except the plates were coated with bacterially-expressed, conformationally correct human recombinant DI, produced by bacterial expression at University College London [41, 42]. Commercially sourced positive control sera (Louisville APL Diagnostics, USA) for IgG and IgM anti-β2GPI antibodies were used as calibrators for anti-DI assay. Calibrators were serially diluted to obtain a standard curve, and arbitrary units were assigned to each point. Anti-DI activity was defined as DI units,

GDIU and MDIU, for IgG and IgM DI antibodies respectively [40], and calculated as described for the anti-CL assay.

2.3. Statistical analysis

Graphical representations were constructed using GraphPad Prism Version 5 for Windows, La Jolla, California USA. All statistical tests were run using the IBM Corp SPSS Version 20 software, Armonk, NY. All results were treated with the Pearson's Chi-squared test, and the Fisher's exact test where a variable had an expected frequency of five or less. For mean comparisons between two groups with continuous variables, the non-parametric Mann-Whitney test was used. The nominal P value <0.05 was considered statistically significant.

3. Results

3.1. Prevalence of positivity for aPL

The relevant demographic and clinical characteristics of the study population groups at the time of sample collection are listed in Table 1. One hundred and twenty seven MS subjects and 92 controls enrolled in the study were screened for the presence of either IgG/IgM aPL antibodies targeting CL, β2GPI and DI of β2GPI by standardised ELISA protocols. Gender was matched between the study group and control group (p value = 0.149). MS and control group were of similar age (mean age for MS was 51.7 ± 12.19 and mean age for controls was 52.1 ± 17.75; p value = 0.585). Patients with multiple sclerosis included in the study did not have any other concomitant disease and they had not presented any APS − related thrombotic episodes or misscarriages. Based on the sera from a population of 92 controls, our cut-offs for positivity in the

ELISA assays are defined by the 99th percentile of the activity of healthy subjects. Overall, 43% of patients with MS tested were positive for at least one aPL antibody as opposed to 3% of controls; p<0.0001 (Figure 1).

Comparing the three antigens, the most prevalent antibodies were anti-CL with the highest percentage of positivity (percentage positivity range 18.1-21.3%). Similarly, levels of anti-DI antibodies were found to be elevated in patients with MS compared to controls. There was a statistically significant association of IgG anti-CL antibodies observed by a subset of 21.3% (n=27) patients with MS found positive for IgG anti-CL antibodies compared to 1.1% of control. Mean titre for IgG anti-CL is shown in Figure 2A (p<0.05). The range of GPLU for MS patients that were positive for IgG anti-CL antibodies was 18 – 144 GPLU (mean GPLU 47). Likewise, for the IgM anti-CL antibodies, 18.1% of MS cases were found positive (n=23) as opposed to 1.1% controls (n=3), p<0.0001 (Figure 2B). The range of MPLU for MS patients that were positive for IgM anti-CL antibodies was 49 – 118 MPLU (mean MPLU 74). Mean activity of MS and controls against DI is shown graphically in Figure 3A&B (p<0.05, for both IgG and IgM anti-DI). IgG anti-DI antibodies in patients with MS were detected at a frequency of 7.1% (n=9) compared to controls (1.1%, n=1) (Figure 3A). For IgM anti-aDI antibodies, there was an incidence of 6.3% (n=8) compared to 1.1% (n=1) of controls (Figure 3B). The range of GDIU for MS patients positive for IgG anti-DI antibodies was 53 – 175 MDIU (mean GDIU 89) and for MDIU was 2 – 21 (mean MDIU 6).

There was no significant difference in titres between MS patients and controls for IgG anti-β2GPI antibodies (illustrated in Figure 4). Out of 127 MS patients, only 3

patients were found positive for IgG anti-β2GPI antibodies and no positive IgG anti-β2GPI antibodies were detected for the controls (Figure 4A). However, for the IgM isotype, there were 6 MS patients found positive as opposed to no positive antibodies in the controls; mean titre is illustrated in Figure 4B (p<0.05).

3.2. Antibody positivity status with respect to type of MS

The frequency of aPL positive results was examined for any association with the type of MS (RRMS, PPMS and SPMS). It is observed that there was seropositivity for IgG anti-CL antibodies among all groups including 18.2% of RRMS and for PPMS but for SPMS there was a positivity of 32.1% (Table 2). There was no significant difference between groups. On the contrary, IgM anti-CL antibody positivity was differentially distributed among groups with a frequency of 10.2% in RRMS, 36.4% in PPMS and 35.7% in SPMS patients (p<0.005), showing elevation of IgM anti-CL antibodies with disease progression (Table 2).

There was no statistically significant association of positive aPL with a specific type of MS for the other two antigens (β2GPI and DI). Nonetheless, it is apparent that there was a trend suggesting a correlation of positive IgG anti-DI antibodies in SPMS and PPMS patients (Table 2).

Interestingly, regarding the positivity across the panel of aPL tested in relation to the type of MS, 71.4% of SPMS patients were positive for at least one aPL with only eight patients found negative for all aPL tested. Moreover, it is observed that 31.8% of RRMS and 54.5% of PPMS were positive for at least one aPL.

4. Discussion

The fundamental findings of this paper are significant associations between the presence of aPL and patients with MS compared to controls. There was a higher percentage of 43% patients with MS tested positive for at least one aPL antibody while the respective frequency in healthy controls was 3%. Koudriavtseva et al also showed that the rate of at least one aPL positivity reached 60% in patients with MS [43]. Antibodies recognizing cardiolipin are the most prevalent autoantibodies in this cohort. To date, this study enrolled the largest number of patients with MS, with most detailed characterization, under long-term follow-up at one centre, to answer different questions. Our presumption is that previous studies thus far report an association between aPL and MS with a wide variability. Notably, a predominance of IgM over IgG subtypes was reported, perhaps due to poor class-switching; this could possibly result from no β2GPI recognition by T cells in MS. A possible explanation for the inconsistencies regarding the prevalence of aPL in the studies reported thus far could be the limited number of patient serum samples tested.

The trend observed for anti-CL antibodies was similar to that found for anti-DI antibodies. IgG and IgM anti-DI levels were 7.1% and 6.3% in MS patients compared to 1.1% in the controls. β2GPI is the major antigen for autoantibodies that cause APS, an autoimmune disease characterized by an increased risk of thrombosis and recurrent fetal loss. It is now well established that even though other domains of the β2GPI may be targeted, the antibodies against DI are more strongly associated with thrombosis than autoantibodies against other parts of this protein [14, 44, 45]. DI is accessible to antibody recognition only after the protein takes its open so-called "hook-like"

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conformation rather than its closed circular form, hence, it is exposed only when the protein is bound to an anionic surface. This is mainly driven by the interaction of the fifth domain of the protein with anionic phospholipids on cellular plasma membranes [46]. After exposure to negatively charged phospholipids, β2GPI binds, opens up, and exposes the epitope of the autoantibodies and the antibodies are able to recognize β2GPI [47]. Hence, β2GPI when coated on an ELISA plate may prevent exposure of cryptic epitopes and explain why we could not detect antibodies against whole β2GPI in contrast to our finding of raised levels of anti-DI antibodies in patients with MS.

To better understand the involvement of aPL in MS, we have correlated aPL positivity with different types of MS. SPMS patients showed a higher frequency for IgG anti-CL antibodies (32.1%) whilst this was true for both primary and secondary progressive MS groups for IgM anti-CL antibodies (36.4% and 35.7% respectively). On the contrary, RRMS patients showed the least frequency for IgM anti-CL, which can signify a relationship between an increase in autoantibody production and disease activity and progression in MS. Up to 20.5% RRMS patients were positive for one aPL. Interestingly, three patients in the study found positive for three aPL simultaneously, belong to this group. In the case of anti-DI antibodies, our results suggest a trend of elevated IgM anti-DI antibodies in PPMS and SPMS which is even higher for IgG anti-DI antibodies. This is further supported by the finding that 71.4% SPMS patients were positive for at least one aPL and 25% were positive for two aPL simultaneously while from the same group only eight patients were negative for all tested aPL. Nevertheless, a larger sample size of PPMS and SPMS patients in future studies could re-enforce the hypothesis of aPL prevalence relating to disease progression.

Nevertheless, we speculate that anti-DI antibodies may occur as one of the arms constituting the expanding inflammatory milieu and their occurrence is a piece of evidence indicating the actual degree of the impairment or possibly a marker that favours the prognosis of the disease. Since there are still unsolved questions regarding the biological and clinical significance of aPL, their occurrence in clinical conditions other than the APS may provide more in-depth knowledge regarding their function. Noteworthy, the general consensus for the APS appears to be that once aPL are generated and sustained, it is thought that a "second hit" is needed for thrombus formation [48, 49]. In line with this observation, it has been suggested that infectious processes and microbes could trigger an inflammatory response by increasing the expression of the aPL target antigen or the expression of antigenic epitopes which might constitute the second hit. This could fit well for our hypothesis for thrombosis – a possible common denominator with MS - as several reports in literature have described an association between cerebral venous thrombosis and MS [50-52].

Interestingly, in a systematic review, Marrie and colleagues reported that stroke was more usually encountered in MS than in the general population [53]. Peeters and colleagues also confirmed a higher risk of venous thromboembolism in MS compared to healthy controls [54]. Furthermore, the possibility of an underlying disrupted venous drainage from the CNS is the third described vascular impairment in MS [55]. Moreover, vascular diseases may be more frequent in patients with MS for several reasons. First they may share pathophysiology including endothelial dysfunction, platelet activation and hypercoagulation [56, 57]. Second, the increased surveillance of patients with MS may increase the likelihood of being diagnosed with vascular disease, although diagnostic difficulties in distinguishing stroke from MS flares may

influence the findings. Thrombotic processes in MS may rather signify the compensating efforts of the innate immune response, either to challenging infections or genetic and environmental disruption of anti-coagulative mechanisms [34]. Hence, we speculate that pro-thrombotic factors, including positivity for aPL, could indicate that the pathogenic mechanisms of MS may, to some extent, involve thrombotic processes.

Our study had a number of limitations. We did not study whether aPL found in all patients are persistently positive, which is a triggering pathogenic factor. Some data is available for a number of patients whose serum was available longitudinally and we have confirmed that the titres remain positive (5 out of 7 patients remain persistently positive for IgG anti-DI and 3 out of 4 patients remain persistently positive for IgG anti-CL; data not shown). aPL titres can fluctuate, sometimes being negative in the acute phase and most studies have not measured aPL sequentially [17]. In future though, it may be worthwhile to carry out a detailed investigation on whether the remaining positive patients in our cohort are persistently positive. Another limitation is the lack of information on lupus anticoagulant which is of clinical importance and on whether the patients had experienced any complications during pregnancy and the number of live births. Hence the relevance of aPL positivity to MS is unclear however it does not lessen their importance as biomarkers in MS.

5. Conclusions

Altogether, our findings showed a significant increase of aPL in patients with MS compared to healthy controls. This is by far, the largest study screening patients diagnosed with definite MS for aPL and investigating the involvement of novel anti-

DI antibodies in MS. In conclusion, we have identified that anti-DI antibodies were found to be positive in our cohort of patients with MS and could be the triggering factor for the occurrence of inflammatory-thrombotic processes in MS.

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Table 1. Demographic and clinical variables of subjects*

	MS	HC
No. of subjects (F:M)	89:38	55:37
Mean age (±SD)	51.69±12.19	52.1±17.75
Clinical history		
RRMS	88	N/A
SPMS	23	N/A
SPMS with relapse	5	N/A
PPMS	11	N/A
Treatment**		
Interferon-β	40	N/A
Natalizumab	11	N/A

* Except where indicated otherwise, values represent the number of subjects. RRMS, relapse-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis; N/A, not available.

** A total of 13 patients were receiving other medication which includes: mitoxantrone, azathioprine, mycophenolate, glatiramer acetate, methotrexate, paroxetine, fingolimod, citalopram, alprazolam.

Table 2. Rate of aPL positivity in the study population groups

aPL	Healthy (n=92) Pos/Total (% pos)	RRMS (n=88) Pos/Total (% pos)	PPMS (n=11) Pos/Total (% pos)	SPMS (n=28) Pos/Total (% pos)
Anti-CL IgG	1/92 (1.1%)	16/88 (18.2%)	2/11 (18.2%)	9/28 (32.1%)
Anti-CL IgM	1/92 (1.1%)	9/88 (10.2%)	4/11 (36.4%)	10/28 (35.7%)
Anti-β2GPI IgG	0/92 (0%)	2/88 (2.3%)	0/11 (0%)	1/28 (3.6%)
Anti-β2GPI IgM	0/92 (0%)	3/88 (3.4%)	0/11 (0%)	3/28 (10.7%)
Anti-DI IgG	1/92 (1.1%)	4/88 (4.5%)	2/11 (18.2%)	3/28 (10.7%)
Anti-DI IgM	1/92 (1.1%)	6/88 (6.8%)	1/11 (9.1%)	1/28 (3.6%)

The cut-off values for positive in each assay was defined as the 99th percentile of the healthy controls: Anti-CL (15.72 GPL or 48.35 MPL/ml for IgG or IgM) and anti-DI (50.72 GDIU or 1.47 MDIU for IgG and IgM). The cut off for the anti-β2GPI assay was zero. aPL, antiphospholipid antibodies; anti-β2GPI, anti-β2glycoprotein; CL, cardiolipin; DI, domain I; RRMS, relapse-remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; pos, positive.
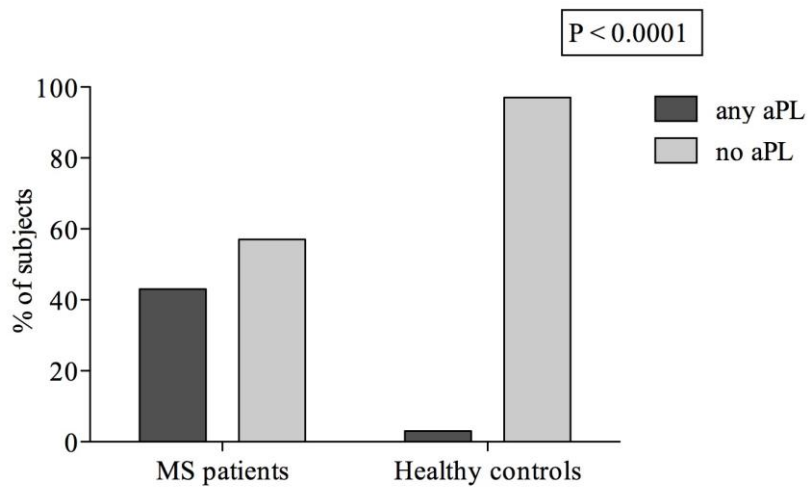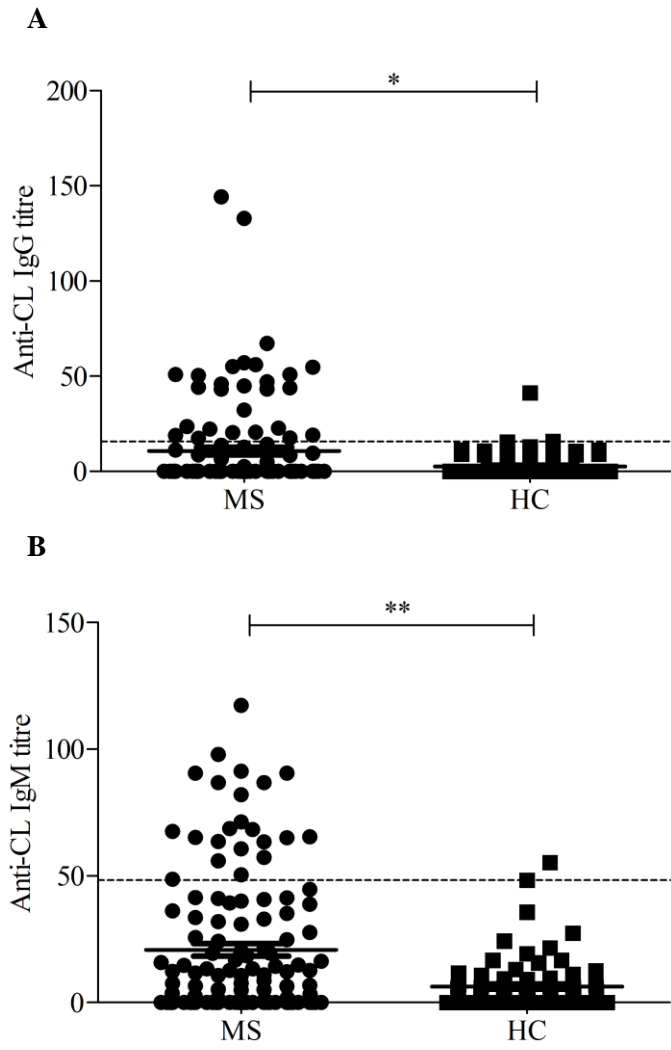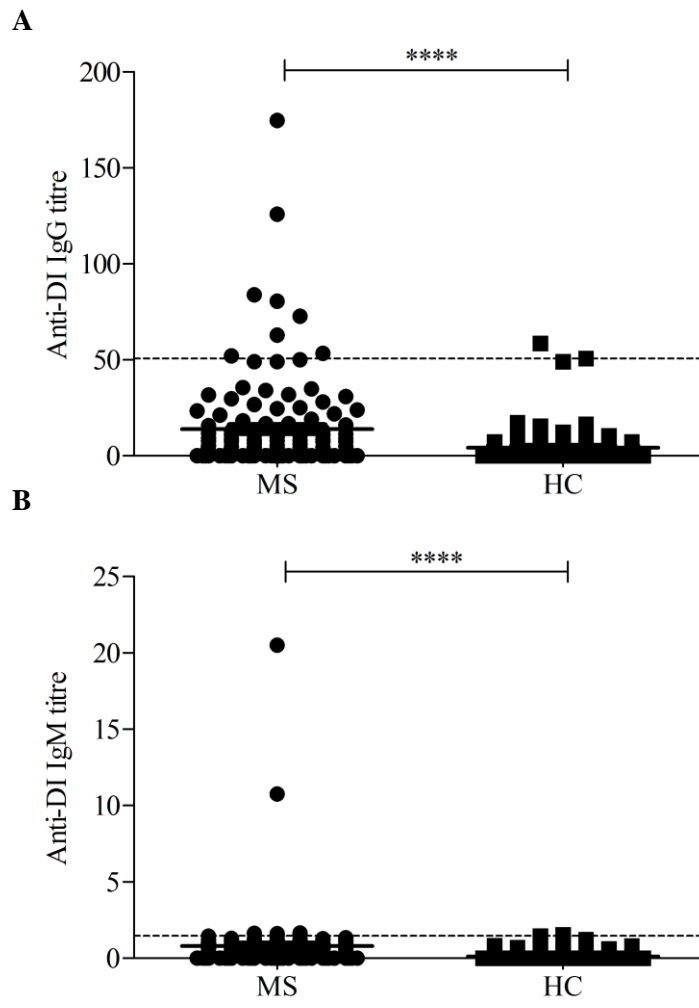
Figure 1.

A

B

Figure 2.

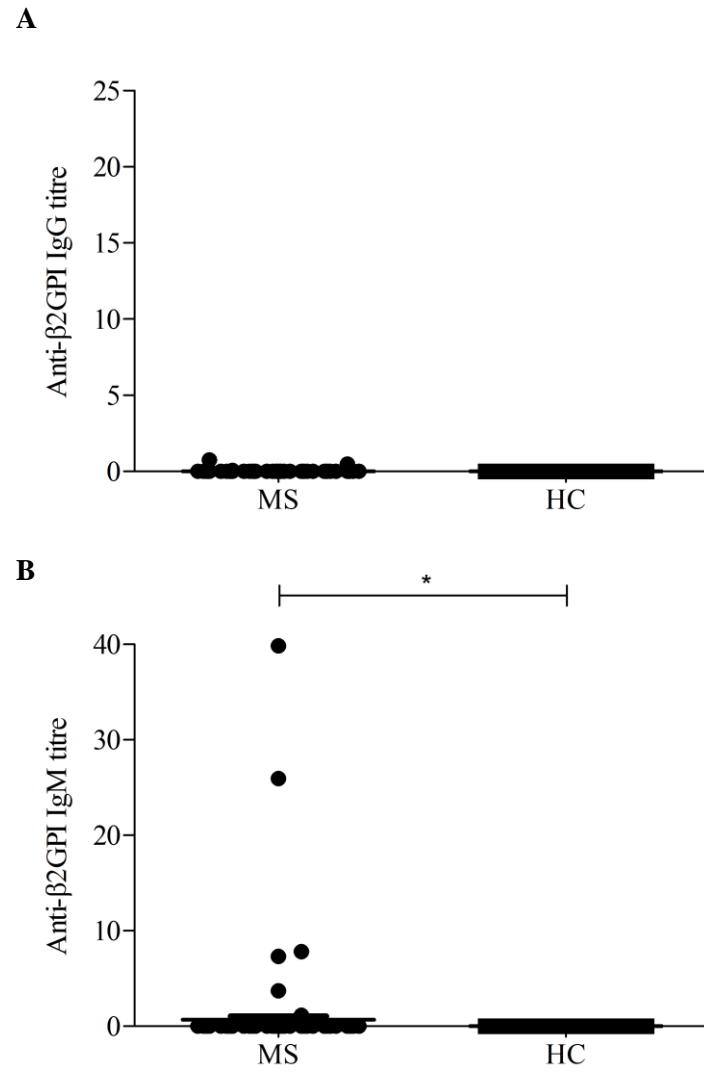**A**

B

Figure 3.

A

B

Figure 4.

Figure Legends

Figure 1. Comparison of positive aPL against the measured antigens in MS subjects versus healthy controls.

Results are shown as percentage of positive subjects. The comparison was performed using Fischer's exact test. p<0.0001.

Figure 2. Detection of IgG and IgM anti-CL antibodies in serum.

Serum from MS patients and from healthy controls (HC) was tested for the presence of IgG (A) and IgM (B) antibodies to cardiolipin. Serum was tested in triplicate at 1:50 dilution, and titres were expressed in GPLU/MPLU (IgG phospholipid units/IgM phospholipid units) in comparison to the binding of polyclonal calibrators. Symbols represent individual subjects; bars show the mean ± SEM (* p<0.05 for IgG anti-CL and ** p<0.005 for IgM anti-CL). Dashed line depicts the cutoff for positivity, defined as values more than in the 99th percentile of the healthy controls (n = 92).

Figure 3. Detection of IgG and IgM anti-DI antibodies in serum

Activities of IgG (A) and IgM (B) antibodies to DI in MS patients and healthy controls. Experiments were performed in duplicate for each sample, and titres were expressed in DI units in comparison to the binding of polyclonal sample. Symbols represent individual subjects; bars represent the mean ± SEM (**** p<0.0001). Dashed line indicates the cutoff for positivity in the assay (the 99th percentile of the healthy control group (n = 92)).

Figure 4. Detection of IgG and IgM anti-β2GPI antibodies in serum.

Serum from MS patients and from healthy controls (HC) was tested for the presence of IgG (A) and IgM (B) antibodies to β2GPI. Titres were expressed in SGU in comparison to the binding of polyclonal calibrators (* p<0.05 for IgM anti-β2GPI).