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Anti-*Borrelia burgdorferi* antibody profile in post-Lyme disease syndrome

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ABSTRACT

Patients with post-Lyme disease syndrome (PLDS) report persistent symptoms of pain, fatigue, and/or concentration and memory disturbances despite antibiotic treatment for Lyme borreliosis. The etiopathogenesis of these symptoms remains unknown and no effective therapies have been identified. We sought to examine the anti-borrelia antibody profile in affected patients with the aim of finding clues to the mechanism of the syndrome and its relationship to the original spirochetal infection. Serum specimens from 54 borrelial seropositive PLDS patients were examined for antibodies to *B. burgdorferi* proteins p18, p25, p28, p30, p31, p34, p39, p41, p45, p58, p66, p93, and VlsE by automated immunoblotting and software-assisted band analysis. Presence of serum antibodies to the 31 kDa band was further investigated by examination of reactivity against purified recombinant OspA protein. Control specimens included sera from 14 borrelial seropositive individuals with a history of early localized or disseminated Lyme disease who were symptom-free (post-Lyme healthy), as well as 20 healthy individuals without serologic evidence or history of Lyme disease. In comparison to the post-Lyme healthy group, higher frequencies of antibody to p28 (p <0.05), p30 (p <0.05), p31 (p <0.0001), and p34 (p <0.05) proteins were found in the PLDS group. Assessment of antibody reactivity to recombinant OspA confirmed the presence of elevated levels in PLDS patients (p <0.005). The described anti-borrelia antibody profile in PLDS offers clues about the course of the antecedent infection in affected patients, which may be useful for understanding the pathogenic mechanism of the disease.

INTRODUCTION

Lyme disease is the most commonly reported tick-borne infection in the United States and is also endemic in Europe and parts of Asia (21, 25). It is caused by bacteria of the *Borrelia burgdorferi* species complex (23). The early phase of the infection is typically associated with a characteristic skin lesion, known as erythema migrans (EM), in the localized stage and with multiple (secondary) EM lesions in the disseminated stage (4). Extracutaneous manifestations of disseminated and late disseminated Lyme disease may affect the joints, heart, and/or the nervous system (24, 29). The most frequent objective neurologic complications include lymphocytic meningitis, cranial neuropathy, and radiculopathy, which usually respond well to antibiotic treatment (12). However, some patients complain of persistent or relapsing symptoms despite treatment and in the absence of objective clinical or microbiologic evidence of ongoing infection, as determined by currently available methods (11, 20). The symptoms in these patients include mild to severe musculoskeletal pain, fatigue, and/or difficulties with concentration and memory (11, 20). The condition, referred to as post-Lyme disease syndrome (PLDS or PLS) or chronic Lyme disease, can be associated with considerable impairment in the health-related quality of life in the affected patient population (16).

Despite several years of debate and a number of treatment trials (10, 16, 17) few clues to the cause of the symptoms of PLDS have emerged. Lack of biomarkers that would correlate with symptoms or treatment outcome in patients has also compounded the problem of understanding the syndrome. There have been no studies to date that systematically examine the antigen specificity of the anti-borrelia immune response in

patients with a history of Lyme disease and persistent symptoms. In this study, we sought to gain clues to the mechanism of PLDS and its relationship to the original *B. burgdorferi* infection by characterizing the antigenic specificity of anti-borrelia antibodies in seropositive patients and control subjects. The described pattern of immune reactivity to proteins of *B. burgdorferi* may help in better understanding the course of preceding acute infection and in gaining clues about the pathogenic mechanism of the syndrome in a large subset of PLDS patients.

MATERIALS AND METHODS

Subjects

Serum samples were from 54 individuals with PLDS who were seropositive by ELISA for IgG antibodies to *B. burgdorferi* (see table 1 for patient group characteristics; see below for assay procedure). The source of samples and selection criteria have been previously described in detail (6, 16). Of the 54 specimens analyzed, 34 were from patients who had a history of single or multiple EM. Documentation by a physician of previous treatment of acute Lyme disease with a recommended antibiotic regimen was also required. Patients had one or more of the following symptoms at the time of enrollment: widespread musculoskeletal pain, cognitive impairment, radicular pain, paresthesias, or dysesthesias. Fatigue often accompanied one or more of these symptoms. The chronic symptoms had to have begun within 6 months after the documentation of infection with *B. burgdorferi*.

The study also included control serum specimens from 14 borrelial IgG ELISA-seropositive individuals who had been treated for early localized or disseminated Lyme disease associated with single or multiple EM with no post-Lyme symptoms after at least 2 years of follow-up (see table 1 for patient group characteristics). The original diagnosis of acute Lyme disease in these currently healthy subjects was confirmed by recovery of *B. burgdorferi* in cultures of skin and/or blood. The source of samples and selection criteria were previously described (6).

Serum samples from 20 healthy subjects without history or serologic evidence of past or present Lyme disease were also included in the study. In addition, serum specimens from 2 individuals who were vaccinated for Lyme disease with the recombinant OspA

protein (Lymerix™) were used as positive controls for experiments aimed at determination of anti-OspA antibody response. This study was approved by the Institutional Review Board of the Weill Cornell Medical College of Cornell University.

Anti-borrelia antibodies

B. burgdorferi Enzyme-linked immunosorbent assay (ELISA). IgG anti-borrelia antibody levels were determined by ELISA as previously described (6).

B. burgdorferi Western blot assay (WB). IgG antibody response to *B. burgdorferi* B31 was further characterized by WB, using commercial prepared blots and the Euroblot automated WB instrument, according to the manufacturer's protocols (Euroimmun, Boonton, N.J.). Briefly, nitrocellulose strips containing electrophoresis-separated *B. burgdorferi* B31 proteins were blocked and then incubated with 1.5 mL of diluted serum sample (1:50) for 30 min. Membrane strips were washed and incubated with AP-conjugated anti-human IgG antibody for 30 min. Bound antibodies were detected using the NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate) staining system (Euroimmun). Quantitative analysis of bands on each blot was carried out using the EuroLinescan software (Euroimmun). Accurate background correction and determination of cutoff values for positivity were carried out by the software for the p18, p25 (OspC), p28, p30, p31 (OspA), p34 (OspB), p39 (BmpA), p41 (FlaB), p45, p58, p66, p93, and recombinant VlsE borrelial protein bands. Determination of IgG positive serology for Lyme disease was based on the CDC criteria, according to which an IgG immunoblot can be considered positive if five or more of the following ten bands are positive: 18 kDa, 25

kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (Fla), 45 kDa, 58 kDa, 66 kDa, and 93 kDa (1, 5).

OspA ELISA. Round bottom 96-well polystyrene plates (Becton Dickinson, Franklin Lakes, N.J.) were incubated with 0.5 µg/well of *B. burgdorferi* recombinant OspA protein (Meridian Life Science, Saco, Maine) in 0.1 M carbonate buffer (pH 9.6) (1.5 h, 37 ^{o}C). Control wells were not coated. Wells were washed and blocked with 1% BSA in phosphate-buffered saline containing 0.05% Tween-20 (PBST) (1.5 h). Incubation with diluted serum specimens (50 µL/well at 1:400 in blocking solution) was done for 1 h. Serum samples from two OspA-vaccinated individuals were included as controls on each plate. Incubation with HRP-conjugated sheep anti-human IgG secondary antibody (GE Healthcare, Piscataway, N.J.) (1:2000 in blocking solution) was done for 1 h. Incubation with developing solution, comprising 27 mM citric acid, 50 mM Na_2HPO_4, 5.5 mM *o*-phenylenediamine, and 0.01% H_2O_2 (pH 5), was done for 20 min. Absorbance was measured at 450 nm and corrected for non-specific binding by subtraction of the mean absorbance of corresponding wells not coated with the OspA protein. Absorbance values were normalized based on the mean for the positive controls on each plate. Cutoff for positivity was assigned as three standard deviations above the mean for the non-Lyme healthy group results.

Data analysis

Group differences were analyzed by two-tailed Welch's t-test or Mann-Whitney U test (continuous data), and Chi-square test or Fisher's exact test (nominal data). Differences with $p<0.05$ were considered to be significant.

RESULTS

Subjects. There was not a significant difference in age, gender, or elapsed time since infection between the PLDS patient group and the post-early Lyme healthy control group (Table 1).

B. burgdorferi ELISA. While all selected post-Lyme samples were seropositive, mean anti-borrelia antibody level (as measured by mean normalized absorbance) was higher for the PLDS group than the post-early Lyme healthy group ($p<0.05$). The difference remained weakly significant when limiting the comparison to those individuals who had presented with EM in each group ($p<0.05$) (Fig. 1). None of the specimens from the non-Lyme healthy control group was positive.

B. burgdorferi Western blot. 47 of 54 (87.0%) ELISA-positive PLDS subjects and 11 of 14 (78.6%) ELISA-positive post-early Lyme healthy subjects were found to be IgG seropositive for anti-borrelia antibodies by immunoblotting as determined by the EuroLinescan software and according to the CDC criteria. None of the healthy control subjects without history of Lyme disease was positive by WB. Significantly more PLDS patients were found to have antibodies to p28 ($p<0.05$), p30 ($p<0.05$), p31 ($p<0.0001$), and p34 ($p<0.05$) proteins than post-Lyme healthy subjects (Fig. 2). When comparing only those in each group with a history of presenting with EM, there was a significantly higher number of PLDS patients with antibodies to p31 ($p<0.001$), while the difference for the number of individuals with antibody reactivity to p28 and p34 approached significance

(p=0.07). In contrast, the frequencies of antibody reactivity to p18, p25, and p45 were either comparable to or slightly higher in the post-Lyme healthy group. Frequencies of antibody reactivity to all bands, with the exception of p41, were significantly higher in the PLDS group than in the group of healthy individuals without history or serologic evidence of Lyme disease.

The significant differences in antibody reactivity to the above four bands were confirmed by comparing the means for the software-calculated band signal intensities (gray-level intensities) for each group. There was a higher mean band intensity for p28 (p <0.05), p30 (p <0.05), p31 (p <0.005), and p34 (p <0.05) in the PLDS group in comparison to the post-Lyme healthy group (Fig. 3). The difference in mean band intensity for p31 remained significant when comparing only those in each post-Lyme group with a history of EM (p <0.005).

OspA ELISA. The observed differential antibody reactivity to p31 in PLDS patients on WB was further investigated by ELISA, using purified recombinant OspA protein. Mean antibody reactivity (as measured by mean normalized absorbance) to OspA in PLDS patients was significantly higher than in post-early Lyme healthy individuals (p<0.0001) (Fig. 4). The difference remained significant when considering only those in each group who had presented with EM (p<0.005). Similarly, the frequency of anti-OspA antibody positivity in PLDS patients (27 of 54, 50.0%) was significantly greater than in post-Lyme healthy individuals (1 of 14, 7.1%) (p<0.05). The difference remained significant when limiting the analysis to those in PLDS and post-Lyme healthy groups who had a history of presenting with EM (15 of 34 vs. 1 of 14; 44.1% vs. 7.1%) (p<0.05).

DISCUSSION

The controversy surrounding PLDS is partially caused by our limited knowledge about the etiology and pathology of the syndrome. A potential source of information about PLDS, namely the specificity the immune response to *B. burgdorferi* in affected patients, has not been systematically characterized before. The availability of serum specimens from 54 seropositive patients fulfilling strict criteria for PLDS provided the opportunity to examine the respective anti-borrelia antibody profiles in detail. The data in this report suggest that a defined pattern of antibody reactivity to borrelial antigens in PLDS may exist and offer novel clues about the etiopathogenesis of the syndrome and its relationship to the original spirochetal infection.

We can consider some possibilities that would explain the observed anti-borrelia antibody response in PLDS and help in understanding its significance and pathogenic relevance to the syndrome. First, it is important to note that the apparently higher overall anti-borrelia antibody response in PLDS was not evenly distributed towards the various *B. burgdorferi* immunodominant proteins. We observed higher than expected frequencies of antibody reactivity to p28, p30, p31, and p34 protein bands in the patient group in comparison to the post-early Lyme healthy group, while the frequencies of antibody reactivity to p18, p25, p41, and p45 were either comparable or slightly higher in the post-early Lyme healthy group. Previous studies have shown that IgG antibodies to p25, p39, p41, and p45 are generated early on in Lyme disease, whereas antibodies against p30, p31, and p34 are more frequent in later stages of infection (2, 8, 14, 19, 22). The IgG antibody response to p31 (OspA), a protein whose expression by the *B. burgdorferi* spirochete

231 diminishes in the early stage of the infection, is particularly rare during this phase, but
232 becomes more common in late Lyme disease (1, 2, 9, 15). Accordingly, the observed
233 antibody profile, especially the significantly higher antibody reactivity towards the p31
234 band on WB and the recombinant OspA protein on ELISA in the PLDS group may be
235 indicative of a longer than assumed course of active infection in affected patients. This
236 may have been caused by a delay in the start of the appropriate course of antibiotic
237 treatment and/or undocumented repeat infection(s) in many PLDS patients. This finding
238 would be in line with previous studies indicating that delayed treatment is associated with
239 increased post-Lyme disease symptoms (20).

240 Second, the observed antibody response may be indicative of persisting attenuated
241 forms of the organism or spirochetal debris in certain tissues after antibiotic treatment,
242 and/or ongoing presence of certain borrelial antigens, possibly retained by antigen-
243 presenting cells, in some PLDS patients. In fact, several studies have demonstrated the
244 persistence of spirochetal DNA or apparently non-infectious forms of the organism in some
245 antibiotic-treated animals (3, 13, 26, 27). One can postulate that while these spirochetal
246 forms or specific antigen remnants of the organism would not be responsive to antibiotic
247 treatment, they might continue to elicit a significant antibody response.

248 Third, the pattern of anti-borrelia antibody reactivity in PLDS may be indicative of
249 differences in host condition, as local environmental changes can affect protein expression
250 by the *B. burgdorferi* spirochete. For example, it has been shown that the expression of
251 OspA by *B. burgdorferi* is significantly increased when inflammation is induced in mice
252 during early infection (7). Therefore, the increased antibody response to OspA in PLDS

patients, even in those who had presented with EM, may be indicative of the concurrent presence of an inflammatory condition during the antecedent infection.

Finally, it is conceivable that certain strains of *B. burgdorferi* can induce higher levels of antibodies against particular antigens in susceptible individuals. Recent work points to the existence of multiple strains of the organism in the United States, most of which have not been studied with regard to their immunogenic potential or specific protein expression levels after introduction into the mammalian host (18, 28, 30, 31). Therefore, the observed differences in the specificity of the anti-borrelia antibody response might be attributed to the higher likelihood of antecedent infection with one strain vs. another in those with persistent symptoms.

As the results show, fewer samples were positive for antibody reactivity to OspA by ELISA than were positive for antibodies to p31 by WB. This could be explained by the 1) generally lower limit of detection in WB, 2) reactivity of PLDS patient antibodies towards additional protein(s) that have the same migration pattern as OspA (appearing at ~31 kDa) on WB, and/or 3) exposure of specific epitopes of the OspA protein in the WB procedure (in which proteins are mostly denatured), but not in ELISA, towards which patients exhibit antibody reactivity. It is worth mentioning that antibody reactivity to the 31 kDa band on WB was also found in some healthy individuals without a history or serologic evidence of Lyme disease. Therefore, the presence of antibodies to the p31 band by itself (i.e., in the absence of antibodies to other *B. burgdorferi*-specific bands) is unlikely to have biomarker utility in the WB system.

A potential limitation of this study is the fact that EM duration was not taken into account in the comparison between patients and controls, as reliable information was not

available for the patient group. While the post-Lyme healthy control subjects were all followed prospectively by one of the investigators (GPW) from the time of diagnosis, the same cannot be said for PLDS patients who were recruited as part of a multi-center clinical trial. Thus, it is conceivable that some PLDS patients in the subgroup with EM also presented with objective clinical manifestations of Lyme disease beyond those of EM. Future prospective studies should pay careful attention to EM history and make an attempt to match patients and controls accordingly.

A second limitation of our study may be perceived to be the exclusion of seronegative patients. In this study, we chose to focus specifically on patients and post-Lyme healthy control subjects who were seropositive by whole-cell ELISA. This was done in order to ensure that all samples in the comparison had the minimal detectable anti-borrelia antibody response necessary for subsequent analyses. Obviously, our findings and conclusions in this report do not extend to the seronegative subpopulation. It should be noted that in the original clinical trial that provided the specimens examined in this study (16), in which there was simultaneous recruitment of both seropositive and seronegative subjects, nearly 40% of the subjects were, in fact, seronegative. The absence of an anti-borrelia antibody response in many PLDS patients points to the heterogeneity of the population under study, but does not diminish the significance of the reported findings. Closer examination of the immune response in seronegative PLDS patients, including the specificity of anti-borrelia antibodies as a function of time since infection, needs to be done in future prospective studies.

While the observed elevated antibody response to specific borrelial proteins in this study adds to earlier evidence for the existence of a differential immune response in PLDS

patients (6) and offers useful clues about the course of the original infection, the data should not be interpreted as having diagnostic value or clinical utility at this point. However, we can conclude that the current data strongly support the continuation of studies aimed at in-depth characterization of the anti-borrelia immune response in the affected patient population. It is expected that the expansion of these studies, with the aim to 1) include additional control groups, such as symptom-free individuals with histories of past Lyme arthritis and neurologic Lyme, and 2) examine the immune response to the entire proteome of the Lyme pathogen, would aid in the development of biomarkers for PLDS and provide important clues to its pathogenesis and potential treatments. This strategy may also prove useful for the study of chronic post-infection symptoms associated with other pathogens.

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Conflict of interest statement

All authors declare that there are no conflicts of interest.

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458 **Table 1. Subject group characteristics.**

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Subject group	History of Lyme disease manifestation prior to treatment	Number of subjects	Gender ratio (F:M)	Mean age (yr) (± SD)	Mean duration of elapsed time since documentation of infection (yr) (± SD)
PLDS		54	25:29	56.3 ± 12.8	4.72 ± 2.81
	Erythema migrans (EM)	34	16:18	54.4 ± 11.8	4.89 ± 2.87
	Single EM	24	11:13	54.0 ± 10.5	4.96 ± 2.97
	Multiple EM	10	5:5	55.4 ± 15.1	4.71 ± 2.77
	Other*	20	9:11	59.5 ± 14.0	4.42 ± 2.76
Post-Lyme healthy					
	Erythema migrans (EM)	14	4:10	51.4 ± 18.0	4.61 ± 3.50
	Single EM	7	0:7	48.0 ± 13.3	4.32 ± 3.98
	Multiple EM	7	4:3	54.4 ± 21.8	4.86 ± 3.34
Non-Lyme healthy		20	12:8	49.7 ± 15.5	

460 *Non-EM manifestations of Lyme disease, including those associated with late Lyme,
461 such as in Lyme arthritis and late neurologic Lyme disease.
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Figure legends:

Figure 1. IgG anti-borrelia antibody levels in PLDS patients and control subjects, as measured by ELISA. While all post-Lyme disease samples were positive by ELISA, mean anti-borrelia antibody level (mean normalized absorbance at 450 nm) was higher for the PLDS group (n=54) than the post-early Lyme healthy group (n=14) ($p<0.05$). The difference remained significant when limiting the analysis to specimens from individuals with a history of EM in both groups (n=34 for PLDS vs. n=14 for post-Lyme healthy) ($p<0.05$). Frequencies of antibody reactivity to all bands, with the exception of p41, were significantly higher in the PLDS group than in the group of healthy individuals without history or serologic evidence of Lyme disease. Error bars represent the standard error of the mean for each group.

Figure 2. Frequencies of positivity for IgG antibodies to *B. burgdorferi* WB bands in PLDS patients and control subjects. Significantly more PLDS patients (n=54) were found to have antibodies to p28 ($p<0.05$), p30 ($p<0.05$), p31 ($p<0.0001$), and p34 ($p<0.05$) bands than post-Lyme healthy subjects (n=14). When comparing only those who had presented with EM in the two groups (n=34 for PLDS vs. n=14 for post-Lyme healthy), there was a significantly higher frequency of PLDS patients with antibodies to p31 ($p<0.001$), while the difference for the frequency of individuals with antibody reactivity to p28 and p34 approached significance ($p=0.07$).

Figure 3. Levels of IgG antibody reactivity to 28, 30, 31, and 31 kDa proteins of *B. burgdorferi* in PLDS patients and control subjects. Means for the software-calculated WB band signal intensities for antibody reactivity with the four proteins were compared between ELISA-seropositive PLDS patients, ELISA-seropositive post-early Lyme healthy subjects, and healthy individuals without history or serologic evidence of Lyme disease. Error bars represent the standard error of the mean for each group.

Figure 4. IgG anti-OspA antibody levels in PLDS and control subjects, as measured by ELISA. Mean anti-borrelia antibody level (mean normalized absorbance at 450 nm) was higher for the PLDS group (n=54) than the post-early Lyme healthy group (n=14) ($p<0.001$). The difference remained significant when limiting the analysis to specimens from individuals with a history of EM in both groups (n=34 for PLDS vs. n=14 for post-Lyme healthy) ($p<0.005$). Error bars represent the standard error of the mean for each group.

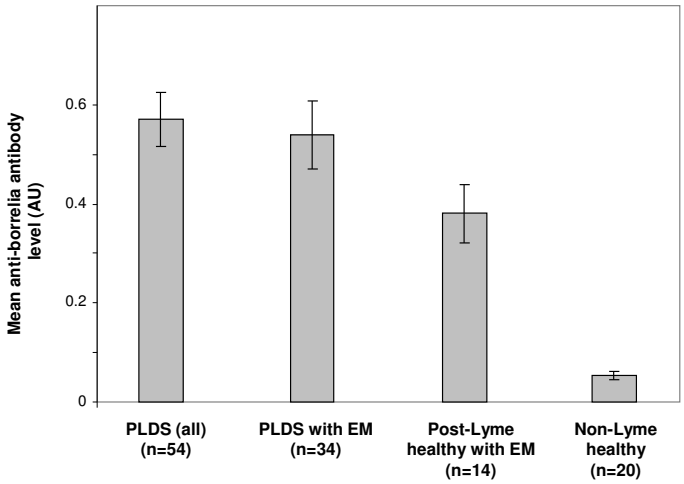

Figure 1

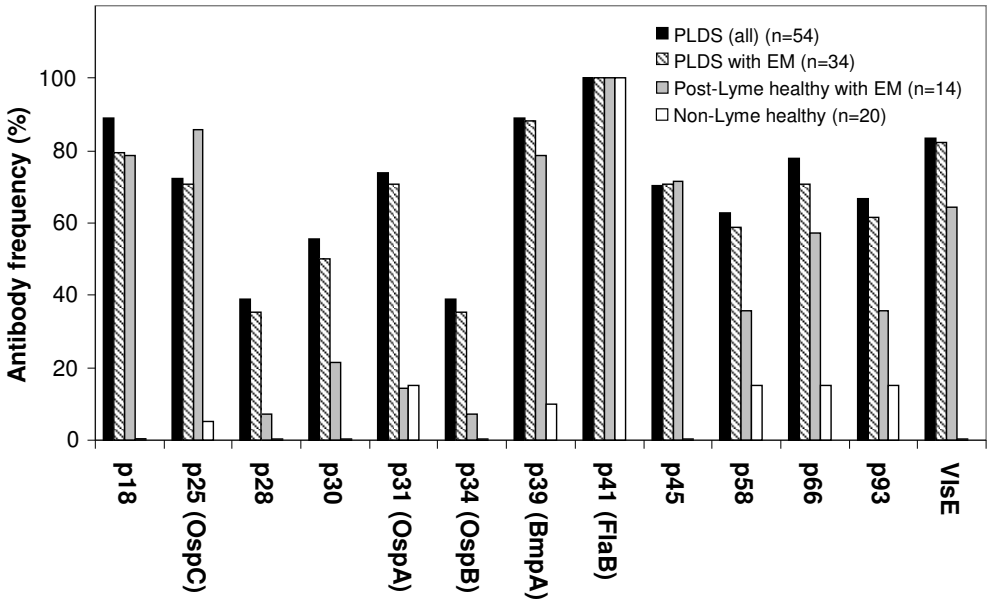

Figure 2

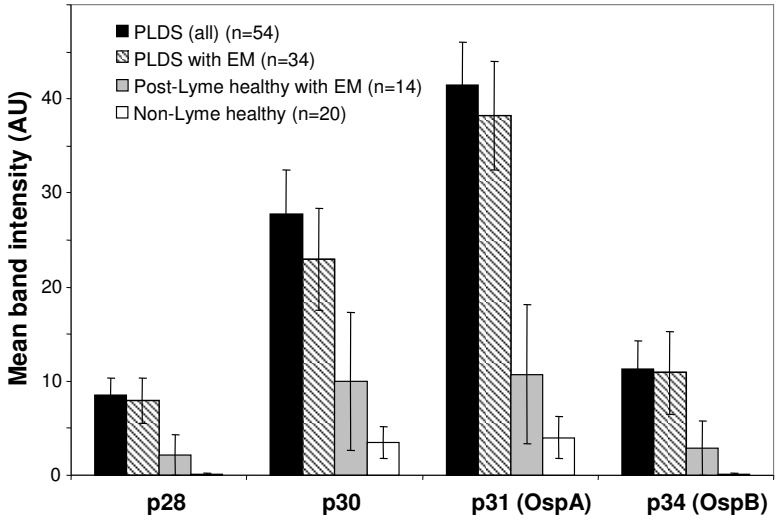

Figure 3

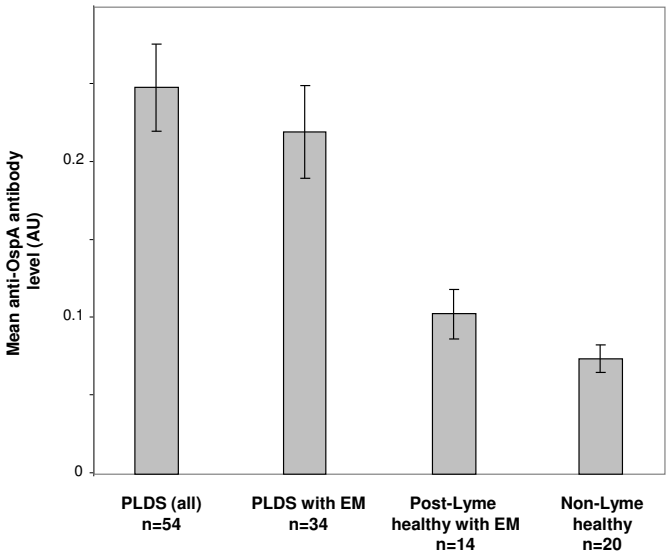

Figure 4