

The immune response against herpesvirus is more prominent in the early stages of MS

P. Villoslada, MD; C. Juste, MD; M. Tintore, MD; V. Llorenç, MD; G. Codina, MD; P. Pozo-Rosich, MD; and X. Montalban, MD, PhD

Abstract—Objective: To assess antibody levels against human herpesvirus type 6 (HHV-6), Epstein-Barr virus (EBV), and *Chlamydia pneumoniae* (CP) in serum from individuals in the early and late phase of MS. **Results:** A strong association was found between anti-HHV-6 immunoglobulin M antibodies and early MS (clinically isolated syndromes at high risk for MS, and short duration active relapsing-remitting MS) when compared with healthy controls and secondary progressive MS. Moreover, in this group of patients, titers for anti-EBV immunoglobulin G were also elevated. The authors found no association between the levels of serum antibodies against CP and MS, nor did they detect the presence of DNA for these pathogens in the serum of patients with MS. Finally, serum from two patients with other inflammatory neurologic diseases also had elevated immunoglobulin M antibodies to HHV-6, indicating that the presence of this antibody is not specific to MS. **Conclusion:** An immune response against herpesviruses such as HHV-6 and EBV is associated with early MS.

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The pathogenesis of MS is not well understood; however, there is strong evidence for an autoimmune component.^{1–3} The involvement of microbial infections in the pathogenesis of MS has long been recognized and substantiated by data from epidemiologic studies (clustering of cases, epidemics in the Faroe Islands, and migration studies) and biologic studies (detection of antibodies or DNA from microbes in MS brain tissue and the presence of oligoclonal bands).⁴ It had been proposed that infections contribute to the etiology of MS but there is no convincing evidence.^{4,5} However, some viruses have been detected in the brain tissue of patients, and an association between the immune response against some microbes and the disease has been described.^{6,7}

Several viruses and bacteria have been associated with MS. Of these, some herpesviruses such as Epstein-Barr virus (EBV)^{8–10} and human herpesvirus type 6 (HHV-6)^{11,12} have been repeatedly associated with MS, either by detection of an increased prevalence of antibodies against such virus in the serum of patients with MS or by detection of the virus in MS brains.^{4,11} Recently, *Chlamydia pneumoniae* (CP) has also been linked with the disease,¹³ although its relation with MS is unclear.¹⁴ The role of microbial infection in the pathogenesis of MS is complex. The analysis of brain tissue, serum, or CSF of patients long after the onset of the disease and the presence

of secondary autoimmune responses might impair identification of early immune responses against a pathogen. We assessed the presence of the humoral immune response specific to EBV, HHV-6, and CP in the early phase of MS.

Methods. Patients and samples. Patients with clinically isolated syndrome (CIS; n = 53) were prospectively selected for inclusion in the study. Patients with CIS were defined as follows: 1) having an episode suggestive of demyelinating disease that was not attributable to another disease and that involved any two or all of the optic nerve, brainstem, and spinal cord; 2) having a risk of developing MS, i.e., the presence of MRI studies suggestive of demyelinating disease (three or four Barkhof criteria¹⁵) or the presence of oligoclonal bands in the CSF in the case of not fulfilling the above Barkhof criteria; 3) being 18 to 50 years old; 4) having onset of the syndrome within 3 months of clinical and MRI examinations and serum collection. Clinical information, including MRI follow-up, from patients with CIS was obtained prospectively (table 1). More clinically detailed information on this cohort was published elsewhere.¹⁶ After a mean follow-up of 43 ± 17 months, 21 out of 53 patients (39%) had a second relapse of their demyelinating disease, becoming clinically definite MS.

Patients with clinically definite MS with relapsing-remitting course (RRMS; n = 49) were selected from a prospective cohort of patients already included in a study of interferon beta (IFNB) treatment.¹⁷ They fulfilled the following criteria: 1) had the RRMS subtype; 2) were between 18 and 50 years old; 3) had been diagnosed with MS for ≤ 5 years; 4) had an active form of the disease and fulfilled clinical criteria for treatment with IFNB in Spain (i.e., two relapses in the last 3 years); and 5) had serum collected before the onset of IFNB treatment. Patients with clinically definite MS with active secondary progressive course (SPMS; n = 50) were selected from placebo groups from two clinical trials running in our center that required active progression in the last years.

From the Neuroimmunology Unit (Drs. Villoslada, Tintore, Pozo-Rosich, and Montalban) and Department of Microbiology (Drs. Juste, Llorenç, and Codina), Hospital Vall d'Hebron, Barcelona; and Department of Neurology (Dr. Villoslada), University of Navarra, Spain.

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Address correspondence and reprint requests to Dr. Pablo Villoslada, Department of Neurology, University of Navarra, Pio XII 36, 31008 Pamplona, Navarra, Spain; e-mail: pvilloslada@unav.es

Table 1 Baseline characteristics of CIS group (n = 53) and ratio of conversion to MS (second relapse)

Characteristics	Value
Age at onset, y, mean (SD)	28.4 (7.6)
F/M	35/18
Initial symptom, n (%)	
Optic nerve	15 (28)
Spinal cord	20 (38)
Brainstem	13 (25)
Other	5 (9)
Baseline EDSS, mean (SD)	2.4 (1.3)
Positive OCB, n (%)	32 (60)
Abnormal MRI at onset, n (%)	42 (80)
Barkoff criteria, n (%)	
0	16 (30)
1	4 (7.5)
2	8 (15)
3	8 (15)
4	13 (24)
Second relapse, n (%)	21 (39)
(follow-up: 43 ± 17 months)	

CIS = clinically isolated syndromes; EDSS = Expanded Disability Status Scale score; OCB = oligoclonal bands.

The healthy control group (HC; n = 50) was selected from volunteers, healthy donors matched by age and sex with the CIS and RRMS group. The demographic and clinical data of each group are shown in table 2.

All patients were recruited from the same geographic area (Catalonia). Serum was obtained by vein puncture and stored at -80 °C until use. We also assured that patients were free of immunomodulatory treatments at the time of the blood drawing. As an assay internal positive control, we included sera from patients with PCR-confirmed infection with HHV-6, EBV, and CP. In addition, in order to assess the specificity of the test with MS, we also tested sera from patients with other neurologic inflammatory diseases (n = 10), including two patients with Guillain-Barré syndrome (GBS), three patients with Miller-Fisher syndrome (MFS), and five patients with chronic idiopathic demyelinating polyneuropathy (CIDP). This study was approved by the Ethical Research Committee in our center. Informed consent was obtained at the time of inclusion of each patient in the original study. This consent also enabled us to store the samples in the tissue bank for future MS research use.

ELISA. The detection of anti-HHV-6 immunoglobulin (Ig)G and IgM; anti-EBV viral capsid antigen (VCA) IgM, early antigen

IgG, and Epstein-Barr nuclear antigen (EBNA) IgG; and anti-CP IgM and IgG was performed by ELISA following the manufacturer's instructions. Anti-HHV-6 IgG and IgM antibodies were assessed using the ELISA kit from PANBIO (Windsor, Australia). Anti-EBV antibodies were assessed using an ELISA kit from Genbio (San Diego, CA) for EBV VCA IgM test and Meridian Diagnostics Inc. (Cincinnati, OH) for EBNA IgG and EBV-early antigen (D component) (EA[D]) IgG test. Anti-*C. pneumoniae* antibodies were assessed using an ELISA kit from LabSystems (Helsinki, Finland). Briefly, 100 µL of sera diluted 1:100 were added to the wells coated with the following antigens: HHV-6 viral lysate for anti-HHV-6 assays; recombinant EBNA-1 antigen, purified EBV-EA(D) extracted from cell cultures of P3HR1 cells, or EBV VCA for anti-EBV assays; and purified elementary *C. pneumoniae* bodies for anti-CP assays. Samples were incubated 20 to 60 minutes at 37 °C and washed five times. One hundred microliters of horseradish peroxidase conjugated antihuman IgM or IgG was added to each well and incubated 20 minutes at 37 °C. After washing five times, 100 µL of TMB or p-NPP was added, incubated 10 to 20 minutes at room temperature, and reaction was stopped and read in an ELISA reader at 405 to 450 nm (depending on each protocol). Each plate contained positive, negative, and cut-off control sera. In addition, the assays were validated in our laboratory by using sera from HHV-6, EBV, or CP PCR-confirmed infected individuals.

Results were expressed using arbitrary units (ELISA titers). Anti-HHV-6 assays were expressed using PanBio units = 10 × absorbance of sample/mean absorbance of cutoff. PanBio units > 20 were considered positive for IgM and PanBio units > 11 were considered positive for IgG. Anti-EBV assays were expressed using arbitrary units = absorbance sera/mean absorbance of reference serum. The tests were considered positive when units > 1.0. Anti-CP assays were expressed using arbitrary units = absorbance sample/absorbance calibrator (×130 for IgG). The tests were considered positive when anti-CP IgG units > 30 and anti-CP IgM units > 1.1.

As a positive control of ELISA test and to have a biologic reference for antibody titers, we tested the titers of such antibodies in individuals with acute infection. The antibody titers of adult patients from our center with confirmed infection with EBV (infectious mononucleosis) (n = 10) were 2.81 ± 0.82 for anti-EBV VCA IgM, and 3.7 ± 0.72 for anti-EBV EBNA IgG. The antibody titers from adult patients with confirmed infection with CP (atypical pneumonia) were 11.14 ± 3.81 for anti-CP IgM and 95.1 ± 14.1 for anti-CP IgG. Antibody titers (PanBio units) using the same ELISA kit in sera from 13 infants with exanthema subitum from Brazil were 43.8 ± 17.9 (range 20.4 to 84.7) for anti-HHV-6 IgM and 36.2 ± 11.4 (range 20.2 to 60.7) for anti-HHV-6 IgG (data provided by Dr. R. Freitas, Evandro Chagas Institute, Brazil¹⁸).

PCR. PCR amplification was performed on serum samples to detect the presence of free virus in blood. HHV-6 and EBV DNA were detected using the Herplex system (Pharma Gen) that amplifies a region of 194 base pairs (bp) from the viral DNA polymerase gene. To enhance sensitivity, the PCR products were hybridized in microtiter plates with a virus-specific probe. A region of 229 bp from the HM-1 and HR-1 primers was amplified from CP DNA as described by Campbell et al.¹⁹ following isolation using the Generation DNA Purification Capture Column (Gentra Systems). Positive and negative controls of the reaction were included. The PCR

Table 2 Main clinical features of the cohort

Group	N	Age at onset, y, mean (SD)	Female/male (ratio)	EDSS, mean (SD)	Duration, y, mean (SD)
CIS	53	28.4 (7.6)	35/18(1.9)	2.4 (1.3)	<1*
RR	49	32.1 (7.1)	32/17(1.8)	1.5 (1)	1.8 (0.9)
SP	49	45 (9.7)	33/16(2.0)	6.8 (1.7)	15.9 (8.3)
HC	50	28.7 (6)	34/16(2.0)	—	—

* Duration of the disease in the CIS group was <3 months by definition of inclusion criteria.

EDSS = Expanded Disability Status Scale score; CIS = clinically isolated syndromes; RR = relapsing-remitting; SP = secondary progressive; HC = healthy controls.

product was detected by hybridization with a CP-specific probe (GEN-ETI-KTM DEIA, DiaSorin).

Statistical analysis. Statistical analyses were performed using SPSS 9.0 software. We compared the differences in titers between groups with the analysis of variance test and post hoc analysis was performed using the Duncan test. Differences in qualitative variables, such as the number of positives or negatives for an antibody test, were assessed using the χ^2 test. Association between titers and quantitative clinical variables were studied using the Pearson linear correlation test. We performed a logistic regression model using the presence of anti-HHV-6 IgM antibodies as a dependent variable and the age at onset, duration, and EDSS score as independent variables. The clinical utility of the anti-HHV-6 IgM test to predict conversion to RRMS was assessed using a logistic regression analysis to compare the positive reaction with antibodies with the presence of a second relapse (main outcome) or the presence of three or four Barkhof criteria in the MRI as the gold standard test.²⁰ We calculated the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy.

Results. Increased humoral responses against HHV-6 and EBV in early MS. We assessed the humoral immune response against HHV-6, EBV, and CP in the early phase of MS by measuring the serum levels of both IgG and IgM antibodies by ELISA. Antibody titers for HHV-6 and EBV were elevated at the early stages of MS when compared with late MS and HC (figure). Patients with CIS and RRMS had higher levels of anti-HHV-6 IgM and anti-EBV EBNA IgG antibody titers than controls ($p < 0.0001$ and $p = 0.041$). The titers of anti-HHV-6 IgM antibodies were 15.56 ± 6.9 (mean \pm SD; $p < 0.001$) in patients with CIS and 15.02 ± 8.6 ($p = 0.002$) in patients with RRMS compared to 10.19 ± 6.4 for the HC group and 11.88 ± 7.5 for the patients with SPMS (figure, A). In addition, the number of patients with anti-HHV-6 IgM antibodies was higher in the CIS (15/53, 30%) and RRMS (8/42, 16%) groups than in the HC (3/50, 5%) and SPMS (5/50, 10%) groups ($p = 0.002$). The titers of anti-HHV-6 IgM in patients with positive antibodies (26.5 ± 7.1 ; range 20.1 to 55.2) were in the lower end of the range found during acute HHV-6 infection (see Methods). No differences were observed in the antibody titer or the percentage of patients with presence of anti-HHV-6 IgG antibody.

Higher titers of anti-EBV EBNA IgG antibodies were observed in the serum of patients with CIS compared to the HC and SPMS groups ($p = 0.012$ and $p = 0.037$) (figure, B). However, the percentage of patients that registered the presence of anti-EBV EBNA IgG antibodies was not different between groups ($p = 0.69$). We found no differences for the anti-EBV VCA IgM or anti-EBV EA IgG antibodies.

We found no association between anti-CP antibodies and MS, either in the titer or in the percentage of patients with anti-CP IgM or IgG antibodies (figure, C). Patients with other neurologic inflammatory diseases were negative for the presence of anti-EBV or CP antibodies. One patient with GBS and another with MFS had positive anti-HHV-6 IgM antibodies.

Finally, we assessed whether the increased humoral response to herpesvirus was related to the presence of the virus in the bloodstream by amplifying viral DNA from serum. We were unable to detect the presence of DNA from HHV-6, EBV, or CP in the serum of any patient tested, even when the serum from patients with anti-HHV-6 IgM antibodies was analyzed (data not shown).

Association between anti-HHV-6 IgM and anti-EBV IgG and clinical variables. Given the association between anti-HHV-6 IgM antibodies and the early phase of MS, it was not surprising that an elevated titer of anti-HHV-6 IgM antibodies correlated with a shorter duration of the disease ($p < 0.0001$), lower age at onset (95% CI 32.26 to 36.86 years, $p < 0.0001$), and lower Expanded Disability Status Scale (EDSS) scores (95% CI 2.5 to 3.2, $p < 0.0001$). No association could be established between the percentage of anti-HHV-6 IgM-positive patients and sex ($p = 0.287$) or the presence of oligoclonal bands ($p = 0.642$). Similarly, we found a correlation between anti-EBV EBNA IgG antibodies and a shorter duration of the disease ($p < 0.0001$), lower age at onset (95% CI 33.26 to 36.86 years, $p < 0.0001$), and lower EDSS scores (95% CI 2.5 to 3.3, $p < 0.0001$). Finally, we found no association between the site of onset in patients with CIS and any of the antibodies tested.

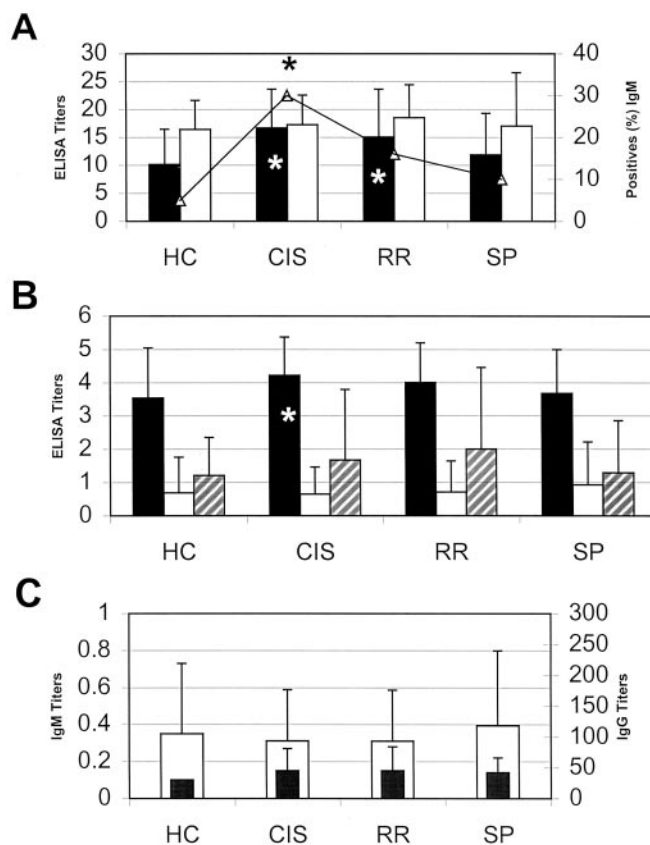


Figure. Microbial specific antibody results measured by ELISA in patients with MS and controls. (A) The results for anti-human herpesvirus (HHV)-6 antibodies in the four groups: healthy controls (HC) and patients with clinically isolated syndromes (CIS), relapsing-remitting MS (RR), and secondary progressive MS (SP). Results are presented as the mean and SD. Immunoglobulin (Ig)M titers (black bars) and IgG titers (white bars) are shown using the arbitrary ELISA units (left axis) given by manufacturers of ELISA kits (see Methods). The percentage of positive patients for anti-HHV-6 IgM antibodies is shown as a line and referred to the right axis. *Significant differences between anti-HHV-6 IgM titers and percentage of positives in CIS and RR groups compared with HC and SP (see text for details). (B) The ELISA titers (see Methods) for anti-Epstein-Barr virus (EBV) Epstein-Barr nuclear antigen (EBNA) IgG (black bars), early antigen IgG (white bars), and viral capsid antigen (VCA) IgM (lined gray bars) antibodies tested in the four groups. *Significant differences between anti-EBV EBNA IgG between CIS and HC groups. (C) Results for anti-Chlamydia pneumoniae IgM (black bars) and IgG (white bars) antibodies for the four groups. No differences between groups were detected.

Value of HHV-6 serology in predicting conversion to MS. Patients with CIS have an elevated risk of developing MS, which has important prognostic and therapeutic consequences. To date, only MRI studies have been shown to improve our capacity to predict which patients will have a second relapse.²⁰ Recently, the combination of MRI studies with biologic data (HLA-DR2 genotype) was shown to provide additional information to assess the risk of developing MS.²¹ Because higher anti-HHV-6 IgM titers were associated with the CIS group, we performed a preliminary analysis to assess whether this test might be useful in predicting conversion to MS.

We found no correlation between the percentage of patients

Table 3 Diagnostic performance of anti-human herpesvirus (HHV)-6 immunoglobulin M (IgM) testing to predict conversion to MS

Test	S	SP	PPV	NPV	A
Anti-HHV-6 IgM	40	75	52	64	50
3–4 Barkoff criteria	68	78	68	78	70
Anti-HHV-6 IgM and 3–4 Barkoff criteria	31	59	35	55	45
Anti-HHV-6 IgM or 3–4 Barkoff criteria	77	60	56	79	65

Values are percentages.

S = sensitivity; SP = specificity; PPV = positive predictive value; NPV = negative predictive value; A = accuracy.

with a positive response for anti-HHV-6 IgM test or patients with elevated titers and the presence of a second relapse in the follow-up period. As expected, we found a correlation in our cohort between the designation of three or four Barkhoff criteria and the development of a second relapse ($p = 0.02$). By contrast, no correlation was seen between the serologic test and the MRI findings at the onset and after 1 year using any of the MRI criteria tested. Testing for anti-HHV-6 IgM antibodies in addition to the requirement of fulfilling MRI criteria increased the sensitivity of the test when compared with the MRI test alone from 68% to 77% in predicting patients who would have a second relapse in the follow-up (table 3). However, testing for anti-HHV-6 IgM antibodies produced a negative impact on the specificity and positive predictive value.

Discussion. In this study, we addressed whether the ability to detect an immune reaction against MS-related virus or bacteria could be correlated with the timing of MS development. We found that an immune response to HHV-6 and EBV could be clearly detected in the early phase of the disease (after the first relapse) and in the first years of the disease. However, we were unable to detect such a response in patients who had had the disease for longer periods of time (SPMS). The bystander activation hypothesis²² for autoimmune diseases suggests that the initial immunologic events degenerate into a more widespread autoimmune reaction with the possible disappearance of the initial trigger. This could account for the difficulties in detecting immune responses to infections in patients with long-term MS. Our results support the idea that studying patients at the early stages of the disease is more informative than studying patients with longstanding MS. Indeed, the recent criteria proposed by The International Panel on MS Diagnosis²³ places emphasis on the early diagnosis of MS.

Our data confirm previous reports that have suggested an association between HHV-6 and EBV and MS. The induction of an IgM response against HHV-6 suggests the presence of an active infection or reactivation of the latent virus. However, in our attempts to amplify DNA from HHV-6, we were unable to detect the free virus in serum. Indeed, previous studies reported an increase in the levels of IgM against HHV-6 in the serum of patients with early

MS.^{11,24} HHV-6 is a common pathogen (more than 80% of adults are seropositive) and remains in a latent state in immune cells, neurons, and oligodendrocytes.⁴ Although it can be detected in the brain of HC it is found mainly in periplaque oligodendrocyte cells,^{25,26} microglia, and infiltrating lymphocytes in MS brains.^{26,27} We hypothesize that the presence of anti-HHV-6 IgM antibodies could be secondary either to a silent primary infection in nonexposed individuals or to a reactivation of latent HHV-6 resulting in the exposure of new epitopes that are able to induce a novel IgM response. The former hypothesis would suggest that patients with MS represent a subset of individuals who were not exposed to the virus in childhood and are experiencing a late infection.²⁸ In the second scenario, predisposed individuals who have a reactivation of a common latent pathogen such as HHV-6 might develop an immune response against critical epitopes.²⁹ Finally, the preliminary results showing the presence of anti-HHV-6 IgM antibodies in some patients with other neurologic inflammatory diseases such as GBS and MFS might suggest a common role for HHV-6 in autoimmune demyelinating diseases that would require further exploration, and suggest that the presence of this antibody is not specific to MS.

The increased levels of EBV EBNA IgG antibodies that we and others^{8,10,30} have found in patients with MS could be primarily triggered by viral antigens from latent EBV in immunized individuals, but could also be a secondary phenomenon resulting from an increase in nonspecific immune reactivity as occurs in MS. An increase in immune reactivity could be induced by EBV reactivation, providing the infectious context to the immune response in a model of the bystander activation process. Ascherio et al.¹⁰ found anti-EBV EBNA IgG antibodies in patients before the onset of MS, highlighting the importance of studying early MS. By contrast, we did not find association with EBV EA IgG in early MS, although a different technique (immunofluorescence) or different populations in terms of viral exposition might account for such differences. Finally, Ascherio et al. did not find a decrease in the prevalence of such antibodies with longer disease period, although their observation period after the onset of the disease was 60 months, which is much shorter than the 15 years' duration of our SPMS group.

We did not find an increase of CP-specific antibodies or bacterial DNA in serum as a free pathogen. This finding does not necessarily rule out a role of CP in MS but may represent population differences or a reduced sensitivity of the technique used. Moreover, because CP is an intracellular pathogen, we would not expect to detect DNA in serum except during active infection.

From a clinical point of view, it is of particular interest to determine which patients with CIS are going to develop a chronic disease in order to start the most effective therapy at the earliest stages. To date, the most useful tool in predicting conversion to

MS is MRI.^{15,20,23} The combination of MRI data with biologic testing is a valuable tool for determining the risk of developing MS at the onset of the disease.^{21,31} Thus, we explored the usefulness of the anti-HHV-6 IgM antibody test in predicting conversion from CIS to RRMS by comparing it with the gold standard test (MRI). Our preliminary study suggested that testing for anti-HHV-6 IgM antibodies increased the sensitivity of the MRI test to identify patients who will develop a second relapse, but with a decrease in the specificity of the test. However, because assessing the possibility of using anti-HHV-6 IgM antibody levels as a predictor for conversion to RRMS was not the primary objective of this study, any conclusion that we might draw could suffer from a lack of power. Thus, we cannot rule out that levels of anti-HHV-6 IgM antibodies may be useful as an indicator of conversion to MS, for which a specifically designed clinical study would be necessary.

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