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Identification of human herpesviruses 1 to 8 in Tunisian multiple sclerosis patients and healthy blood donors

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Abstract Members of the human *Herpesviridae* family are candidates for representing the macroenvironmental factors associated with multiple sclerosis (MS) pathogenesis. To verify the possible role of human herpesviruses (HHVs) as triggering or aggravating factors in relapsing–remitting multiple sclerosis clinical outcome, we studied the prevalence of all eight human herpesviruses in whole blood samples collected from 51 MS patients and from 51 healthy controls. The presence of DNA of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8) was searched by specific nested polymerase chain reaction. HHVs were significantly more prevalent in the blood of MS patients than in those of the controls ($P<10^{-4}$). HSV-1, HSV-2, HCMV and HHV-8 were negative in both MS patients and controls samples. In MS patients, EBV, HHV-7, HHV-6 and VZV were detected in 31.3%, 33.3%, 5.8% and 7.8% of samples, respectively, compared with 3.9%, 9.8%, 1.96% and 1.96%, respectively, of samples from controls. We found a statistically significant difference only for EBV DNA and for HHV-7 DNA prevalence ($P<0.001$ and $P=0.03$). Although these results indicate lack of apparent association in terms of gender, type of diagnosis, symptoms, disease score and β interferon treatment between EBV or HHV-7 to MS among Tunisian patients, heterogeneity related to genetic polymorphism as well as geographical distribution of the disease and of pathogens may be of significance.

Keywords MS · Nested PCR · EBV · HHV-7 · Genetic polymorphism

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Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system characterized by an autoimmune pathogenic process in genetically predisposed individuals (Sospedra and Martin 2005). MS usually affects young adults and women more frequently than men and is clinically characterized by the dissemination in space and time of relapses which refer to the occurrence of neurological symptoms and signs. About 80% of MS patients start with a relapsing–remitting course that, over time, transforms into a secondary progressive course. In a smaller group of patients (20%), MS begins with a primary progressive (Compston and Coles 2008).

The mechanism that triggers this autoimmune disorder is still not clear; however, the hypothesis that viral infections might play an aetiological or cofactorial role in the pathogenesis of MS, both on the onset of the disease or during acute episodes, has been repeatedly suggested.

Accumulating data, including animal models, human models of virus inducing demyelination, epidemiologic, and laboratory findings, have demonstrated that viruses and host genetic factors can interact to cause immune-mediated demyelination (Giovannoni and Ebers 2007). While many viruses have been postulated as a possible cause of MS, to date, no "MS associated virus" has been definitively shown to be linked with this disease. Alternatively, ubiquitous viruses are being considered environmental "triggers", which may be involved in the MS disease process (Giovannoni et al. 2006). Herpesviruses are particularly interesting as they are prone to localize in the central nervous system. They share the property to persist during the host lifetime, have the ability to be reactivated by several stimuli and most of them are common causes of diseases. The majority of human herpesviruses (HHVs) have been studied as possible environmental factors related to MS. In recent years, several reports in the literature have suggested the possible involvement of herpes simplex virus (HSV) (Hawkes et al. 2006; Bello-Morales et al. 2005), Epstein– Barr virus (EBV) (Nielsen et al. 2007; Cepok et al. 2005), varicella zoster virus (VZV) (Mancuso et al. 2007; Sotelo et al. 2007), cytomegalovirus (HCMV) (Zivadinov et al. 2006) and human herpesvirus type 6 (HHV-6) (Alvarez-Lafuente et al. 2007; Gardell et al. 2006) in the aetiology of the disease, but no definite conclusion has been reached.

Tunisia is considered as a low zone of prevalence for multiple sclerosis. Consequently, only very few studies have taken interest in this disorder in North Africa. To our knowledge, this is the first report studying the association between herpesviruses and multiple sclerosis in the Tunisian population. The aim of this study was to establish the prevalences of HSV, VZV, EBV, HCMV, HHV-6, HHV-7 and HHV-8 in the whole blood of MS patients and controls, to evaluate the possible involvement of these viruses in the pathogenesis of MS.

Materials and methods

Patients and samples

We analysed 102 whole blood samples: 51 from patients with defined relapsing–remitting multiple sclerosis (RRMS), enrolled at the department of Neurology, Fattouma Bourguiba Hospital, Monastir, Tunisia, and 51 from healthy controls who had donated blood at the Regional Center for Blood Transfusion, Monastir, Tunisia (Table 1). In the patient group, 18 were having an MS relapse when blood was drawn (48 h after onset of symptoms), and 33 were in remission. There were 18 men (mean age, 43.31 years; age range, 24–64; SD 16.970) and

Table 1 Demographic and clinical data of MS patients and controls

Group	Subjects			Age of recruitment, mean±SD (range)			Age at onset, mean±SD (range)			Duration, mean±SD (range)			EDSS, mean±SD (range)		
	T	M	F	T	M	F	T	M	F	T	M	F	T	M	F
MS	51	18	33	38.26±6.36 (19–64)	43.31±16.97 (24–64)	35.81±6.36 (19–54)	32.81±14.84 (16–58)	37.56±13.43 (16–58)	30.51±14.84 (17–51)	5.46±8.48 (1–15)	5.87±3.53 (1–12)	5.27±8.48 (1–15)	2.92±1.41 (1–8)	2.94±3.53 (1–8)	2.9±4.94 (1–8)
HBD	51	23	28	36.26±8.56 (18–58)	38.26±9.36 (20–52)	34.26±7.47 (19–49)	–	–	–	–	–	–	–	–	–

33 women (mean age, 35.81 years; age range, 19–54; SD 6.363). The mean age at disease onset was 32.81 years (SD 14.849, range 16–58), the mean disease duration was 5.46 years (SD 8.485, range 1–15) and mean Extended Disability Status Scale (EDSS) score was 2.921 (SD 1.414, range 1–8). Diagnostic criteria incorporate magnetic resonance imaging, cerebrospinal fluid and evoked potentials testing. Disability was assessed with EDSS.

Among MS patients, 25 were receiving interferon β treatment, but none received steroid treatment prior to blood sampling. In the control group of healthy blood donors, there were 23 men (mean age, 38.26 years; age range, 20–52; SD 9.36) and 28 women (mean age, 34.26 years; age range, 19–49; SD 7.47). This study was approved by the local ethics committee, and all of the participants gave informed consent before the experimental procedures.

DNA extraction and PCR assays

After collection, DNA was extracted from 200 μL of total blood using spin column technique of QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and eluted in 100 μL of water. Two negative controls, consisting of reagents only, were processed with each set of ten samples.

All specimens were examined for the presence of HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6, HHV-7 and HHV-8 DNA by specific nested polymerase chain reaction (n-PCR) using the appropriate set of primers (Table 2). About 100 ng of extracted DNA of each sample was initially amplified in a reaction solution of 50 μL, containing 1.5 mM of $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP, 1× PCR buffer and 0.5 U Taq polymerase (Applied Biosystems, Foster City, CA).

The PCR conditions for herpesviruses are shown in Table 2. In all reactions, the primary PCR mixture was heated for 5 min at 94°C and subjected to amplification for 35 cycles according to the data shown in Table 2; the PCR reaction ended with an elongation step at 72°C for 10 min.

HHV-6 variants were characterized by restriction enzyme cleavage, as previously described by Di Luca et al. HHV-6 strains segregate in two different variants (HHV-6A and HHV-6B), closely related to each other but clearly distinguishable on the basis of biological and molecular characteristics (Di Luca et al. 1996).

In each PCR reaction, we performed at least two negative controls using sterile double-distilled water instead of the sample. PCR for detecting human β actin gene was performed on 10 ng of all samples, to ensure that they were suitable for DNA amplification. Aliquots of the PCR reactions were electrophoresed in 2% agarose gels and analysed with ethidium bromide staining.

Statistical analysis

The results were analysed through standard statistical methods applied in case–control studies. The correlation of each of the studied characteristics with viral infection was evaluated by Pearson's χ^2 analysis or Fisher's exact test where indicated (expected frequencies <5).

In all cases, $P<0.05$ was considered statistically significant. The analyses were performed using SPSS software, version 12 (SPSS Inc., Chicago, IL, USA).

Results

All 102 samples from controls and MS patients were positive for the β actin gene. The results on the prevalence of HHV DNA are shown in Table 3. Viral DNA was detected by PCR in 38 of 102 total blood samples for at least one member of the *Herpesviridae* family. A statistically significant difference ($p<10^{-4}$) was found when we compared the whole prevalence of herpesviruses in MS patients (58.82%) and controls (15.68%). VZV DNA was found in 4 of 51 MS patients (7.84%) and in 1 of 51 healthy control (1.96%). EBV DNA was detected in 16 of 51 MS patients (31.37%) and in 2 of 51 healthy controls (3.92%) ($p=0.00027$). HHV-6 DNA was present in 3 of 51 MS patients (5.88%) and in 1 of 51 healthy controls (all of them were variant B, as determined by endonuclease cleavage of PCR products by HindIII and HinfII). HHV-7 DNA was harboured in 17 of 51 MS patients (33.33%) and in 5 of 51 healthy control (9.8%) ($p=0.0038$). Positive signals for VZV, EBV, HHV-6 and HHV-7 were present only after nested PCR, suggesting that low amounts of virus were present in positive samples.

Finally, no positive samples to HSV-1, HSV-2, HCMV or HHV-8 were detected in MS patients or controls. The simultaneous presence of two DNA viruses was detected as follows: EBV and HHV-7 in six MS patients (11.76%), whereas the combination of HHV-6 and HHV-7 was found in two cases (3.92%) and the combination of EBV and HHV-6 in only one case (1.96%), Finally, concurrent detection of VZV and EBV was found in only one case (1.96%).

There was only one case of simultaneous presence of EBV and HHV-7 in healthy controls. Patients with concurrent detection of EBV and HHV-7 were more likely to have worst prognosis (high EDSS score, attack phase more than two times a year and a duration of the disease more than 5 years).

Within the MS group, β interferon-treated patients did not differ from the untreated patients in viral prevalences of any of the studied herpesviruses (Table 3). Statistical analysis did not reveal any association between the

Table 2 Oligonucleotide primer sequences and polymerase chain reaction conditions used in this study

Target	Genome region	Primer sequences	PCR conditions	Amplification size (bp)
HSV1	DNA POL	Outer primers		329
		5′-AAC AAG GAG GAG GTC GAC AG-3′	94°C/5 min; 35 cycles	
		5′-GTG GAC AGG TCG TAG AGC AG-3′		
		Inner primers		
		5′-ACC GAC GTG TAC TAC TAC GA-3′	94°C/30 s, 55°C/30 s, 72°C/30 s; 72°C/7 min	
		5′-ATG AGC TTG TAT GCC GGT AG-3′		
HSV2	UL48	Outer primers		289
		5′-CCA GCA TCT CCA AGT CGA AG-3′	94°C/5 min; 35 cycles	
		5′-ACA TTC GAG AGC ACC TGA AC-3′		
		Inner primers		
		5′-GGC GTC ATA TCC ACC TCC TC-3′	94°C/30 s, 55°C/30 s, 72°C/30 s; 72°C/7 min	
		5′-TTA CGG ATC GAC AAT CGA GG-3′		
VZV	ORF62 gene for IE62 transactivator	PCR1		443
		5′-CTC CCG TTC CGC ATG TAG GC-3′	95°C/5 min; 35 cycles	
	ORF06 gene for IE62 transactivator	5′-GGT CCG TCA AGT GGC ATC GTT ATT-3′	95°C/20 s, 65°C/30 s, 72°C/30 min; 72°C/15 min	
		PCR2		
		5′-ACG GAC ATA GTT TAA GAC TGC C-3′	95°C/5 min; 35 cycles	
		5′-GAT GGC GAT ACA CTT TGA TGA T-3′	95°C/20 s, 58°C/30 s, 72°C/3 min; 72°C/15 min	
EBV	Seminested latent membrane protein-1 gene	Outer primers		283
		5′-GCG ACT CTG CTG GAA ATG ATG-3′	95°C/5 min; 35 cycles	
		5′-TGA TTA GCT AAG GCA TTC CCA-3′	94°C/1 min, 55°C/1 min, 72°C/30 s; 72°C/10 min	
		Inner primers		
		5′-AAA ATT GAC GGA AGA GGT TGA-3′	95°C/5 min; 20 cycles	
		5′-TGA TTA GCT AAG GCA TTC CCA-3′	94°C/1 min, 57°C/1 min, 72°C/30 s; 72°C/10 min	
CMV	Major immediate early antigen	Outer primers		110
		5′-AAG CGG CCT CTG ATA ACC AAG CC-3′	94°C/5 min; 35 cycles	
		5′-AGC ACC ATC CTC CTC TTC CTC TGG-3′	94°C/30 s, 57°C/30 s, 72°C/30 s; 72°C/7 min	
		Inner primers		
		5′-AGT GTG GAT GAC CTA CGG GCC ATC G-3′	94°C/5 min; 25 cycles	
		5′-GGT GAC ACC AGA GAA TCA GAG GAG C-3′	94°C/30 s, 59°C/30 s, 72°C/30 s; 72°C/7 min	
HHV6	KAL3 conserved herpesvirus transactivator U42	Outer primers		525
		5′-ACC TTA CAA CGG AGA CGC C-3′	94°C/5 min; 35 cycles	
		5′-ACG ATG GAC ATG GCT TGT TG-3′	94°C/1 min, 63°C/1 min, 72°C/1 min; 72°C/10 min	

Table 2 (continued)

Target	Genome region	Primer sequences	PCR conditions	Amplification size (bp)
		Inner primers		
		5′-CAA AAC AAC GCA TCC GAG AC-3′	94°C/5 min; 35 cycles	
		5′-AGG TTT ACC GCA GAG TTG CC-3	94°C/1 min, 61°C/1 min, 72°C/1 min; 72°C/7 min	
HHV7	KAL3 conserved herpesvirus transactivator U42	Outer primers		574
		5′-AAG CTG CAA GAC GGA GTT GT-3′	94°C/5 min; 35 cycles	
		5′-AGT ATT CCG GTG AAG CAC GA-3′		
		Inner primers		
		5′-CGC GTA TAG ACT GAG GTT GT-3′	94°C/1 min, 58°C/1 min, 72°C/1 min; 72°C/10 min	
		5′-CCA GCT CAT AGG ATT CGA GA-3′		
HHV8	Minor capsid protein homolog ORF26 gene	Outer primers		160
		5′-GCC GAA AGG ATT CCA CCA T-3′	94°C/5 min; 35 cycles	
		5′-TCC GTG TTG TCT ACG TCC AG-3′	94°C/1 min, 58°C/1 min, 72°C/1 min; 72°C/10 min	
		Inner primers		
		5′-ACG GAT TTG ACC TCG TGT-3′	94°C/5 min; 35 cycles	
		5′-AAT GAC ACA TTG GTG GTA TAT-3′	94°C/1 min, 56°C/1 min, 72°C/1 min; 72°C/7 min	

Table 3 Total prevalence of human herpesviruses in peripheral blood of MS patients and healthy blood donors and breakdown according to disease course, gender and β interferon treatment

	HSV1	HSV2	VZV	EBV	CMV	HHV6	HHV7	HHV8	HHVs
MS patients (n=51)	0	0	4	16	0	3	17	0	40
			7.84%	31.37%		5.88%	33.33%		78.43%
Healthy controls (n=51)	0	0	1	2	0	1	5	0	9
			1.96%	3.92%		1.96%	9.8%		17.64%
p value	NS	NS	0.16	0.00027	NS	0.3	0.0038	NS	10^{-4}
MS patients in relapse (n=18)	0	0	3	6	0	1	5	0	15
			16.66%	33.33%		5.55%	27.77%		83.33%
MS patient in remission (n=33)	0	0	1	10	0	2	12	0	25
			3.03%	30.30%		6.06%	36.36%		75.75%
p value	NS	NS	0.08	0.82	NS	0.94	0.53	NS	0.17
MS ♂ patients (n=18)	0	0	1	6	0	1	5	0	13
			5.55%	33.33%		5.55%	27.77%		72.22%
MS♀ patients (n=33)	0	0	3	10	0	2	12	0	27
			9.09%	30.3%		6.06%	36.36%		81.81%
p value	NS	NS	0.65	0.82	NS	0.94	0.99	NS	0.42
MS-treated patients (n=25)	0	0	2	9	0	2	8	0	21
			8%	36%		8%	32%		84%
MS-non-treated patients (n=26)	0	0	2	7	0	1	9	0	19
			7.69%	26.92%		3.84%	34.61%		73.07%
p value	NS	NS	0.96	0.48	NS	0.52	0.84	NS	0.34

presence of herpesvirus DNA and MS disease activity, gender, age of onset or score (Table 3). Radiologic data did not show any correlation with viral prevalence in our MS population.

Discussion

The *Herpesviridae* family is a good candidate to play the role of environmental factor in the pathogenesis of MS; it comprises neurotropic viruses establishing latency after primary infection that can reactivate and are capable of producing demyelination. We designed our study to investigate the prevalence of all human herpesviruses types 1–8 in the blood of MS patients and healthy blood donors from Tunisia and to study the relationship between virus presence and clinical MS parameters.

The findings described here show that HHVs infection is more frequent in the blood of MS patients than in healthy blood donors ($p<10^{-4}$); however, when we analysed the prevalence of each of the viruses, we found a statistically significant difference only for EBV and HHV-7. EBV has been already suspected of being involved in MS as a potential aetiopathogenic factor (Haahr et al. 1995; Munch et al. 1998a). The data obtained in the present study show a high frequency of EBV DNA in peripheral blood mononuclear cells (PBMCs) of RRMS with a statistically significant difference compared to healthy controls indicating that EBV may be involved in the pathogenesis of MS. The published results on EBV DNA in PBMCs from relapsing MS patients are contradictory. Ferrante et al. (1997) collected serial blood samples beginning at the onset of an exacerbation and frequently detected EBV DNA in PBMCs early in the relapse. However, Alvarez-Lafuente et al. (2006) and Sotelo et al. (2007) did not detect any increase in EBV DNA in relapse compared with remission. Lunemann et al. (2006) and Lindsey et al. (2009) measured EBV DNA levels in peripheral blood lymphocytes and found that the levels in MS were non-significantly higher than controls. Also our results fail to show any correlation between EBV presence and MS disease activity.

In our study, HHV-7, along with EBV, was the virus most commonly detected in MS patients. A possible association between human herpesvirus 7 (HHV-7) and MS has not been extensively studied, and few reports are available. The virus has been found to be equally prevalent in a latent form in peripheral blood mononuclear cells from MS patients and healthy controls (Rotola et al. 2000; Alvarez-Lafuente et al. 2002). Soldan et al. (2000) measured the lymphoproliferative response to HHV-7-infected cell lysate and found no significant difference between patients and controls. Also, Taus et al. (2000) reported no relationship between MS and HHV-7.

In the present study, we found that 33% of MS patients harboured HHV-7 DNA in their peripheral blood versus 9.8% in healthy controls. This result is unexpected; in fact the scientific literature concordantly describes a very high prevalence of HHV-7 infection in PBMCs of the healthy population, with percentages higher than 70% (Tomsone et al. 2001). At the moment, we do not know if this lower prevalence is characteristic of the Tunisian population, and the result needs to be confirmed. Nevertheless, the higher prevalence of HHV-7 DNA observed in MS patients does not correlate with MS activity.

HHV-6 can induce neurological diseases (Huang et al. 1991; Ishiguro et al. 1990), and it has been suggested as an aetiological agent of MS (Alvarez-Lafuente et al. 2002; Soldan et al. 1997). In our study, we did not observe significant differences in the frequency of HHV-6 DNA between MS patients (5.8%) and healthy controls (1.9%). Our results are compatible with previously published data, with the exception of the low prevalence of infection detected in our samples. For example, Rotola et al. (2000) found a prevalence of 41% among MS patients compared with 29% in healthy blood donors (with a nested PCR of a ten-molecule sensitivity); Kim et al. (2000) found a 20.6% prevalence among MS patients and 0% among donors (sensitivity of the nested PCR not indicated); moreover, Alvarez-Lafuente et al. (2002) found a 49% prevalence among MS patients and 21.6% among donors (sensitivity of the nested PCR ten copies).

Although VZV has been postulated as a possible candidate to participate in MS (Mancuso et al. 2007; Sotelo et al. 2007), the results of most epidemiological or serological studies failed to confirm this link leaving uncertain the participation of VZV in the aetiology of MS. A report evaluating 40 studies indicated that there is insufficient evidence to support the association of MS with VZV infection (Marrie and Wolfson 2001). In the present study, we found 7.84% of MS patients with positive VZV DNA in their peripheral blood versus 1.96% in controls. No statistical significance was reached, but the numbers analysed were relatively low. Interestingly, a recent study analysed a very large number of patients, showing that subject experiencing a herpes zoster attack had a significantly higher risk of developing MS within 1 year (Kang et al. 2011). Therefore, a potential role for VZV in MS cannot yet be dismissed.

No result was obtained for the other herpesviruses; in fact HSV-1, HSV-2, HCMV and HHV-8 were absent both in patients' and in controls' PBMCs. Our negative results differ from previously published data that reported the presence, with similar prevalences between MS patients and controls, for HSV (31% vs 32%) (Ferrante et al. 2000), HCMV (26% vs 23%) (Alvarez-Lafuente et al. 2002) and HHV-8 (12% vs 9%) (Rotola et al. 1999). However, even

the mentioned studies did not suggest any association between herpes simplex, HCMV and HHV-8 presence and MS.

At the moment, we are unable to explain the apparent discrepancy between the low prevalence of human herpesviruses in the Tunisian healthy population and previous results obtained in different populations (Alvarez-Lafuente et al. 2002; Rotola et al. 1999, 2000). Presently, we do not know whether the difference is a population-based phenomenon, or due to inter-laboratory technical discrepancies. Our preliminary data should be confirmed with a larger study to draw a firm conclusion.

However, this observation cannot be explained by a low sensitivity of our analysis, since we performed nested PCRs with detection limits close or lower than ten total viral copies. To our knowledge, this is the first time that all HHVs are analysed in a Tunisian population, and therefore, it might be hypothesized that the low prevalence might be a feature characteristic of this specific geographical area. It is interesting to mention that a seroepidemiologic survey of pregnant women from different parts of the world highlighted a low prevalence of HHV-6 in the Moroccan population (Ranger et al. 1991). Further studies are necessary to confirm these results and to support the hypothesis of lower HHVs prevalence in the North African region.

Although HHV DNA was detected in blood samples of 58% MS patients compared to 15% of control individuals, with high statistical significance, no difference was observed within the MS patients in terms of gender, type of diagnosis, symptoms, disease score and β interferon treatment. This might indicate that herpesviruses do not play a significant role in the initiation of MS. It is possible that other factors are responsible for the onset of the disease since the samples are obtained from a geographical population different than previously published reports and MS has a considerable heterogeneity (Lucchinetti et al. 2000). In addition, gene–environment interactions have recently been suggested to be relevant in the context of MS. For example, a genetic polymorphism in the MHC2TA gene has been associated with HHV-6 infection (Martınez et al. 2007). Similar results were obtained associating specific EBV genotypes in MS patients (Munch et al. 1998b), although contradictory results have recently been reported (Lindsey et al. 2008). This observation has prompted investigators to propose individualized treatment of MS (Gold and Hartung 2005).

In conclusion, the screening of human herpesviruses in blood samples from multiple sclerosis patients and healthy controls, performed for the first time in the Tunisian population, showed an overall prevalence of herpesvirus infection significantly higher than healthy controls (58% vs 15%, $p<10^{-4}$). EBV and HHV-7 were the only herpesviruses showing higher prevalence in MS than controls. These observations could suggest either that these two viruses may be involved in the pathogenesis of MS or, more generally, that MS might affect herpesvirus latency or reactivation, without any direct viral involvement in pathogenesis. Additional studies are needed before drawing any definitive conclusion.

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Conflict of interest The authors declare that there are no conflicts of interest.

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