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ORIGINAL ARTICLE

Bacterial DNA findings in ruptured and unruptured intracranial aneurysms

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ABSTRACT

Objective Chronic inflammation has earlier been detected in ruptured intracranial aneurysms. A previous study detected both dental bacterial DNA and bacterial-driven inflammation in ruptured intracranial aneurysm walls. The aim of this study was to compare the presence of oral and pharyngeal bacterial DNA in ruptured and unruptured intracranial aneurysms. The hypothesis was that oral bacterial DNA findings would be more common and the amount of bacterial DNA would be higher in ruptured aneurysm walls than in unruptured aneurysm walls. **Materials and methods** A total of 70 ruptured ($n = 42$) and unruptured ($n = 28$) intracranial aneurysm specimens were obtained perioperatively in aneurysm clipping operations. Aneurysmal sac tissue was analysed using a real-time quantitative polymerase chain reaction to detect bacterial DNA from several oral species. Both histologically non-atherosclerotic healthy vessel wall obtained from cardiac by-pass operations (LITA) and arterial blood samples obtained from each aneurysm patient were used as control samples. **Results** Bacterial DNA was detected in 49/70 (70%) of the specimens. A total of 29/42 (69%) of the ruptured and 20/28 (71%) of the unruptured aneurysm samples contained bacterial DNA of oral origin. Both ruptured and unruptured aneurysm tissue samples contained significantly more bacterial DNA than the LITA control samples (p-values 0.003 and 0.001, respectively). There was no significant difference in the amount of bacterial DNA between the ruptured and unruptured samples. **Conclusion** Dental bacterial DNA can be found using a quantitative polymerase chain reaction in both ruptured and unruptured aneurysm walls, suggesting that bacterial DNA plays a role in the pathogenesis of cerebral aneurysms in general, rather than only in ruptured aneurysms.

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Introduction

The mechanisms behind intracranial aneurysm development, structural weakening and rupture have been studied widely.[1–3] Classical risk factors for aneurysm development and rupture include female gender, smoking, high blood pressure, excessive alcohol consumption and a family history of subarachnoidal haemorrhage (SAH).[4–6] Oral infections have been associated with cardiovascular disease and periodontitis is accepted as an independent risk factor, but the causality remains debatable.[7–10] Periodontitis is the most common chronic oral bacterial disease and the incidence of severe periodontitis is ~20% in the Finnish population.[11] Poor dental hygiene, dental operations and bacterial endocarditis have been found to be pre-disposing factors in the formation of rare mycotic intracranial aneurysms.[12,13] To the best of our knowledge, bacterial infections have not been earlier associated with either the formation or the rupturing of an intracranial saccular aneurysm.

Before the intracranial aneurysm ruptures, the wall undergoes inflammatory changes, such as apoptosis, T-cell and macrophage infiltration and complement activation, but the aetiology of these inflammatory changes remains unknown.[1–3] In our previous study, we detected bacterial DNA of oral origin and the activation of bacterial receptors cluster of differentiation (CD14) and Toll-like-receptor 2 (TLR2) in ruptured intracranial aneurysm sac tissue samples, supporting the hypothesis of a bacteria-driven inflammation in aneurysm tissue.[14] Ruptured aneurysm walls have been widely studied and inflammation seems to play a role in aneurysm rupture.[1,2] The histology differs between ruptured and unruptured aneurysms: loss of endothelium and mural cells (i.e. vascular smooth muscle cells, myofibroblasts and fibroblasts), breakdown of the collagen matrix and partial hyalinization of the wall are associated with a ruptured aneurysm wall.[1,3] The inflammatory cell infiltration is more prominent in ruptured walls than in unruptured walls.[1] In our previous report we

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showed that TLR2 and CD 14—receptors for recognizing bacterial infection—were actively expressed at the rupture site.[14] This suggests that bacteria entrapped in the wall of the aneurysm may be involved in the inflammation, leading to the rupture of the aneurysm. However, TLRs also recognize oxidation-damaged molecular complexes and necrotic cell debris. Upon stimulation, TLRs and especially TLR2 and TLR4 initiate intracellular signalling cascades that ultimately promote pro-inflammatory gene expression and cytokine production, leading to an inflammatory response.[15–17]

The pathophysiology of rare infectious (mycotic) aneurysms differs from saccular, non-infectious aneurysms: bacteria from septic emboli invade the adventitia causing polymorphonuclear leucocyte infiltration in both the muscular and the internal elastic layer.[18] Infectious aneurysms are, thus, pseudoaneurysms with a highly fragile aneurysm wall and a fragile parental artery.[18] Mycotic aneurysms are often more distally located, fusiform shaped and multiple and the aneurysm size can change within the follow-up.[12,18–20] Patients with mycotic aneurysms also usually have a predisposing infection, such as bacterial endocarditis, and they are younger than patients with saccular aneurysms.[12,19,21,22] Diagnosis of a mycotic aneurysm can be confirmed with aneurysm wall staining to detect bacteria.[12,18]

The aim of this study was to measure the presence of oral and pharyngeal bacterial DNA in both ruptured and unruptured intracranial aneurysms.

Materials and methods

Specimens from ruptured and unruptured aneurysm walls were obtained perioperatively after prompt microsurgical clipping of the saccular aneurysm under sterile conditions. The specimens were collected between June 2010 and June 2014. Aneurysms clipped by experienced neurosurgeons were considered suitable for this study if the specimen could be taken with microscissors after the aneurysm was clipped without any risk to the patient. The inclusion criteria for patients were: being aged over 18 years, having a saccular aneurysm wall that was clipped and it was technically possible to take the sample. Patients whose aneurysms were not treated by clipping and whose aneurysm wall was not safely excised were excluded. Aneurysm samples were collected using sterile techniques. An arterial blood sample via arterial cannula was obtained from each patient during the procedure to be used as a negative control (i.e., reference sample) for bacterial DNA analysis. The specimens were frozen at −70°C after collection. Five non-atherosclerotic left internal thoracic artery (LITA) samples were collected to be used as an additional negative control and to validate the results.

All patients gave their informed consent for the study. The study was approved by the Hospital Ethics Committee.

Detection of bacteria

For the real time quantitative polymerase chain reaction (RT-qPCR), bacterial DNA was extracted from the samples using a commercially available QIAamp DNA Mini Kit (Qiagen, CA, Valencia) according to the instructions provided. The extraction of DNA was controlled by measuring the amounts of human DNA with the qPCR using the human housekeeping gene, RNaseP (Applied Biosystems, Foster City, CA). The presence of the candidate bacterial DNA for endodontic bacteria (*Streptococcus sp.*, mainly the *Str. mitis* group, *Str. mitis*, *Str. oralis*, *Str. sangui* & *Str. gorgonii* and the *Streptococcus anginosus* group, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and periodontal bacteria (*Porphyromonas gingivalis*, *Aggregatibacter* (previously *Actinobacillus*), *actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Dialister pneumosintes*, *Parvimonas micra* and *Treponema denticola*) was identified using a RT-qPCR and the ABI PRISM 7900 sequence detection system (Applied Biosystems). The total amount of bacterial DNA in the samples was determined by using universal bacterial primers and probes, with human housekeeping gene, RNaseP (Applied Biosystems) used as a reference gene. Details of the measurement are presented in our earlier study.[14] Briefly, the relative amounts of these organisms in the specimens were calculated by the comparative critical threshold cycle (*Ct*) method ($\Delta\Delta Ct$, $\Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}}$), with a simplification. The control sample was the individual's own arterial blood (inner control). The calculations were repeated using LITA, i.e., the mean value (ΔCt) from five LITA samples was calculated and used as an additional (external) control. The bacterial DNA positivity of the samples was determined by using a cut-off level Ct 32 for the universal bacterial DNA measurement. The sample was marked as positive if $2^{-\Delta\Delta Ct} \geq 2$*SD of the sample gene copies.

Statistical analyses

Differences in the bacterial findings and patient characteristics between ruptured and unruptured groups were assessed with Fisher's exact test using SPSS software (IBM SPSS Statistics for Windows, version 19.0. Armonk, NY: IBM Corp.). Differences in the amount of bacterial DNA between the groups were assessed with the Mann-Whitney test using R software (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.) The figures were created with R graphics and ggplot2-package (H. Wickham. ggplot2: elegant graphics for data analysis; Springer, New York, 2009)

Results

Patient characteristics

The characteristics of the 70 patients (42 patients with ruptured aneurysms and 28 with unruptured aneurysms) are shown in Table 1.

Of the 42 SAH patients, 12 were previously healthy without any regular medication at the time of haemorrhage. Sixteen SAH patients had hypertension. All the other patients had diseases such as depression, hypercholesterolemia, hypothyreosis, migraine, myoskeletal problems, heart disease, diabetes, asthma, a history of head trauma, a history of cervical fracture, and reflux disease.

Only three patients of the 28 patients with unruptured aneurysms were previously healthy. Fifteen patients had hypertension. All the other patients had diseases such as depression, myoskeletal problems, heart disease, migraine, hypercholesterolemia, benign tumour, hypotyreosis, asthma and atrial fibrillation. Seven patients with unruptured aneurysms had a history of previous SAH. All the other unruptured aneurysms were incidental findings.

Molecular microbiological findings in aneurysm walls

Using RT-qPCR, bacteria were detected in 49/70 (70%) of the aneurysms (Table 2). The prevalence of bacteria was 69% in the ruptured aneurysm wall samples and 71% in the unruptured aneurysm wall samples (NS). In the ruptured group, endodontic and periodontal pathogens were identified in 25/42 (60%) and 18/42 (43%) of aneurysms, respectively. Figure 1 shows the frequencies of bacterial DNA-positive findings in all cases (42 ruptured and 28 unruptured samples). In ruptured aneurysm wall samples, 13 out of 42 (31%) contained the DNA of both periodontal and endodontic bacteria. In the unruptured aneurysm wall samples, 43% contained both types of bacteria. Patients with an unruptured aneurysm but with a history of previous SAH had similar bacterial findings as the patients without a history of SAH (total positive = 71% and 83%, respectively).

Bacterial DNA from the *Streptococcus mitis* group and *Fusobacterium nucleatum* were the most common pathogens. We calculated two separate n-fold values for the amount of bacterial DNA in each aneurysm sample; the first used the mean amount of bacteria from the LITA samples as a control and the second used the individual's own blood sample as a control. Figure 2 shows the strong correlation between these two values ($r = 0.84$; $p < 0.001$). The total amount of bacterial DNA in the aneurysm tissue samples was 12.2-times higher than that found in their control blood samples (mean = 12.2; SD = 25.9; median = 4.1). The amount of bacterial DNA was 9.1-times higher in the aneurysm tissue samples than that found in the LITA samples (mean = 9.1; SD = 16.8; median = 3.5). Figure 3 shows that both ruptured and unruptured aneurysm tissue samples contained significantly more bacterial DNA than the LITA control samples (p-values = 0.003 and 0.001, respectively).

Table 1. Patient and aneurysm character.

	Ruptured aneurysms ($n = 42$)	Unruptured aneurysms ($n = 28$)	p-value
Mean age (years)	50.9	57.8	0.009
Female/Male	29/18	14/16	0.24
Heavy alcohol consumption	6 (14%)	4 (14%)	1.00
Smoking	27 (64%)	12 (43%)	0.09
Positive family history of SAH	6 (14%)	4 (14%)	1.00
Location			
ICA	0	0	1.00
MCA	40 (95%)	28 (100%)	0.50
ACoA	4 (10%)	1 (4%)	0.64
Pericallosa	0 (0%)	1 (4%)	0.40
ACA	1 (2%)	0 (0%)	1.00
Mean fundus size (mm)	11.4	9.6	0.18

Table 2. The prevalence of bacterial DNA in the samples.

Bacterial DNA	Positive	Difference of the positive findings (p-value)
Ruptured aneurysm wall	29 (69%)	
Unruptured aneurysm wall	20 (71%)	1.00
Total	49 (70%)	

Discussion

Bacterial infection was previously associated only with rare mycotic (infectious) intracranial aneurysms, not saccular, non-infectious aneurysms. We detected oral bacterial DNA and bacteria-driven inflammation associated with ruptured saccular aneurysm wall samples in our previous study.[14] The spread of infection in mycotic aneurysms can be endovascular, as in infective endocarditis, or uncommonly contiguous extravascular, as in meningitis or cavernous sinus thrombophlebitis.[18] Endovascular infection is caused either by septic microemboli to the vasa vasorum or by bacterial escape from a septic embolus that occludes the vessel.[18] In infective endocarditis the bacterial load is extremely high and this embolic event causes mycotic aneurysms to be located distally where the diameter of the artery is small.[18] However, patients without endocarditis or another severe infection constantly have a subclinical amount of bacteria in the circulation all the time after tooth brushing, chewing, etc., and this small amount of bacteria could possibly attach to a vessel wall with a predisposing local haemodynamic state, as in vessel bifurcations or in atherosclerotic plaques. To study the relation between bacteria and saccular aneurysms more extensively, we compared the bacterial DNA findings in ruptured and unruptured aneurysm walls. We hypothesized that ruptured aneurysm walls contain the bacterial genome more frequently and in greater amounts, which could be causative factors for aneurysm rupture. We found statistically significantly more bacterial DNA in both ruptured and unruptured aneurysm tissue samples than in the control samples. There was no difference in the amount of bacterial DNA between the ruptured and unruptured samples.

Chronic oral infections are common; the prevalence of severe periodontitis is ~20% in the Finnish population.[11] Periodontitis causes systemic inflammation in an otherwise healthy population.[23] There is a prevalence of periapical lesions at the apex of the tooth in up to 27% of the Finnish population.[11] Clinically relevant oral and other pathogens have previously been detected in atherosclerotic coronary plaques and abdominal aortic aneurysms [24–27] and in 2013 we published the first study to identify oral bacteria in ruptured intracranial aneurysm walls.[14] In addition, we have recently highlighted that it is not only oral pathogens that can be detected in atherosclerotic lesions; oral streptococcal DNA positivity in the thrombus aspirates of patients with clinical myocardial infections also occurred together with dental periapical lesions.[28] In the present study, the *Streptococcus mitis*-group and *Fusobacterium nucleatum* were the most commonly found pathogens in both ruptured and unruptured

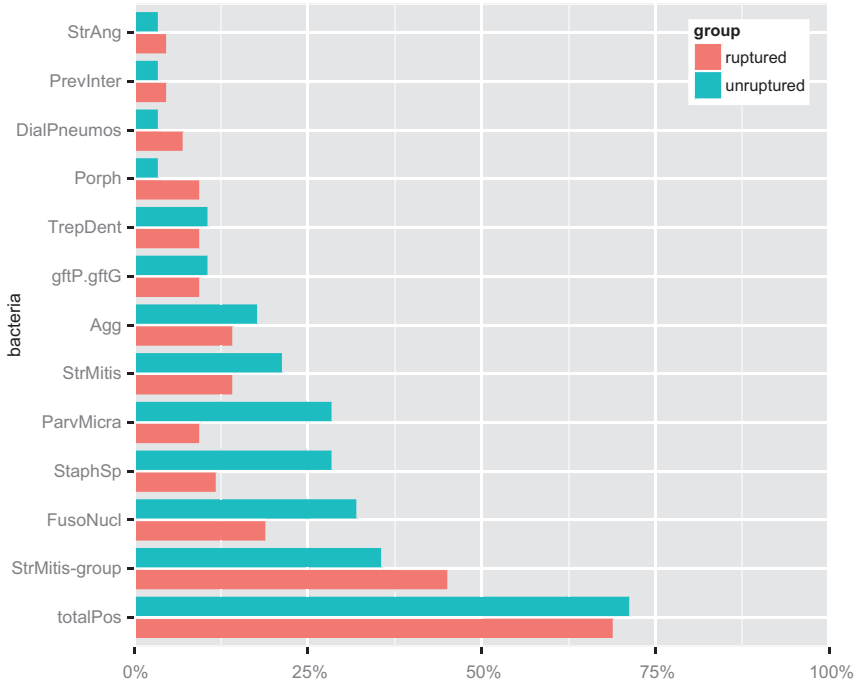

Figure 1. Distribution of bacterial DNA findings in all specimen (42 ruptured and 28 unruptured aneurysm wall) using specific primers and probes in RT-qPCR. StrAng, *Str. anginosus* group (*Str. anginosus, Str. milleri, Str. constellatus, Str. intermedius*); PrevInter, *Prevotella intermedia*; DialPneumos, *Dialister pneumosintes*; Porph, *Porphyromonas gingivalis*; TrepDent, *Treponema denticola*; gftP.gftC, virulence factors of gftP and gftG, i.e., recognition of *Str. sangui and Str. gordonii*; Agg, *Aggregatibacter actinomycetemcomitans*; StrMitis, *Streptococcus mitis*; ParvMicra, *Parvimonas micra*; StaphSp,*Staphylococcus aureus, S. epidermidis*; FusoNucl, *Fusobacterium nucleatum;* StrMitis-group, *Streptococcus mitis*- group (*Str. mitis*, *Str. salivarius*, *Str. gordonii*, *Str. sangui*, *Str. pneumoniae*, *Str. oralis*), *Str. thermophilus*, *Lactobacillus lactis*; totalPos, positive result from one or more measurements.

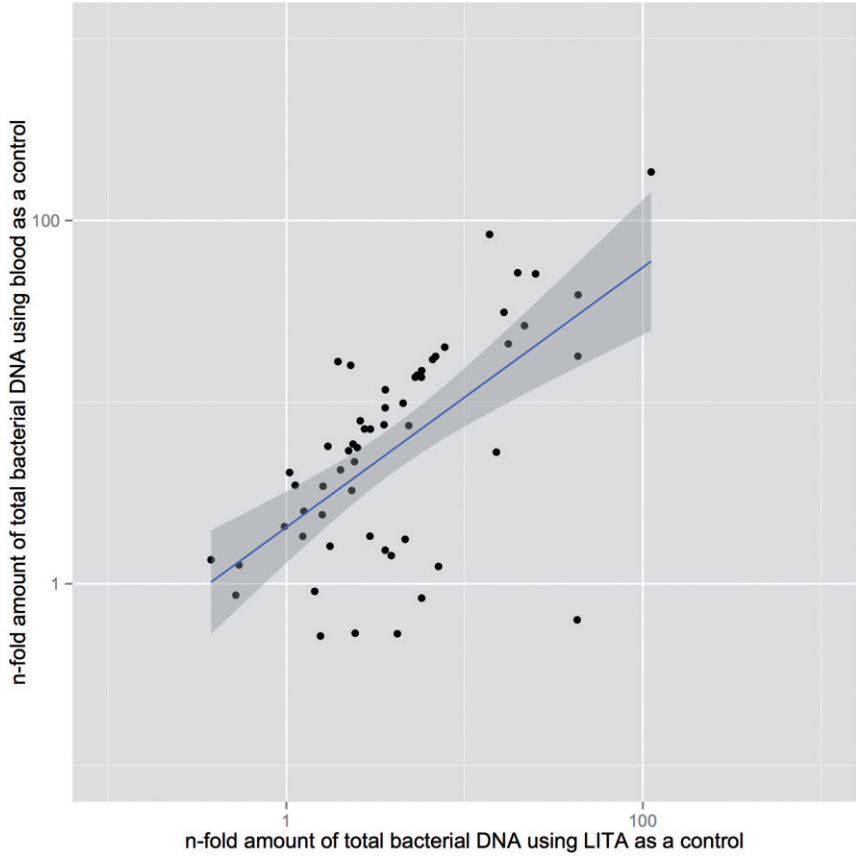

Figure 2. The correlation between the n-fold values of the bacterial DNA amount using different control samples (i.e., LITA and individual's own blood).

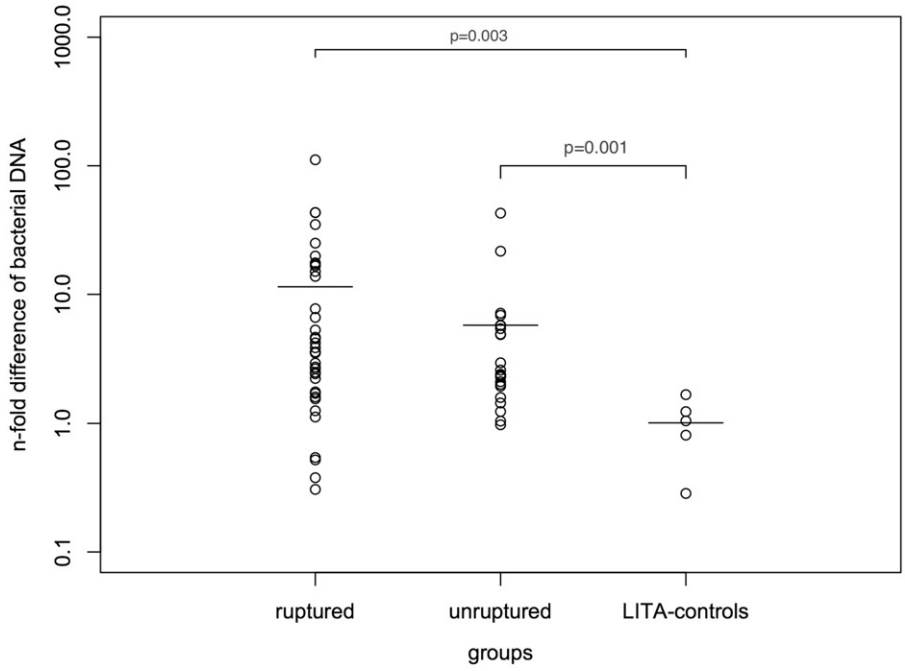

Figure 3. The n-fold differences of total amount of bacterial DNA in both ruptured and unruptured aneurysm samples using LITA as a control.

aneurysms, and these bacteria are also clinically important and common bacteria in dental infections. Although the sample size in this study is small, the findings suggest that these bacteria may have a role in the pathophysiology of intracranial aneurysm disease. Since there was no difference between the bacterial DNA content of the ruptured and unruptured aneurysm specimens, this suggests that oral bacteria may also play a part in the formation as well as the rupture of an intracranial aneurysm. On the other hand, if the bacteria have a major role in the initiation of the rupturing process, the inflammatory process itself might destroy them. This is highly speculative and further studies concerning the role of the bacteria are definitely needed.

In addition to the small sample size, the second major limitation of our study is the lack of a separate control group. In our previous study we used a piece of contralateral intracranial artery from an autopsy case as a control sample, in addition to blood samples from the patients.[14] The blood samples were used to exclude the possible bacterial background due to the sampling method and the possible circulating bacterial DNA of the peripheral blood. We have used blood samples as an inner control in our earlier studies as well.[14,28] In this study, peripheral arterial blood during the procedure served as internal negative controls for each subject. Since it was not relevant to obtain a piece of healthy intracranial artery from the aneurysm patient, we used atherosclerosis-free LITA control samples as an additional control group to confirm the result of the inner control group, i.e., the blood samples. N-folds of total bacterial DNA were similar using either the LITA or the blood as a reference, suggesting the reliability of our analyses. It is shown in Figure 2 that, when the relative amount of bacterial DNA in the aneurysm sample is high compared to the blood sample, it is high when the same sample is compared to the LITA sample. By using the internal control and comparative critical threshold cycle methods,[29] we confirmed that the amount of bacterial DNA in the aneurysms differed significantly from that in the control samples within the same individual. As a limit of the true finding for the samples, a two-times fold difference was used.[30,31] Therefore, all the presented positive results are true findings corrected with the inner control. The third major limitation of this study is the selection bias. The patient selection was made by the surgeon who had to decide whether it was possible to obtain the tissue sample without causing any additional risk to the patient. The fourth limitation of this study is that the oral status of the study participants is not included. Larger studies with microbiological, clinical and radiological examinations are needed.

In conclusion, bacterial DNA was detected in 70% of the specimens, supporting our previous finding that dental infection could be a part of the pathophysiology of intracranial aneurysm disease. Bacterial genomes were found in both unruptured and ruptured aneurysm walls. In some ruptured aneurysms, the amount of bacterial DNA was considerably large. This preliminary finding is extremely interesting and further studies—with larger sample sizes—are needed concerning the role of the bacteria.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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