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Oral *P. gingivalis* infection alters the vascular reactivity in healthy and spontaneously atherosclerotic mice

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Abstract

Background: Considering that recent studies have demonstrated endothelial dysfunction in subjects with periodontitis and that there is no information about vascular function in coexistence of periodontitis and atherosclerosis, we assessed the impact of oral inoculation with the periodontal pathogen *Porphyromonas gingivalis* on vascular reactivity in healthy and hypercholesterolemic apolipoprotein E-deficient (ApoE) mice. *In vitro* preparations of mesenteric arteriolar bed were used to determine the vascular responses to acetylcholine, sodium nitroprusside and phenylephrine (PE).

Results: Alveolar bone resorption, an evidence of periodontitis, was assessed and confirmed in all infected mice. Acetylcholine- and sodium nitroprusside-induced vasorelaxations were similar among all groups. Non-infected ApoE mice were hyperreactive to PE when compared to non-infected healthy mice. *P. gingivalis* infection significantly enhanced the vasoconstriction to PE in both healthy and spontaneous atherosclerotic mice, when compared to their respective controls.

Conclusions: This study demonstrates that oral *P. gingivalis* affects the alpha-adrenoceptor-mediated vascular responsiveness in both healthy and spontaneous atherosclerotic mice, reinforcing the association between periodontitis and cardiovascular diseases.

Background

Periodontal disease is a chronic inflammatory disease that affects the gum tissue and other structures supporting the teeth. It begins as gingivitis and, if left untreated, can progress to periodontitis, where destruction of connective tissue attachment and alveolar bone can lead to tooth loss. Beyond its local effects, periodontal disease may also interfere with other systems of the body, and many epidemiological studies have associated periodontitis with atherosclerosis. However, a causal relation between both diseases remains controversial [1].

Investigators have proposed that periodic transient bacteremia, which leads to invasion of vascular cells and increase of the levels of circulating cytokines, accelerates the atherogenic process [2]. Also, it has been reported

that *P. gingivalis*, the periodontopathogen associated with the most common form of periodontal disease, accelerates atheroma formation [3-5], increases systemic inflammatory markers [4,6-8], invades endothelium and vascular smooth muscle cells [9,10] and appears to alter endothelial function [11-15].

Endothelial dysfunction appears to be an early event in the development of atherosclerosis [16] and also predicts plaque instability [17]. Support for the clinical importance of vascular reactivity and endothelial dysfunction is provided by several studies demonstrating increased cardiovascular disease risk in patients with vascular dysfunction in coronary and peripheral arteries [17-22].

On the basis of these observations, we aim to investigate whether oral *P. gingivalis* infection alters the vascular responsiveness in spontaneous atherosclerosis in apolipoprotein E-deficient (ApoE) mice compared with C57BL/6 (C57) wild-type mice.

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Methods

Experimental groups

Experiments were conducted on adult (30-week-old) male C57 and ApoE mice from the Laboratory of Transgenes and Cardiovascular Control of the Federal University of Espirito Santo. Animal care and treatment were approved by the institutional Ethics Committee for Use of Animals (CEUA-Emescam, Protocol # 020/2007) and were conducted in conformity with institutional guidelines, in compliance with international laws and policies. C57 mice were used as animals systemically healthy. C57 and ApoE animals were randomly divided in control (Ct) or infected (Pg) groups: C57 Ct, C57 Pg, ApoE Ct, ApoE Pg.

Preparation of bacterial culture and oral infection

P. gingivalis strain ATCC 33277 was obtained from the Collection of Microorganisms of Reference (INCQS, Fiocruz, Brazil) and cultured in blood-agar supplemented with hemin/menadione, under an anaerobic condition [23]. At age of 18 weeks randomly selected mice were infected with *P. gingivalis* as follows. 10^9 CFU of live bacteria (optical density of ≈ 0.8 at 660 nm) in 100 μ L of PBS with 2% carboxymethylcellulose was administered via oral topical application three times at 2-day intervals. Control mice received carboxymethylcellulose in PBS. Infected mice were kept in microisolated cages (Beiramar, Brazil).

Cholesterol and systemic inflammation analysis

At 30 weeks of age, under anesthesia with thiopental (40 mg/kg i.p.), mice were subjected to the axillary plexus isolation and samples of blood were obtained by puncture [24]. For each mouse two blood samples were collected. Serum was obtained to determine the levels of plasma total cholesterol by chromogenic assays (Bioclin, Brazil). Blood was used to perform leucogram (Beckman Coulter MAXM HMX, USA). Systemic host inflammatory response or systemic inflammation was assessed by neutrophils/lymphocytes ratio [25].

"In Vitro" preparation of the mesenteric arteriolar bed

Isolated mesenteric arteriolar beds were obtained 12 weeks after *P. gingivalis* inoculation of the animals and after blood samples collection. Briefly, the superior mesenteric artery was cannulated (PE 50, Becton Dickinson, Sparks, MD, USA), and the intestine was immediately severed from the body. The mesenteric vascular bed was perfused at a constant flow of 2 mL/min with oxygenated (mixture of 95% O₂ and 5% CO₂, 37°C) physiological salt solution (130 NaCl, 4.7 KCl, 1.6 CaCl₂·2H₂O, 1.8 KH₂PO₄, 4.7 MgSO₄, 1.17 H₂O, 14.9 NaHCO₃, 0.026 EDTA, and 11.1 glucose; mmol/L), using a roller pump (Harvard Apparatus, USA). The

bowel was removed to prevent the return of the Krebs' solution through the venous system. After passing through the vascular bed, the perfusate was artificially drained out from the preparation. After a 40-min stabilization period, the experimental protocol was initiated. Mean perfusion pressure (MPP) was measured; as a result of maintenance of a constant flow, changes in the MPP represented changes in vascular resistance. The vasoconstrictor response was assessed by stimulation of α -adrenoceptors evoked by phenylephrine (PE; 0.001-300 μ g; Sigma-Aldrich). To study endothelium-dependent and endothelium-independent vasodilation, responses to acetylcholine (ACh; 10^{-10} to 10^{-3} mol/L; Sigma-Aldrich) and sodium nitroprusside (SNP; 10^{-10} to 10^{-3} mol/L; Sigma-Aldrich) respectively were determined as percentages of reductions in the precontractions induced by 10^{-5} mol/L PE (concentration that induces 60% to 80% of the maximal effect).

Quantification of atherosclerotic lesion area

After bowel removal the heart was perfused through right atrium with 50 mL of PBS (0.1 M; pH 7.4), following by 50 mL of formaldehyde (4%). Aorta was removed and stored in buffered fixative solution, and cryostat sections were prepared, as previously described [26]. Atherosclerotic lesion area was quantified using a microscope (Olympus, Japan) interfaced with a videocamera (Hitachi, Japan) and an image analysis system (Image J, USA). Mean lesion area per mouse was calculated by an investigator blinded to the experimental protocol and was expressed as μ m².

Quantification of alveolar bone loss

Alveolar bone between first and second molars of the left mandible was assessed by a morphometric method. Briefly, after 15 minutes in boiling water mandible was mechanically defleshed, exposed overnight in 3% hydrogen peroxide and immersed in bleach for 1 minute. The bone level, that is, the distance from the cementum-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured under a dissecting microscope ($\times 40$) with a total of 2 measurements per mouse. The measurements were repeated two times per site in random and blinded protocols. Because a greater distance from the CEJ-ABC indicates less alveolar bone, bone levels were converted to relative amounts of bone by the following calculation: 1/distance from CEJ-ABC [23]. The values were normalized by converting bone values of each mouse to a percentage of the mean value for respective control mice group.

Statistical Analysis

Vascular responses are given as percentage of dilation relative to the PE-induced precontraction level. Values

are expressed as means \pm SEM. For each dose-response curve, the maximum effect (E_{max}) and the dose of agonist that produced one-half of E_{max} (ED_{50}) were estimated using nonlinear regression analysis (GraphPad Software Inc., San Diego, CA). The sensitivity of the agonists is expressed as pED_{50} . One- and two-way ANOVA, followed by Bonferroni t test, or unpaired Student's t test when appropriated, were used for statistical analyses. $p < 0.05$ was considered statistically significant.

Results

Alveolar bone loss produced by oral infection with *P. gingivalis*

To determine the degree of local periodontal destruction, we dissected the mandibles after euthanasia and measured alveolar bone loss. As summarized in Figure 1, infected mice displayed significantly increased alveolar bone loss compared with the respective control, indicated by a decrease in relative amounts of bone in infected mice (C57 Ct: 100 ± 2 versus C57 Pg: $48 \pm 6\%$; ApoE Ct: 103 ± 6 versus ApoE Pg: $63 \pm 2\%$, respectively; $p < 0.05$ for all comparisons).

Effects of the oral *P. gingivalis* infection on serum cholesterol levels and systemic inflammatory response

We investigated if the oral infection with *P. gingivalis* modulated established risk factors for atherogenesis in this murine model. No differences in total plasma cholesterol were observed between infected and control groups in both C57 (Ct: 78 ± 6 versus Pg: 85 ± 3 mg/dL) and ApoE groups (Ct: 523 ± 64 versus Pg: 629 ± 41 mg/dL) groups (Table 1). When systemic inflammation was analyzed we observed an increase (~ 3 -fold) in

Table 1 Effects of Oral Infection with *P. gingivalis* on Serum Cholesterol and Body Weight

| Parameters | C57 Ct (8) | C57 Pg (8) | ApoE Ct (7) | ApoE Pg (6) |
|---------------------------|--------------|--------------|------------------------|-------------------|
| Body weight (g) | 35 ± 0.6 | 33 ± 0.6 | 34 ± 0.8 | 33 ± 1.5 |
| Serum cholesterol (mg/dL) | 78 ± 6 | 85 ± 3 | $523 \pm 64^{\dagger}$ | $629 \pm 41^{\S}$ |

Values are mean \pm SEM.

† vs C57 Ct; § vs C57 Pg; $p < 0.05$.

neutrophils/lymphocytes ratio from mice with atherosclerosis compared to C57 in both control (ApoE: 0.17 ± 0.01 versus C57: 0.06 ± 0.01 ; $p < 0.05$) and infected (ApoE: 0.17 ± 0.02 versus C57: 0.05 ± 0.001 ; $p < 0.05$) animals (Figure 2). However, oral *P. gingivalis* inoculation did not change ($p > 0.05$) the systemic inflammatory status in both healthy and atherosclerotic mice, when compared to their controls (Figure 2). Body weight was also monitored and was similar among all groups (Table 1).

Effects of the oral *P. gingivalis* infection on atherosclerotic lesion area

To evaluate if the oral infection with *P. gingivalis* amends the progression of atherosclerotic lesions, morphometric analyses were used and demonstrated that there were no differences ($p > 0.05$) in mean atherosclerotic lesion area in infected mice compared with controls in both C57 (Pg: 0.75 ± 0.2 versus Ct: $0.66 \pm 0.2 \mu m^2 \times 10^3$) and ApoE (Pg: 57.5 ± 4.3 versus Ct: $41.25 \pm 0.9 \mu m^2 \times 10^3$) groups (Figure 3).

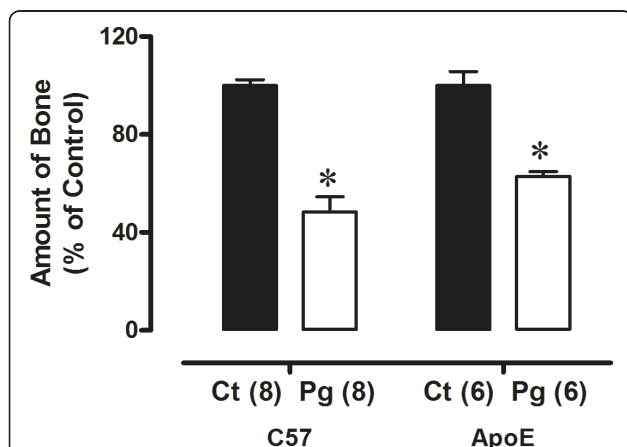


Figure 1 Alveolar bone loss produced by oral infection with *P. gingivalis* in C57 and ApoE mice. C57 represents animals systemically healthy and ApoE represents animals with spontaneous atherosclerosis. The relative amounts of bone are significantly less in infected (Pg) than in control (Ct) mice. Values are means \pm SEM. * $p < 0.05$ vs Ct group.

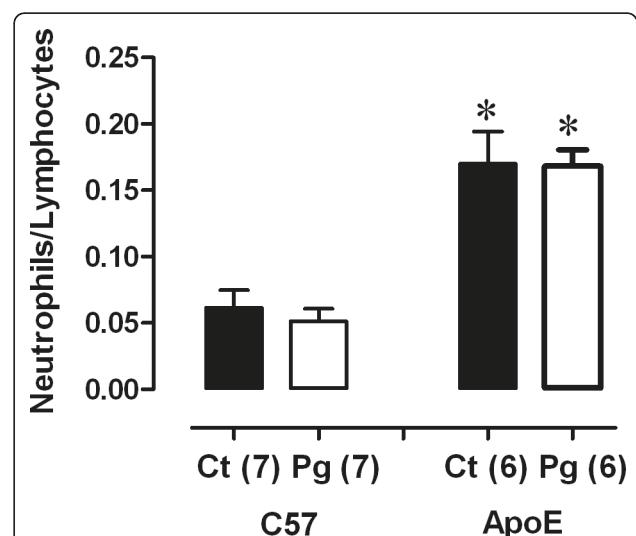
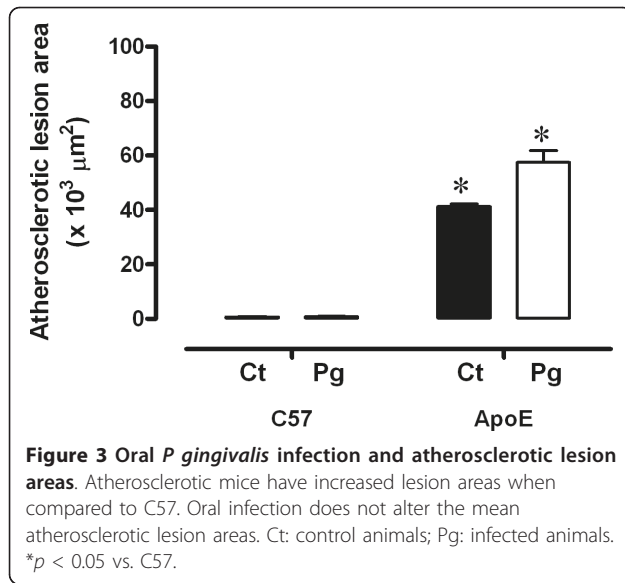


Figure 2 Oral *P. gingivalis* infection and systemic inflammation. Atherosclerotic mice have increased neutrophils/lymphocytes ratio when compared to C57. Ct: control animals; Pg: infected animals. * $p < 0.05$ vs. C57.

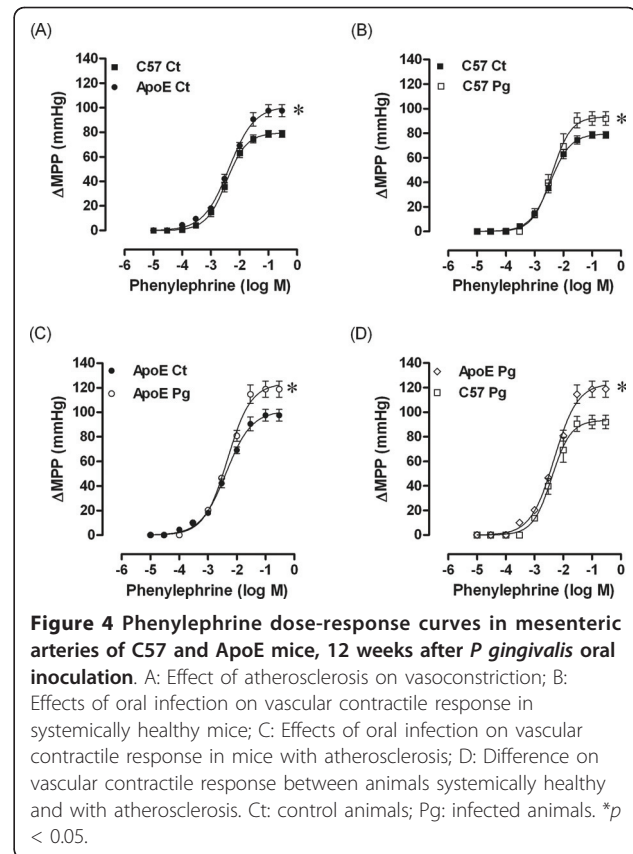


Effects of the oral *P. gingivalis* infection on mesenteric arteriolar bed responsiveness

We examined the effects of *P. gingivalis* oral infection on mesenteric arteriolar bed responsiveness to phenylephrine, acetylcholine and sodium nitroprusside. As summarized in Figure 4 (panel A) the spontaneous atherosclerotic mice were hyperreactive to phenylephrine when compared to systemically healthy mice (ApoE Ct: 98 ± 5 mmHg versus C57 Ct: 79 ± 3 mmHg; $p < 0.05$). Periodontitis caused by *P. gingivalis* oral inoculation enhanced ($p < 0.05$) the maximal response to phenylephrine in all groups when compared to the respective control group in both C57 (Pg; 92 ± 6 versus Ct: 79 ± 3 mmHg) and ApoE (Pg; 119 ± 7 versus Ct: 98 ± 5 mmHg) groups (Figure 4B and 4D). Figure 4 (panel D) shows the preservation of the vascular hyperreactivity to phenylephrine of atherosclerotic mice (119 ± 7 mmHg) compared to healthy mice (92 ± 6 mmHg; $p < 0.05$) even after *P. gingivalis* infection. Despite of changes in vasoconstrictor responses, neither endothelium-dependent nor endothelium-independent vasodilations were changed by *P. gingivalis* oral infection (values shown in Table 2).

Discussion

The present study was designed to test whether oral challenge with an established periodontal pathogen amends vascular responsiveness in a murine model of spontaneous atherosclerosis. Surprisingly, our data show that oral *P. gingivalis* infection not only increases the vascular contractile response to phenylephrine in atherosclerotic mice but also in systemically healthy mice. The ApoE model of atherosclerosis used in this study is well established [27,28]. In agreement with previous



studies we detected high levels of plasma cholesterol in ApoE mice fed a standard chow diet [28-31]. We also noted that the systemic inflammation observed in this study is consistent with the fact that atherosclerosis is a systemic inflammatory disease [32]. An important finding of this study is that oral *P. gingivalis* inoculation did not change the systemic inflammatory status in atherosclerosis, a result consistent with that described by

Table 2 Effects of Oral Infection with *P. gingivalis* on Vascular Reactivity to PE, ACh and SNP

| Parameters | C57 Ct (8) | C57 Pg (8) | ApoE Ct (7) | ApoE Pg (6) |
|-----------------------------|-----------------|-----------------|----------------------|------------------------|
| PE | | | | |
| E_{max} (mmHg) | 79 ± 2.7 | $92 \pm 5.5^*$ | $98 \pm 5^{\dagger}$ | $119 \pm 7^{*\dagger}$ |
| pED_{50} | 2.5 ± 0.09 | 2.37 ± 0.06 | 2.40 ± 0.07 | 2.3 ± 0.05 |
| ACh | | | | |
| E_{max} (% of relaxation) | 76 ± 2.3 | 74.5 ± 1.4 | 75.5 ± 2.9 | 75 ± 3 |
| pED_{50} | 6.15 ± 0.17 | 6.25 ± 0.1 | 6.12 ± 0.27 | 6 ± 0.18 |
| SNP | | | | |
| E_{max} (% of relaxation) | 92 ± 1.3 | 89 ± 0.8 | 91 ± 2.5 | 92 ± 2 |
| pED_{50} | 6.6 ± 0.07 | 6.71 ± 0.04 | 6.90 ± 0.10 | 6.7 ± 0.08 |

Values are mean \pm SEM.

*Pg versus Ct; † versus C57 Ct, ‡ versus C57 Pg; $p < 0.05$.

Miyamoto et al. [33]. In healthy animals it was not detected systemic inflammatory response induced by oral *P. gingivalis* infection, although some authors have reported an increase in systemic inflammatory markers such as C-reactive protein, interleukin 6 and neutrophils in subjects with periodontitis [6-8]. Therefore, more studies are necessary to elucidate this controversial data. One consideration and limitation is that in the present study we investigated the effects of only one periodontal pathogen, while human periodontitis encloses several microorganisms.

The oral *P. gingivalis* infection did not influence the serum cholesterol levels. This result is in agreement with those found in mice infected with *P. gingivalis* [4] and humans [11] with periodontitis. Although literature reports an increase in the area of atherosclerotic lesion caused by *P. gingivalis* in animal models [4,5,33,34], in this study we did not find changes in the atherosclerotic lesion area. We attribute this difference to different methodologies used in the studies. Li et al. [5] reported increase in the area of injury only after 14 intravenously weekly inoculations of *P. gingivalis* (10^7 CFU) in atherosclerotic mice. Animals inoculated for 10 weeks did not show any change in the area of plaque [5]. In our study we performed only three inoculations, and to mimic normal conditions for which periodontal pathogens could reach the circulatory system, the inoculation of *P. gingivalis* was oral topical, and not intravenously as described by those authors. Lalla et al. [4] also observed increased aortic lesions in ApoE mice after 15 inoculations (10^{12} CFU) with *P. gingivalis*. Again, the number of inoculations in that study was high and was performed by oral gavage and anal topical application; the latter mode used to establish a cycle of oral reinfection, because mice use to be coprophagic. In the present study, however, the extent of the injury may not have changed because we used smaller burden of pathogens (10^9 CFU), the inoculations were less sparse and smaller in number, and because we used only oral topical inoculation, that is more compatible with periodontitis in humans. Despite of a great difficulty in establishing a model of periodontitis similar to periodontal disease in humans, *P. gingivalis* inoculation reproduces the periodontal tissue destruction found in humans, making this model well accepted to study periodontitis.

Endothelial dysfunction has been considered one of the early steps in atherosclerosis [32]. Although endothelial dysfunction has been frequently considered when an impaired endothelium-dependent vasodilation is observed, the localized modulation of vascular endothelium to a nonadaptive functional state can be termed as endothelial dysfunction [35]. In ApoE mice, endothelial dysfunction, taken as an impaired endothelium-dependent dilation, is controversial. Endothelial

dysfunction can be detected or not in ApoE mice depending on the type of diet, age, gender, and type of vessel [36-45]. Recently it was shown in the mesenteric vascular bed from male ApoE mice vascular dysfunction, characterized by increased pressor responsiveness to norepinephrine, despite of normal endothelium-dependent and -independent relaxations [44]. Similarly, we observed a hyperreactive response to phenylephrine in atherosclerotic animals without changes in endothelium-dependent and -independent vasodilations to acetylcholine and sodium nitroprusside, respectively. At present, the mechanism by which hypercholesterolemia alters vascular responsiveness in mesenteric arteriolar bed is unknown and further studies will try to elucidate this issue.

Interestingly, the hyperreactivity to the α -adrenoceptor agonist in ApoE mice infected with *P. gingivalis* was exacerbated when compared with noninfected ApoE animals. In the systemically healthy mice, the response to phenylephrine was also increased, but if we compare its maximal responses, the hyperreactivity was more pronounced in ApoE mice. The mechanism by which oral *P. gingivalis* infection interferes with the reactivity to phenylephrine is unknown. However, based on the finding of an increased production of endothelin in crevicular fluid in subjects with periodontitis [46] and that the actions of endothelin include cell proliferation, migration and contraction [47], we speculate that one of the possible mechanisms by which periodontitis leads to exacerbated pressor response to α -adrenoceptor agonists could be the increase of systemic levels of endothelin.

Conclusions

In conclusion, in the present study we demonstrate that oral *P. gingivalis* infection amplifies the vasoconstrictor hyperreactivity to phenylephrine in mice with spontaneous atherosclerosis. Moreover, in healthy mice the oral *P. gingivalis* infection also produces increased vasoconstrictor response to this α -adrenoceptor agonist. This finding supports the hypothesis that oral infection with *P. gingivalis* is one of several risk factors of cardiovascular diseases.

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Authors' contributions

RBP conceived the study, carried out the animal experiments, analysis of data, statistics and drafted the manuscript. ECV and IS participated in the co-supervision of the study and in the critical revision of the manuscript. SSM participated in design and supervision and in the critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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