Research Article

Study on Biochemical Perspectives of Antioxidant and Oxidant Indices in Oral Squamous Cell Carcinomas (OSCCs) and Other Oral Potentially Malignant Disorders (OPMDs)

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ABSTRACT:

Background-Oxidative stress in biological systems is a complex process that is characterized by an inequity between the production of free radicals (FR) and the ability of the body to eliminate these reactive species through the use of endogenous and exogenous antioxidants. The pathogenesis of oral cancer has been linked to alterations in the antioxidant defense mechanism.

Materials & Methods- Saliva from twenty patients with OSCC, forty patients with OPMDs and twenty healthy subjects in the age group of thirty five to seventy five years was analyzed for levels of nitric oxide, vitamin C, total sialic acid and GSH using spectrophotometry.

Results - The levels of salivary vitamin C and glutathione were significantly reduced and those of nitric oxide and sialic acid were raised in patients having OPMD's and oral squamous cell carcinoma. The co-relation between the AOI and calculated ratios indicated that antioxidant potential of the saliva was decreased and was statistically related to (p <0.001) development of OPMD, which further may progress to oral cancer, notably OSCC.

Conclusion- The current study demonstrated that the estimation of vitamin C, nitric oxide, sialic acid and GSH in saliva could be used as an early potential diagnostic biomarker in the screening of oral cancer. The antioxidant-oxidant indices (AOI's) can be used as a reliable tool for predicting the oral microenvironment and its predicted change towards development of oral cancer. This optimized developed protocol was also found to be simple and cost effective.

KEYWORDS: oral cancer; saliva; AOI; spectrophotometry

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INTRODUCTION:

Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. Head and neck cancers (HNCs) have emerged as a leading cause of cancer-related mortality and morbidity worldwide. It is estimated that more of 90% of all oral neoplasms are OSCC.[1] Saliva is one of the vital fluids secreted in human beings. It is of importance to understand that the use of saliva is and will be a most promising detection method for the diagnosis of oral cancer. Obtaining saliva samples is non-invasive; there is a lower risk of infection, while direct contact of saliva with oral pathologies can contribute to the earlier detection of relevant diseases. Although the oral cavity is frequently examined, 60% of intra oral carcinomas

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are in advanced stage at the time of detection. Persistent difficulties arising in oral cancer are late diagnosis, poor response of tumor to chemotherapy, lack of reliable biomarkers for early diagnosis and post-therapeutic monitoring.[2]

Biomarkers of oxidative stress have been a major source of debate related to monitoring oxidative stress and the possible resulting damage. Biomarkers of oxidative stress have been measured in plasma, whole blood, urine, respired gases, muscle, and other skeletal tissues. Saliva is an attractive bio-specimen for a number of reasons including the ease of its collection and the copious amount the human body is capable of producing for examination. Saliva is increasingly used and well validated in diagnosing. Moreover, saliva is reported to be suitable to detect the body's oxidative stress level.[3]

The present study was done as a part of biochemical evaluation using standardized materials and methods to assess the antioxidant–oxidant indices (AOI) in patients having oral potentially malignant disorders and oral cancer. This was a research-based study with non-invasive laboratory experimentation on patients suffering with OSCC and OPMD with those of normal controls. The study was aimed to evaluate and compare the salivary levels of nitric oxide, vitamin C, total Sialic acid and GSH in patients with oral potentially malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC) with healthy controls. We determined the antioxidant to oxidant indices (AOI) of above mentioned biomarkers in saliva of OPMD and those of OSCC patients. Lastly, we correlated changes in AOI of vitamin C, nitric oxide, Glutathione (GSH) and sialic acid in saliva of patients with OPMD with OSCC and control patients.

MATERIALS & METHODS:

The data was collected non-invasively from patients visiting the Jawaharlal Nehru cancer center, Bhopal and Dept. of Oral Medicine and Radiology, RKDF Dental College and Research Centre, Bhopal. Informed consent was obtained from all the patients before collection of samples. The study was performed in the Department of Biochemistry, RKDF Dental College and Research Centre, Bhopal after obtaining approval from the university ethical committee.

Method Of Collection of Data **-** Saliva from twenty patients with OSCC, forty patients with OPMDs and twenty healthy subjects in the age group of thirty five to seventy five years was analyzed for levels of nitric oxide, vitamin C, total sialic acid and GSH using spectrophotometry.

a. **Study group 1***:* Patients who were histopathologically diagnosed with OSCC (n = 20)
b. **Study group 2*:*** Patients who were histopathologically diagnosed with OPMD (n = 40)
c. **Control group***:* Normal healthy individuals with clinically normal oral mucosa (n = 20).

All the patients were examined using the mouth mirrors and probes under the artificial light. Their history and clinical findings were recorded using the standard proforma after informed consents.

Inclusion criteria-

1. Histopathologically diagnosed new cases of OSCC & OPMD were included in the study group.

Exclusion criteria-

1. Patients undergoing treatment such as chemotherapy and radiotherapy;
2. Past history of any major illness such as liver disease, tuberculosis, diabetes and hypertension;
3. Any history of malignancy other than oral cancer;
4. Recurrent or secondary lesions;
5. Subjects on antioxidants/multivitamin preparations;
6. The healthy controls had no habit of tobacco, alcohol, were devoid of any chronic illness and were not on any long-term medication.

Armamentarium-

- Dental oral mirror (odontoscope)
- Straight dental probe
- Plain test tubes
- Normal Saline
- Sterile container for saliva
- Cooling centrifuge
- Micropipettes with plastic disposable pipette tips
- Cuvettes
- Water bath
- Distilled water
- Beaker
- Stirrer
- Measuring cylinder
- Test tube stand
- Reagents for vitamin C, NO, GSH & Sialic acid.
- Spectrophotometer
- Auto analyzer

Sample collection and processing of saliva:

5ml of unstimulated salivary sample was collected from each patient after rinsing and spitting with normal saline (0.9% v/v) for a period of one minute between 10 am to 12 pm to avoid circadian variations. Two milliliters of saliva was collected and transferred for biochemical analysis.

Patients were given detailed information about the collection protocol:

(a) Refrain from eating or drinking at least 90 minutes prior to salivary collection.
(b) To sit in a comfortable position with eyes open and head tilted slightly forward.
(c) Avoid swallowing and oral movements during collection.

Saliva samples were immediately centrifuged (1000g for 10 minutes) at 4°C to remove cell debris. The resulting supernatants were immediately transferred to 4 separate aliquots. The sample was finally centrifuged for about 15 minutes at 16,000 rpm for 5 minutes to remove the cellular components

i. First group of aliquots were used for estimating vitamin C
ii. Second group of aliquots were used for estimating nitric oxide
iii. Third group of aliquots were used for estimating sialic acid
iv. Fourth group of aliquots were used for estimating glutathione peroxidase.

Antioxidant-Oxidant Index (AOI) was calculated using the ratio between the levels of nitric oxide (NO), vitamin C, total sialic acid and GSH peroxidation levels. Post hoc Bonferroni's test analysis was used for the comparison of the two study groups to the control group. The data is expressed as mean ± SD. The statistical significance of the results was analyzed using post hoc Bonferroni's test. Correlation between the groups was done using Pearson's correlation coefficient test.

RESULTS:

The study comprised of 80 subjects, there were 27 females (34%) and 53 males (66%) in the study group. There were fifty-three males (controls-10; OPMD-30; OSCC-13) and twenty-seven females (controls-10; OPMD-10; OSCC - 07). The mean age was 36.4±1.58 years in control group, 39.30±10.57 years in OPMDs and 49.25±15.58 years in OSCC.

Out of 40 OPMD patients, 5 (12.5%) were without any habit, 18 (45%) with tobacco chewing, 10 (25%) with smoking and 7 (17.5%) with both tobacco chewing and smoking habits.

Among 20 OSCC patients, 1 (5%) was without any habit, 6 (30%) with habit of tobacco chewing, 4 (20%) with smoking and 9 (45%) with both smoking and tobacco chewing.

Our results showed that, among 40 OPMD patients, leaukoplakia was seen in 25 (62.5%) cases, followed by oral submucous fibrosis in 9 cases (22.5%) and lichen planus in 6 (15%) cases.

7 (35%) cases revealed buccal mucosal lesions, 6 (30%) patients showed tongue lesions, 3 (15%) cases showed lesions on tonsil, 2 (10%) showed lesions involving alveolus. Palatal and retromolar trigone had 1 (5%) case each.

Descriptive Statistics of Variables-

The data was recorded and analyzed statistically using SPSS software version 20.0 using one way ANOVA and post hoc Bonferroni's tests. Mean of controls and patients were compared using Student's t-test. The difference was considered statistically significant when *p*-value were 0.001 or less.

The mean salivary vitamin C level was 30.32±4.34 µmol/l in OSCC group whereas; it was 38.20±8.45 µmol/l in OPMDs group and 48.76±2.60µmol/l in control group.

The mean nitric oxide level was 27.34±5.51 µmol/l in OSCC group, 22.5±2.33 µmol/l in OPMD group and 10.11±0.88 µmol/lin control group.

The glutathione reductase activity in control patients was found to be 0.0915 U/ml under optimal pH, temperature and K_m. In OPMD group the GR activity was found to be 0.0515 U/ml. Similarly, the activity in the OSCC group was found to be 0.0292 U/ml.

The total sialic acid (TSA) in the saliva of control patients was found to be 41.241±5.3312 μg/mL. In the case of OPMD patients it was 64.25 ± 4.33 μg/mLand in the OSCC patients it was, $^{79.60 \pm 6.93}$ μg/mL As seen from the graphs above, the levels of salivary vitamin C and glutathione were significantly reduced [Graph 1 & Graph 2] and those of nitric oxide and sialic acid were raised [Graph 3 & Graph 4] in patients having OPMD's and oral squamous cell carcinoma. The antioxidant to oxidant index (AOI) was measured between NO and vitamin C; NO and GSH; total sialic acid and vitamin C and lastly, total sialic acid and GSH. The following indices are tabulated below with the corresponding values. (Tables 1,2,3,4).

It can be seen from the analysis that there was a statistically significant difference between the reduced levels of vitamin C and GSH to those that of raised nitric oxide and sialic acid levels in patients

Table 1: AOI in controls and study groups. *ANOVA, *p*-value <0.001 considered statistically significant

variable	group	n	mean	Std. Deviation	*p*-value
AOI NO / VIT.C	Control	20	0.207	0.023	<0.001
	OPMD	40	0.589	0.166	
	Cancer	20	0.901	0.279	

Table 2: AOI in controls and study groups. *ANOVA, *p*-value <0.001 considered statistically significant.

variable		group	n	mean	Std. Deviation	*p*-value
AOI	NO / GSH	Control	20	0.11	0.012	<0.001
		OPMD	40	0.436	0.122	
		Cancer	20	0.936	0.289	

Table 3: AOI in controls and study groups. *ANOVA, *p*-value <0.001 considered statistically significant.

variable		group	n	mean	Std. Deviation	*p*-value
AOI	TSA / VIT.C	Control	20	0.845	0.093	<0.001
		OPMD	40	1.687	0.466	
		Cancer	20	2.625	0.812	

Table 4: AOI in controls and study groups. *ANOVA, *p*-value <0.001 considered statistically significant

variable		group	n	mean	Std. Deviation	*p*-value
AOI	TSA / GSH	Control	20	0.45	0.052	<0.001
		OPMD	40	1.247	0.351	
		Cancer	20	2.72	0.842	

Table 5: Pairwise comparison of AOI index in control and study groups. *Post-hoc Bonferroni's analysis with ANOVA, *p*-value <0.001 considered statistically significant.

Dependent variable	(i) group	(J) group	mean Difference (i-J)	*p*-value
AOI NO / VIT.C	Control	OPMD	-0.382*	0.001
		Cancer	-0.694*	0.001
	OPMD	Control	0.382*	0.001
		Cancer	-0.312*	0.001
	Cancer	Control	0.694*	0.001

Table 6: Pairwise comparison of AOI index in control and study groups. *Post-hoc Bonferroni's analysis with ANOVA, *p*-value <0.001 considered statistically significant.

Dependent variable	(i) group	(J) group	mean Difference (i-J)	*p*-value
AOI NO / GSH	Control	OPMD	-0.326*	0.001
		Cancer	-0.826*	0.001
	OPMD	Control	0.326*	0.001
		Cancer	-0.500*	0.001
	Cancer	Control	0.826*	0.001

having or suffering from OPMD's and those having histopathologically proven cancer of oral cavity. The data where p-value <0.001 is statistically significant.

Post – hoc Bonferroni's analysis was performed for pairwise comparison between the controls, OPMD and OSCC patients. The analysis clearly indicates

Table 7: Pairwise comparison of AOI index in control and study groups. *Post-hoc Bonferroni's analysis with ANOVA, *p*-value <0.001 considered statistically significant.

Dependent variable	(i) group	(J) group	mean Difference (i-J)	*p*-value
AOI TSA / VIT.C	Control	OPMD	-0.842*	0.001
		Cancer	-1.420*	0.001
	OPMD	Control	0.842*	0.001
		Cancer	-0.938*	0.001
	Cancer	Control	1.420*	0.001

Table 8: Pairwise comparison of AOI index in control and study groups. *Post-hoc Bonferroni's analysis with ANOVA, *p*-value <0.001 considered statistically significant.

Dependent variable	(i) group	(J) group	mean Difference (i-J)	*p*-value
AOI TSA / GSH	Control	OPMD	-0.797*	0.001
		Cancer	-2.270*	0.001
	OPMD	Control	0.797*	0.001
		Cancer	-1.473*	0.001
	Cancer	Control	2.270*	0.001

Table 9: *p* –value significance chart between OPMD and OSCC group

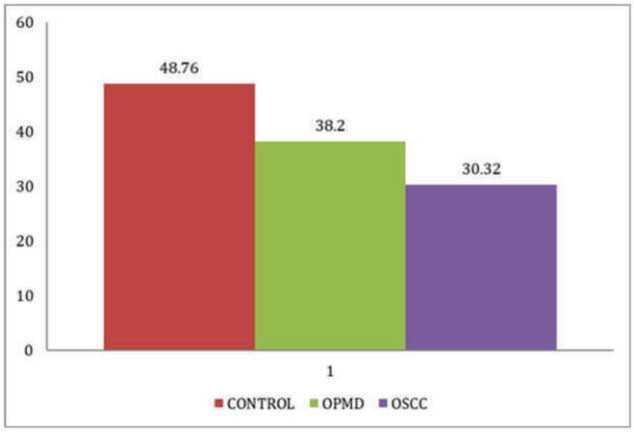
Variables	(A) Group	(B) Group	Mean Difference (A-B)	Standard error	*p*-value
VITAMIN C	Control	OPMD	10.56	2.489	0.003
	Control	OSCC	18.44	2.489	0.003
	OPMD	OSCC	7.88	1.889	0.001
NITRIC OXIDE (NO)	Control	OPMD	-12.39	1.81	0.001
	Control	OSCC	-17.23	1.81	0.001
	OPMD	OSCC	-4.44	1.344	0.001
GLUTATHIONE	Control	OPMD	0.04	0.0177	0.003
	Control	OSCC	0.0623	0.0177	0.003
	OPMD	OSCC	0.0223	0.0177	0.003
TOTAL SIALIC ACID	Control	OPMD	-23.009	2.389	0.001
	Control	OSCC	-38.359	2.389	0.001
	OPMD	OSCC	-15.349	1.891	0.001

statistical difference between the co-relation between the values of raised NO levels to those of vitamin C. This significant statistical relationship where p-value is less than 0.001 indicates changes in the biochemical nature of saliva with patients having mucosal potentially malignant disorders as well as oral cancer. (Tables 5,6,7,8).

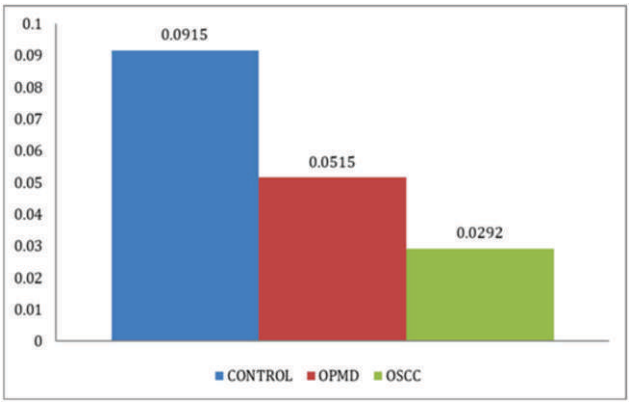
Our results were consistent with the grouped analysis between various salivary biomarkers as can be from the tabulated data above. There was significant increase in AOI [NO/Vit.C] from control group (0.023), OPMDs (0.167) and OSCC group (0.279) (Table 1)

And AOI [NO/GSH] from control group (0.012), OPMDs (0.122) and OSCC group (0.289) (Table 2)

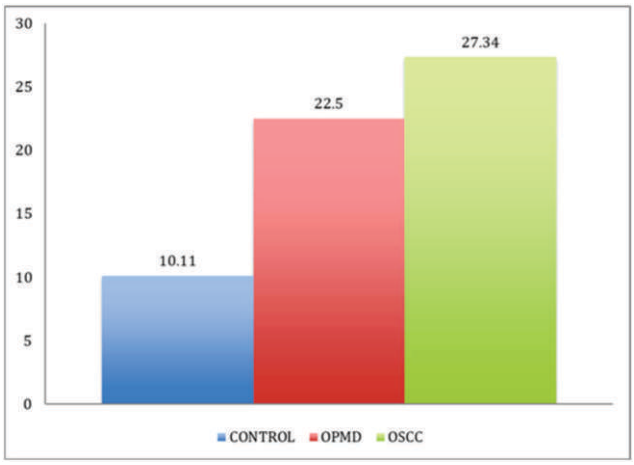
Similarly the increase was seen in AOI [TSA/Vit.C] from control group (0.093), OPMDs



GRAPH 1: Mean concentration of Vitamin C (µmol /L).

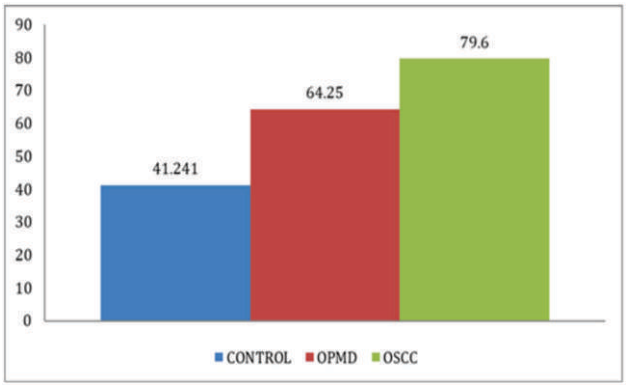


GRAPH 2: Mean concentration of salivary glutathione (U/ml).



GRAPH 3: Mean concentration of nitric oxide - NO (µmol /L).

(0.467) and OSCC group (0.812)(Table 3). The AOI [TSA/GSH] showed increase from control group (0.052), OPDs (0.351) and OSCC group (0.842)(Table 4). On comparing salivary vitamin C levels and GSH levels with those of the nitric oxide and sialic acid, the difference was highly significant (p<0.003). Though the levels were not significant between OPMD and OSCC group (p>0.001) (Table 9).



GRAPH 4: Mean concentration of salivary TSA (µg/mL)

The levels of salivary vitamin C and glutathione were significantly reduced and those of nitric oxide and sialic acid were raised in patients having OPMD's and oral squamous cell carcinoma.

DISCUSSION:

A biomarker is defined as a pharmacological or physiological measurement that is used to predict a toxic event; a specific molecule in the body, which has a particular feature that makes it instrumental for measuring disease progression or the effects of treatment. Biomarkers are by definition suitable to develop new diagnostic tools, alone or in combination with traditional methods (Brinkman and Wong, 2006). [4]

The immune system of an individual works in a very well-organized manner for the sustenance of the normal equilibrium, thus helping in achieving a disease-free state. Free radicals are generated as a by-product of normal cellular metabolism and the increase in the levels of ROS may be caused either due to increased production or decreased destruction of these formed free radicals by the enzymatic and nonenzymatic antioxidants. To control the influx of ROS, aerobic cells have developed their own defense system – the antioxidant protection system, which includes enzymatic and nonenzymatic components that function interactively and synergistically to neutralize free radicals.[5]

Nitric oxide (NO) is a highly reactive oxygen radical found in normal and malignant tissues, however its levels are much higher in malignant tissue. Its generation is thought to be by a family of enzymes called nitric oxide synthase (NOS). NOS are available in three isoforms NOS1 or type 1 or nNOS (neuronal), NOS2 or type 2 or iNOS (inducible), NOS3 or type 3 or eNOS (endothelial). Out of these, iNOS produces continuous NO and is shown to be expressed in many malignant tumors.[6]

Vitamin C is a major water-soluble antioxidant. Generally, vitamin C is a six carbon

organic acid with structural similarity to glucose. It acts as a potent reducing agent and its laevo (l-) form is generally more active. Vitamin C has been shown, together with some other antioxidant agents, to be an endogenous modulator of the metabolism of nitric oxide (NO) and subsequent endothelium-dependent vasodilation. The difference in NO production at the periodontal level is probably different from NO in the bloodstream: In the mouth, it is an antibacterial defense, whereas systemically, it impacts endothelial function. [7,8]

Glutathione occurs in high concentrations (0.5 to 10mmol/L) in virtually all cells. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress and many pathological conditions. Salivary glutathione levels may be an index of oxidative stress at the level of the upper airways and in particular of oral cavity and pharynx. Therefore, high salivary glutathione may be an epidemiological marker to identify subjects with an increased risk of developing HNSCC, to submit to strict follow-up and chemoprevention. Metabolic alterations of saliva could be both an epidemiological marker and a target for chemoprevention of oral and oropharyngeal carcinogenesis.[9,10] Sialic acid plays a significant role in cancer due to increased sialylation and sialyltransferase activity.[11]

In our study, levels of nitric oxide were lowest in control group but increased significantly in OPMDs and OSCC groups. The levels of nitric oxide in OPMDs and OSCC were comparable. This is in accordance with the study done by Juneja S et al[12], who also reported increased level of nitric oxide in OPMD and OSCC patients.

Serum levels of vitamin C were highest in control group and reduced significantly in OPMDs and OSCC group in the present study. There was statistically significant difference in the levels of vitamin C in OPMDs and OSCC also. Vitamin C reduces the degradation of Vitamin E thus enhancing chemotaxis, phagocytosis and collagen synthesis. It inhibits the formation of nitrosamines and causes reduction in oncogene expression. Vitamin E maintains the integrity of membranes thus inhibiting the growth of cancer cell and differentiation. It also inhibits mutagenicity and formation of nitrosamines. Synergistic action between Vitamin E, selenium and ascorbate hinders DNA and RNA protein synthesis in the cells. [13]

Glutathione participates in detoxification at several different levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, glutathione provides the cell with multiple defences not only against ROS but also against their toxic products. In the study done by us, the levels of salivary glutathione reductase were lower in OSCC when compared to OPMD and the difference was statistically significant ($p<0.003$)

In our study, the total sialic acid (TSA) in the saliva of control patients was found to be 41.241±5.3312 μg/mL. In the case of OPMD patients it was 64.25 ± 4.33 μg/mLand in the OSCC patients it was, 79.60 ± 6.93 μg/mL We also found significantly higher levels of free sialic acid in well-differentiated OSCC patients compared to those of moderately differentiated cases. This suggests correlation of elevated salivary sialic acid levels to the progression of OSCC. There is elevated salivary sialic acid level in moderately /poorly-differentiated squamous cell carcinoma without any significant change in well-differentiated squamous cell carcinoma. This finding was consistent with the study by Rajaram S et al.[14]

CONCLUSION:

The findings of the present study indicate that estimation of Vitamin C, nitric oxide, GSH and sialic acid can be suitably used and could assist in the early diagnosis of potentially malignant disorders and oral cancer using saliva. OSCC increases oxidative stress and may trigger mutations, suggesting that it may play a role in the initiation and development of multistage carcinogenesis. Understanding the function of reactive oxygen species (ROS) as key mediators in signaling pathways may open up new avenues for pharmacological intervention.

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Conflicts of interest
There are no conflicts of interest.

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