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ORIGINAL ARTICLE

Assessment of bone metabolism biomarkers in serum and saliva of cirrhotic patients

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Abstract

Objective To assess the serum and salivary levels of biomarkers related to bone metabolism in cirrhotic patients as well as the evidence of osteoporotic changes on panoramic radiographs.

Materials and methods Thirty-eight cirrhotic patients underwent anamnesis and physical examination. Specimens of blood and saliva were collected for evaluation by using Luminex™ xMAP technology to quantify RANKL, OPG, IL-1β, IL-6 and TNF-α. Panoramic radiographs were evaluated based on the mandibular cortical index (MCI) and the resulting data were compared to the expression of biomarkers in serum and saliva. Descriptive data analysis was performed and the Mann–Whitney's test and Spearman's correlation were used.

Results Most of the sample consisted of males (68.4%) who had cirrhosis mostly resulting from alcoholism (28.9%). Median concentration values of RANKL (74.44 pg/mL), IL-1 β (45.91 pg/mL), IL-6 (67.69 pg/mL) and TNF-α (5.97 pg/mL) in saliva were higher than those observed in serum. In 72.7% of the panoramic radiographs, MCI was found to be suggestive of osteoporotic changes. No statistically significant correlation was observed between salivary and serum expressions of biomarkers or between biomarkers and MCI.

Conclusion RANKL, OPG, IL-1β, IL-6 and TNF-α are expressed differently in serum and saliva and the concentration of these biomarkers is not related to MCI.

Clinical relevance This study contributes to the study of the mechanisms of osteoporosis in cirrhotic individuals.

Keywords Hepatic cirrhosis · Osteoporosis · Bone biomarkers · Saliva · Panoramic radiography

Introduction

Hepatic cirrhosis is often a clinical consequence of several chronic hepatic diseases and is characterised by extensive tissue fibrosis and formation of nodules in the liver structure [1].

Cirrhotic individuals on the liver transplant waiting list face several complications related to hepatic functional deficit, such as hepatic osteodystrophy. This condition includes relatively rare alterations (e.g. osteomalacia) as well as more common ones (e.g. osteoporosis) [2], which can lead to spontaneous fractures as a result of low-intensity trauma [3]. Although its pathogenesis is unknown, it seems to be multifactorial as genetic factors, nutritional deficiency, low concentrations of calcium and vitamin D, low growth factor (similar to type-1 insulin) and excessive consumption of alcohol are involved [4].

Even though dual-energy X-ray densitometry is the gold standard for diagnosis of changes in bone mineral density (BMD), alternatives can be used as early indicators in the diagnosis of osteoporosis. In fact, several linear and qualitative radiomorphometric indices have been proposed to detect low BMD. A recent systematic review showed that the Klemetti index, which assesses the mandibular cortical erosion, has sensitivities of 0.789 and 0.806 in the detection of low BMD, respectively [5]. In addition, other studies have

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shown that these radiomorphometric indices are correlated with biochemical markers of bone turnover in osteoporotic individuals, such as alkaline phosphatase and N-terminal telopeptides of type I collagen and of type II collagen [6–10].

In the context of bone metabolic disorders, such as osteoporosis, the osteoblasts and osteoclasts are significantly affected by a system of proteins consisting of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B (RANK) and receptor activator of nuclear factor kappa-B ligand (RANKL) [10]. RANKL is mainly produced by osteoblasts and is capable of binding to the RANK present in the membrane of osteoblasts and precursor hematopoietic cells, thus promoting osteoclastogenesis and bone resorption [11]. On the other hand, OPG works as potent inhibitor of this interaction, since this protein can bind to RANKL [11]. It has been suggested that individuals with chronic hepatic disease have changes in their serum levels of RANKL/OPG, indicating that these proteins may play a role in the modulation of bone loss [4]. In addition to playing an important role in the bone metabolism, the RANK-RANKL-OPG system participates in the immune system homeostasis [12] and imbalance in this protein system has already been studied in several conditions, such as process of metastasis and malignant cell invasion [13], rheumatoid arthritis [14] and osteoporosis [15].

Concomitantly, pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) have been target of study as they participate not only in the processes of inflammation, steatosis, fibrosis and carcinogenesis, but also in the process of bone loss [16, 17]. It is also important to highlight that cirrhotic individuals present significant dysregulation in their immune responses as the liver is the main organ in the reticuloendothelial system and because of the sequestration of leukocytes by the spleen [18].

These biomarkers of bone metabolism and pro-inflammatory cytokines have been extensively studied in the blood [3], and more recently, their behaviour has also been investigated in the saliva. This occurred because saliva has been shown to be an excellent fluid for diagnosis and detection of systemic diseases [19, 20], being in many cases as effective as blood [21, 22].

In view of the above, the objective of this study was to assess the presence of proteins RANKL, OPG, IL-1β, IL-6 and TNF-α in saliva and serum of cirrhotic individuals as well as to compare the results to evidence of osteoporotic changes on panoramic radiographs.

Materials and methods

Ethical aspects

This study was approved by the local research ethics committee according to protocol number 3065652 and conducted according to the STROBE recommendations for observational studies. All the participants in the study signed a free informed consent form following the principles set by the Helsinki declaration.

Study design, sample and inclusion/exclusion criteria

This is a cross-sectional, quantitative, observational study conducted with cirrhotic individuals on the liver transplant waiting list.

The sample consisted of male and female individuals who were on waiting list for liver transplant. Sample size was calculated by using the Power and Sample Calculation® software considering the incidence of low BMD in the general population (43.9%) [23] and in individuals with chronic hepatic disease (70.8%) [23]. Sample power of 90% and confidence interval of 95% were used to obtain an ideal sample of 38 individuals.

All cirrhotic individuals older than 18 years old who were on the liver transplant waiting list were included in this study. Those individuals with specimens of saliva and serum inadequate for laboratory analysis were excluded.

Clinical assessment

All the participants underwent clinical examination for collection of the following data: gender, age and presence of chronic systemic diseases. With regard to hepatic cirrhosis, the following data were collected: aetiology (i.e. viral hepatitis, alcoholism, others), MELD score (model for end-stage liver disease) and presence of cirrhotic complications (e.g. hepatocellular carcinoma, collateral circulation, changes in red and white blood cells, coagulopathy, upper gastrointestinal bleeding, hepatic encephalopathy, spontaneous bacterial peritonitis and ascites). Presence of spontaneous gingival bleeding was also recorded.

Radiographic evaluation

Of the 38 participants, 22 had panoramic radiographs available for study. Panoramic radiographs from patient dental records taken in the past 6 months were assessed in order to measure the mandibular cortical index (MCI) and to rate the patient according to the index proposed by Klemetti and collaborators (1994) [24]. This index assesses the lower cortex of the mandible located posteriorly to the mental foramen on both sides as follows: C1 (endosteal cortical margin is clear and sharp on both sides); C2 (endosteal margin of the cortex showing semi-lunar defects—lacunar resorption—or endosteal cortical residues); and C3 (formation

of dense layers of endosteal cortical residues which are clearly porous). The radiograph analysis was performed by two examiners with experience in maxillofacial radiology who were calibrated (J.R.T. and N.T.D.) (inter-examiner kappa = 0.8; intra-examiner kappa = 0.85) and a third (K.L.O.) was consulted in case of disagreement.

Blood and saliva collection

Blood and saliva were collected in the presence of the following biomarkers: RANKL, OPG, IL-1β, IL-6 and TNF-α. Whole unstimulated saliva collection was performed in which the patients were instructed to avoid food, beverages and oral hygiene 60 min before the examination. For saliva collection, the patient was asked to spit into a funnel placed inside a scaled plastic tube so that the whole saliva could be spontaneously produced within 10 min. Next, the saliva was immediately distributed in safe-lock tubes and stored in freezer at − 80 °C. After saliva collection, blood was collected through peripheral venous access and then stored in a test tube containing coagulant activator and left under refrigeration at 4 °C for 30 min. Next, the tubes were centrifuged at 3000 rpm for 15 min. Serum was collected with a pipette and stored into safe-lock tubes before being placed in a freezer at − 80 °C.

Luminex analysis

Luminex™ xMAP (multiple analyte profiling) technology was used because it is an immunoassay using microspheres stained with fluoride phosphate. The assays were conducted based on the conventional "sandwich" methodology, in which the mix of microspheres is incubated in a 96-well plate. Next, the biotinylated detection antibody was added and the signal was amplified through incubation with streptavidin–phycoerythrin conjugate. Reading was made with a Luminex 200 device (Software xPonent/Analyst version 4.2).

Statistical analysis

Data were analysed by using the Statistical Package for the Social Science software (SPSS® for Windows, version 22.0, SPSS Inc., Chicago, IL, USA). Shapiro–Wilk normality test was used to assess the non-parametric distribution of the data. Mann–Whitney's test was used for comparison of data distribution between MCI and independent variables (i.e. biomarkers measured in saliva and serum). The correlations between the values of proteins in saliva and blood, including between these proteins and independent variables, were checked by using Spearman's correlation. All statistical analyses were conducted at a significance level of $P \leq 0.05$.

Results

The study sample consisted of 38 cirrhotic individuals on the liver transplant waiting list, with the majority being males ($n = 26$; 68.4%), mean age of 50.61 years old (range from 20 to 71 years old; median = 52.5), MELD score of 18 (range from 11 to 30; median = 17) at the examination and mean time on the waiting list of 21.34 months (range from 1 to 75 months; median = 9). The main aetiology of cirrhosis was alcoholism ($n = 11$; 28.9%) followed by hepatitis C ($n = 09$; 23.7%). Among the complications of cirrhosis, the most frequently found were changes in red and white blood cells ($n = 37$), coagulopathy ($n = 36$) and presence of collateral circulation ($n = 34$) (Table 1).

The biomarkers of bone metabolism investigated in the present study were found at different levels in all saliva and blood specimens. RANKL and pro-inflammatory cytokines were found at higher levels in saliva than in serum (Table 2).

No statistically significant correlations were observed ($P < 0.05$) between serum and salivary levels of the biomarkers in question as well as between their levels (serum and saliva) and age, comorbidities, time on the transplant waiting list, aetiology of the cirrhosis, MELD score and complications of hepatic cirrhosis.

Of the 38 participants, 22 had panoramic radiographs available for study. Analysis showed that the majority of the sample presented some changes in the mandibular cortex suggestive of osteoporotic alterations ($n = 16$; 72.7%) according to MCI, since 13 patients were rated as C2 (59.1%) and three as C3 (13.6%) (Fig. 1). No statistically significant correlations were observed between MCI and the variables studied (i.e. age, gender, MELD score, cirrhosis aetiology, cirrhotic complications and comorbidities) ($P > 0.05$).

The indices were dichotomised into normal (C1) and altered (C2 and C3) MCI so that they could be correlated with serum and salivary biomarkers. Patients with altered MCI showed low levels of RANKL and OPG in their blood compared to those patients with normal MCI. In saliva, RANKL and OPG were at higher levels in patients with altered MCI compared to those with normal MCI. Among the inflammatory biomarkers, one can observe that patients with altered MCI had higher level of IL-6 only in the blood compared to saliva, where the levels of all inflammatory biomarkers were higher. However, no statistically significant correlations were found between MCI and none of the biomarkers (Table 3).

One can observe higher levels of IL-6 and RANKL in the saliva of patients with spontaneous gingival bleeding by correlating this symptom with the proteins studied here, a finding which was statistically significant (Table 4).

Table 1 Characterisation of the study sample. Demographic data, MELD score, aetiology of cirrhosis, cirrhotic complications, comorbidities and spontaneous gingival bleeding ($n=38$)

Variables	*n* (%)
Age (in years)	50.61 ± 14.38
Gender	
Male	26 (68.4)
Female	12 (31.6)
MELD score (current)	18.00 ± 4.93
Aetiology of cirrhosis	
Alcoholic cirrhosis	11 (28.9)
Hepatitis C	09 (23.7)
Cryptogenic cirrhosis	06 (15.8)
Autoimmune hepatitis	04 (10.5)
Non-alcoholic steatohepatitis cirrhosis	02 (5.3)
Budd-Chiari syndrome	02 (5.3)
Sclerosing cholangitis syndrome	01 (2.6)
Schistosomiasis	01 (2.60
Hepatitis B	01 (2.6)
Secondary biliary cirrhosis	01 (2.6)
Complications of cirrhosis	
Changes in red and white blood cells	37 (97.4)
Coagulopathy	36 (94.7)
Hepatic encephalopathy	34 (89.5)
Collateral circulation	34 (89.5)
Upper gastrointestinal bleeding	22 (57.9)
Changes in white blood cells	22 (57.9)
Ascites	18 (47.4)
Hepatocarcinoma	05 (13.2)
Bacterial peritonitis	04 (10.5)
Comorbidities	
Diabetes	11 (28.9)
Nephropathies	05 (13.2)
Pulmonary comorbidities	05 (13.2)
Cardiopathies	01 (2.6)
No comorbidities	16 (42.1)
Spontaneous gingival bleeding	
Presence	06 (15.8)
Absence	32 (84.2)

Discussion

Imbalance in bone metabolism is one of the complications of hepatic cirrhosis and osteoporosis is more prevalent in these patients, who are at higher risk of fracture compared to the general population [25, 26]. These changes in bone mineral density involve multiple aetiological factors and, at least theoretically, might be identified by radiographic techniques as well as by alterations in biomarkers related to bone metabolism [2].

One of the ways to detect changes in BMD is by measuring the panoramic radiomorphometric indices. Even though panoramic radiographs should not be obtained for diagnosing BMD specifically, when they are available in the dental records of the patient, they can and must be used as a detection tool and the patient should be referred to a physician for medical evaluation, if necessary. Although there are various radiomorphometric indices, in the present study, we opted to use MCI because of its effectiveness [27, 28]. By using this index, we found that more than 72% of the cirrhotic patients showed changes compatible to decreased BMD.

Assessment of serum concentrations of biomarkers such as RANKL, OPG and pro-inflammatory cytokines is another way to investigate the bone metabolism. Measurement of these proteins has been widely used in studies on osteoporosis [29]. In the present study, the presence of these biomarkers was assessed in serum and saliva in order to verify whether the latter might have results equivalent to those of the former (which is the gold standard).

The serum values of OPG found in the present study, which were significantly higher than those of RANKL, are numerically resembling those reported by Azizieh and collaborators (2019) [30] in post-menopausal women with osteoporosis. However, lower levels of RANKL were similar to those of patients with normal BMD.

Table 2 Descriptive analysis of the values of RANKL, OPG, RANKL/OPG, IL-1β, IL-6 and TNF-α in specimens of serum and saliva ($n=18$)

	Variable	Mean	SD	Min	Max	Percentile		
						25	50	75
Serum	RANKL	7.45	19.50	0.31	99.85	0.87	1.46	3.77
	OPG	468.10	229.93	191.00	1244.00	275.50	442.00	570.50
	RANKL/OPG	0.018	0.054	0.000	0.328	0.001	0.003	0.011
	IL-1β	0.64	1.02	0.18	6.52	0.31	0.39	0.59
	IL-6	19.30	30.72	1.83	172.00	4.92	9.95	18.88
	TNF-α	2.43	2.43	0.40	15.94	1.38	2.08	2.71
Saliva	RANKL	74.44	181.96	0.27	1122.00	7.20	26.62	75.31
	OPG	342.71	384.84	44.65	1903.00	116.00	216.00	342.25
	RANKL/OPG	0.337	0.415	0.000	1.432	0.027	0.137	0.579
	IL-1β	45.91	49.90	1.07	240.00	8.38	27.80	68.16
	IL-6	67.69	89.36	6.30	464.00	20.50	32.53	80.10
	TNF-α	5.97	5.37	0.68	23.82	2.58	4.14	7.54

Values in pg/mL. *SD* standard deviation; *Min* minimum value; *Max* maximum value

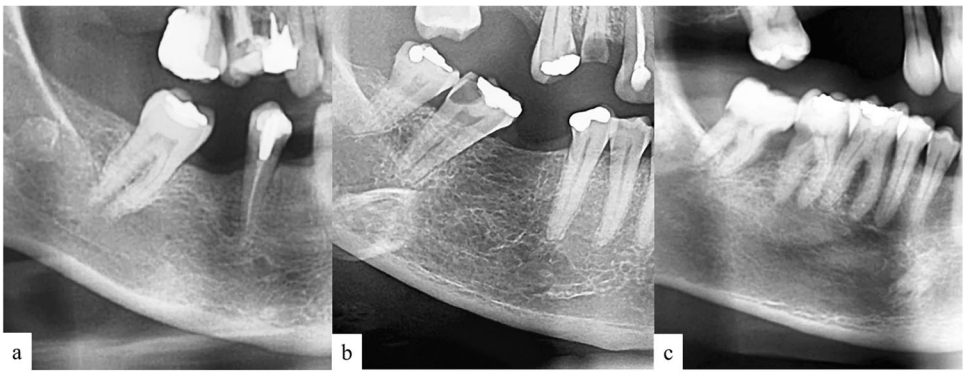

Fig. 1 Mandibular cortical index: **a** C1, endosteal cortical margin is clear and sharp on both sides; **b** C2, endosteal margin of the cortex showing semi-lunar defects or endosteal cortical residues; and **c** C3, formation of dense layers of endosteal cortical residues which are clearly porous

It is believed that cirrhotic patients develop a systemic inflammatory condition during the natural course of the disease, independently of its aetiology, with high expression of pro-inflammatory cytokines and possible interference with the expression of RANKL [29, 31]. On the other hand, Moschen and collaborators (2005) [4] state that the presence of small amounts of RANKL does not mean necessarily a low osteoclastic activity (since pro-inflammatory cytokines also activate osteoclasts), but a reflection of a low osteoblastic activity as RANKL is produced by osteoblasts. Still, according to these authors, the increased values of OPG work as a feedback mechanism in an attempt to prevent bone loss and maintain BMD.

Table 3 Mean and standard deviation of the values of the biomarkers according to the mandibular cortical index ($n=22$)

Variables	MCI	Mean	Standard deviation	P*
RANKL—serum	Normal	9.51	14.62	0.231
	Altered	1.48	1.36	
OPG—serum	Normal	608.33	369.78	0.367
	Altered	431.69	193.11	
RANKL/OPG—serum	Normal	0.019	0.026	0.407
	Altered	0.004	0.004	
IL-1β—serum	Normal	0.48	0.25	0.971
	Altered	0.46	0.24	
IL-6—serum	Normal	9.22	5.33	0.294
	Altered	19.59	22.39	
TNF-α—serum	Normal	4.66	5.57	0.367
	Altered	2.32	1.10	
RANKL—saliva	Normal	27.24	21.59	0.858
	Altered	115.44	274.86	
OPG—saliva	Normal	149.04	53.94	0.098
	Altered	373.67	309.63	
RANKL/OPG—saliva	Normal	0.234	0.219	0.858
	Altered	0.406	0.507	
IL-1B—saliva	Normal	32.88	35.23	0.494
	Altered	43.18	39.15	
IL-6—saliva	Normal	44.34	41.21	0.747
	Altered	71.93	84.56	
TNF-α—saliva	Normal	4.28	1.50	0.971
	Altered	8.02	7.53	

*Mann–Whitney's test

Therefore, at a quick glance at this information (i.e. panoramic radiograph and serum biomarkers) and in light of the literature, we might believe that a high incidence of altered BMD corresponds clinically to the serum expression of osteoporosis-related biomarkers. However, statistical analysis found no correlation between serum expression of biomarkers and MCI, perhaps because panoramic radiographs show the result of a long process of bone resorption/apposition while biomarkers are linked to a transient bone metabolic condition which can change under different stimuli in cirrhotic patients.

By comparing the values of biomarkers in serum and saliva, we found no correlation between them. In addition, the levels of biomarkers were always higher in the saliva than in the blood. It is possible that the peculiarity of the oral microenvironment involving the co-existence of regional inflammatory conditions (e.g. gingivitis and periodontal diseases) has an influence. Previous studies showed that prevalence, extension and severity of periodontal disease are more likely to occur in patients with cirrhosis than in healthy individuals, with the former presenting greater loss of insertion and more gingival recession [32]. This fact can explain the differences in the expression of biomarkers in the present study as the salivary concentration of pro-inflammatory cytokines increases depending on the progression of periodontal disease, being also associated with a greater loss of insertion [33, 34]. In fact, we observed that the salivary concentrations of RANKL and IL-6 were statistically higher in patients with gingival bleeding, which might point to the presence of a local inflammatory process developing concomitantly with an existing systemic condition. Moreover, a recent systematic literature review showed that IL-β and IL-6 are important salivary biomarkers which are increased in patients with gingivitis and periodontitis [35], highlighting that higher levels of these proteins in saliva are indicative of local inflammatory process. For García-Valdecasas-Campelo and collaborators (2006) [36], pro-inflammatory cytokines

Table 4 Correlation between the presence of spontaneous gingival bleeding and concentrations of RANKL, OPG, RANKL/OPG, IL-1β, IL-6 and TNF-α in saliva and blood

Variable	RANKL—serum	OPG—serum	RANKL/OPG—serum	IL-1B—serum	IL-6—serum	TNF-α—serum	RANKL—saliva	OPG—saliva	RANKL/OPG—saliva	IL-1B—saliva	IL-6—saliva	TNF-α—saliva
Gingival bleeding												
No	6.42 ± 18.31	456.90 ± 230.50	0.02 ± 0.06	0.49 ± 0.29	19.77 ± 32.70	2.53 ± 2.62	38.69 ± 46.83	348.04 ± 398.63	0.26 ± 0.36	49.57 ± 53.38	62.13 ± 93.38	5.77 ± 5.18
Yes	12.90 ± 26.30	527.83 ± 238.16	0.02 ± 0.03	1.43 ± 2.49	16.77 ± 18.51	1.86 ± 0.72	265.12 ± 423.80	314.31 ± 331.08	0.74 ± 0.50	26.41 ± 14.79	97.36 ± 61.47	7.01 ± 6.77
*P**	0.689	0.378	0.673	0.630	0.984	0.603	0.015	0.904	0.022	0.779	0.049	0.968

*Mann–Whitney's test. Values expressed in mean and standard deviation

such as IL-β, IL-6 and TNF-α have pro-osteoclastogenic and pro-resorption potentials.

Nevertheless, in the present study, the other pro-inflammatory cytokines (i.e. IL-β and TNF-α) were not found to be statistically correlated with gingival bleeding. It is probably happened because sample calculation was not performed to verify the correlation between biomarkers and gingival or periodontal disease. For this reason, it is necessary to take caution when interpreting these results. Therefore, further studies are needed to assess the presence of these same biomarkers in cirrhotic patients with and without periodontal disease.

This is the first study investigating the proteins RANKL, OPG and pro-inflammatory cytokines in blood and saliva of cirrhotic patients in which one attempted to detect associations with osteoporotic alterations. However, this study has some limitations. Because it is an observational cross-sectional study, the results presented here require further longitudinal assessment in order to test the same hypotheses as well as to determine the cause-effect relationships between the presence of the studied proteins and presence of changes suggestive of osteoporosis during the natural course of cirrhosis. Similarly, future longitudinal studies are needed to monitor the progression of the periodontal disease concomitantly and clinically, including the levels of the studied proteins.

Therefore, it is possible to conclude that the biomarkers RANKL, OPG, IL-1β, IL-6 and TNF-α are differently expressed in saliva and blood and that their concentrations in these fluids are not correlated with mandibular cortical defects on panoramic radiographs.

Author contribution Conceptualisation—Jefferson Rocha Tenório, Karem López Ortega. Methodology—Jefferson Rocha Tenório, Nathália Tuany Duarte, Mariana Lobo Bergamini. Formal analysis and investigation—Jefferson Rocha Tenório, Natália Andrade, Ana Carolina Mamana Fernandes de Souza. Writing (original draft preparation)—Jefferson Rocha Tenório, Natália Andrade, Karem López Ortega. Writing (review and editing)—Jefferson Rocha Tenório, Paulo Henrique Braz-Silva, Karem López Ortega. Funding acquisition—Paulo Henrique Braz-Silva; Supervision: Karem López Ortega.

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Declarations

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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