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# Lymphatic Vessel Density and VEGF-C Expression are Significantly Different Among Benign and Malignant Thyroid Lesions

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**Abstract** Thyroid cancer is the most frequent endocrine neoplasia worldwide. The route for metastasis and loco-regional invasion preferentially occurs by lymphatic vessels. For this reason, the assessment of lymphatic vessel density (LVD) is supposed to represent both a prognostic parameter and also a potential therapeutic target. In order to evaluate the value of LVD in benign and malignant thyroid

lesions, we analyzed 110 thyroidectomy specimens using D2-40, a specific marker for lymphatic vessels and vascular endothelial growth factor C (VEGF-C), the most potent molecule of lymphatic proliferation. LVD was significantly different between papillary and follicular carcinomas in total ( $p=0.045$ ) and peritumoral area ( $p=0.042$ ). Follicular adenoma and follicular carcinoma showed an important difference of intra- ( $p=0.019$ ) and peritumoral ( $p=0.033$ ) LVD. VEGF-C was more markedly expressed in malignancies than in benignant lesions ( $p=0.0001$ ). Almost all cancers with high positive VEGF-C expression also exhibited increased peritumoral LVD ( $p=0.049$ ) when compared with the benignant lesions. Indeed, the high peritumoral LVD of malignant thyroid lesions is an important finding for surgery planning and supports the practice of total thyroidectomy in malignant thyroid neoplasm's since the lymphatic peritumoral vessels definitely are an escape path for tumor cells.

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## Introduction

Several mechanisms can mediate tumor cell dissemination, including hematogenous or lymphatic spread, local invasion, and direct seeding of body cavities or surfaces [1]. Increased lymphatic vessel density (LVD) is commonly associated with more aggressive behavior of malignant neoplasias which commonly escape from the primary site preferentially through lymphatic vessels due to its structural features without cohesive pericytes and thin endothelial wall [2]. Evaluation of lymphangiogenesis in cancer is relatively recent when compared to angiogenesis, mainly due to the lack of specific

markers to identify lymphatic endothelial cells. D2-40 monoclonal antibody [3, 4] proved to be a selective marker for lymphatic endothelium, allowing identification of lymphatic vessels in formalin-fixed paraffin-embedded tissue, and consequently facilitating the studies of LVD in solid tumors [4, 5]. This is important because identification of lymphatic vessels on morphological basis alone is not consistent due to overlap with blood vessels capillaries [6–8]. Furthermore, adequate distinction between these two types of vessels is crucial for understanding the biology of interaction between tumors and neoformed vascular structures, potentially contributing to the specific tailoring of therapeutical strategies in the future. This approach highlights the important differences between blood and lymphatic vessels regarding their receptors, with potential effects on diagnostic evaluation as well as prognostication [2].

In recent years, a number of evidences provided better understanding of the role and the molecular mechanisms related to development and maintenance of lymphatic vessels and also their role in different pathologic conditions [1, 9]. The vascular endothelial growth factor C (VEGF-C) is involved in proliferation and migration of endothelial cells, increased vascular permeability and lymphangiogenesis [10, 11]. VEGF-C and its receptor VEGFR-3 were shown to be essential to lymphatic vessels development in embryos [12] and in the maintenance of lymphatic endothelial cells in adults [13]. Some reports showed VEGF-C expression in several types of human tumors and a correlation between VEGF-C expression in primary tumors and nodal metastasis [8, 14, 15].

Thyroid carcinomas are the most frequent endocrine malignancy, mainly affecting women, with an estimated 37,200 new cases and 1,630 deaths due to the thyroid cancer in United State of America in 2009 [16]. Most thyroid tumors follow an indolent clinical course with favorable prognosis [17–20]. Although it has been already well characterized that metastatic dissemination differs between papillary and follicular carcinoma [18, 21], studies on differential distribution of these two types of vascular structures in common benign and malignant thyroid lesions are relatively scant in literature. LVD and expression of VEGF-C in malignant and benign thyroid lesions are poorly studied and are still far to be fully understood [21]. In addition, intra- and peritumoral LVD show different patterns and prognostic significance related to the tumor behavior in different types of tumors [22]. However, few data are available regarding LVD and VEGF-C expression in thyroid lesions, and for the majority of them, the results are conflicting [21, 23].

The objective of the present study was to determine the LVD pattern assessed by D2-40 antibody in malignant and benign thyroid lesions, exploring potential differences between LVD distributions among the different types of thyroid lesions and correlated them with VEGF-C expression and clinicopathological features.

## Materials and Methods

The casuistic comprised a series of formalin-fixed, paraffin-embedded tissues from 110 patients submitted to thyroidectomy. The frequency of different thyroid lesions was: 34 (30.9%) goiters, 17 (15.5%) thyroiditis, six (5.5%) follicular adenomas, 49 (44.5%) papillary carcinomas, and four (3.6%) follicular carcinomas (none of them were classified as minimally invasive). The papillary carcinoma samples comprised 33 samples of papillary carcinoma of conventional form, 11 samples of follicular variant of papillary carcinoma, one sample of tall cell variant of papillary carcinoma and four samples of papillary microcarcinoma. For statistical purposes, all papillary carcinomas were grouped as a single type. All patients were submitted to surgical resection by the same surgeon (RSC) and the diagnoses were categorized according World Health Organization classification [24] by one of the authors (KSES).

Pathological features herein evaluated included macroscopic presentation, principal site of the tumor, histological type, status of resection margins, and lymph node status.

## Immunohistochemistry

Thyroid tissue samples were analyzed for D2-40 immunohistochemical expression using monoclonal D2-40 clone (DAKO Corporation, Carpinteria, CA, USA) specific antibody. Comparisons of D2-40 expression in intra- and peritumoral neoplastic areas were quantified. Also, D2-40-LVD from benign lesions as adenoma, goiter, and thyroiditis were evaluated.

The lymphatic molecular player VEGF-C was analyzed in all non-malignant and malignant cases with rabbit polyclonal anti-VEGF-C antibody (Zymed Laboratories, San Francisco, CA, USA). Slides from all 110 specimens were reviewed and mapped, and tissue microarrays were building using manual tissue arrayer (MTA-1 Beecher Instrument, Silver Spring, MD, USA). Representative areas of the thyroid lesions were selected and cores of 1.0 mm in diameter were twice sampled and arranged at 0.3 mm, one from each other, in the recipient paraffin block. A database was build for every block produced, including the coordinates of each core and case of origin. The positive expressions of VEGF-C were semi-quantitatively assessed.

## D2-40 and VEGF-C Immunohistochemistry

D2-40 and VEGF-C protocol followed Longatto-Filho et al. [25] recommendations with slight modifications. Brief-

ly, deparaffinized and rehydrated sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated at 98°C for 20 min for epitope–antigen retrieval. Subsequently, endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol. Primary antibody incubation step was done with 1:200 dilution for D2-40 and VEGF-C, overnight at 4°C. The amplification method used was Novolink system (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK). Visualization was developed with 3,3'-diamino-benzidine (DAKO Corporation, Carpinteria, CA, USA) and counterstaining with Harris' hematoxylin (Merck, Darmstadt, Germany). Negative controls were obtained by omitting the primary antibody incubation step; and tonsil was used as positive controls.

### Immunohistochemical Evaluation

Immunohistochemical reaction for D2-40 was considered positive when the staining decorated the cytoplasm of lymphatic endothelial cells. The evaluation was performed blindly and LVD was assessed as postulated by Weidner et al. [26] with slight modifications. LVD was defined as a single endothelial cell or a cluster of endothelial cells positive for D2-40, sitting around a visible lumen clearly separate from adjacent microvessels and from other connective tissue components. Additionally, as lymphatic vessels could generally appear as distorted and overlapped structures in cancer setting, the packed vessels

were assumed as one lymphatic unit. Number of vessels was determined at 200× magnification, with the mean and median for ten fields calculated and defined as LVD.

Expression of VEGF-C by neoplastic and non-neoplastic samples was semi-quantified under 200× magnification, considering membrane and/or cytoplasmic staining of normal, follicular, or malignant thyroid cells. The evaluation was performed blindly and the tissue microarray spot of each sample were fully analyzed. The following grading system was used: negative (0) absence of expression, + positive staining with expression in up to 10% of cells, ++ positive expression in over 10% up to 25% of cells, +++ positive expression in over 25% up to 50% of cells, and ++++ positive expression in over 50% of cells. For statistical analyses purposes, VEGF-C expression was dichotomized in two categories: negative (0 to up 10% of positivity) and positive (over 10% of positive reaction).

### Statistic Analysis

The data were obtained from the available information on patient's files and tissue samples revised. Accordingly, the statistics were based on these variables.

The relationship between D2-40 expression, VEGF-C, and clinicopathologic features were examined using Pearson's chi-square ( $\chi^2$ ) test or Fisher's exact test when appropriate and considered significant when  $p < 0.05$ . The Mann–Whitney test was used for continuous variables.

**Table 1** Summary of clinico-pathologic features

		Frequencies	Percent
Gender ( $n=110$ )	M	23	21%
	F	87	79%
Age (years, $n=104$ )	<45	55	52.9%
	≥45	49	47.1%
Mean age ( $\pm$ SD, $n=104$ )		45.9+14.9	
Diagnosis ( $n=110$ )	Goiter	34	30.9%
	Thyroiditis	17	15.5%
	Follicular adenoma	6	5.5%
	Papillary thyroid carcinoma	49	44.5%
	Follicular carcinoma	4	3.6%
Capsular invasion ( $n=35$ )	Present	16	45.7%
	Non-detected	19	54.3%
Vascular invasion ( $n=44$ )	Present	12	27.30%
	Non-detected	32	72.70%
Peri-thyroidal soft tissue compromise ( $n=34$ )	Present	6	17.6%
	Non-detected	28	82.4%
Lymph node status ( $n=35$ )	Negative	21	60%
	Positive	14	40%

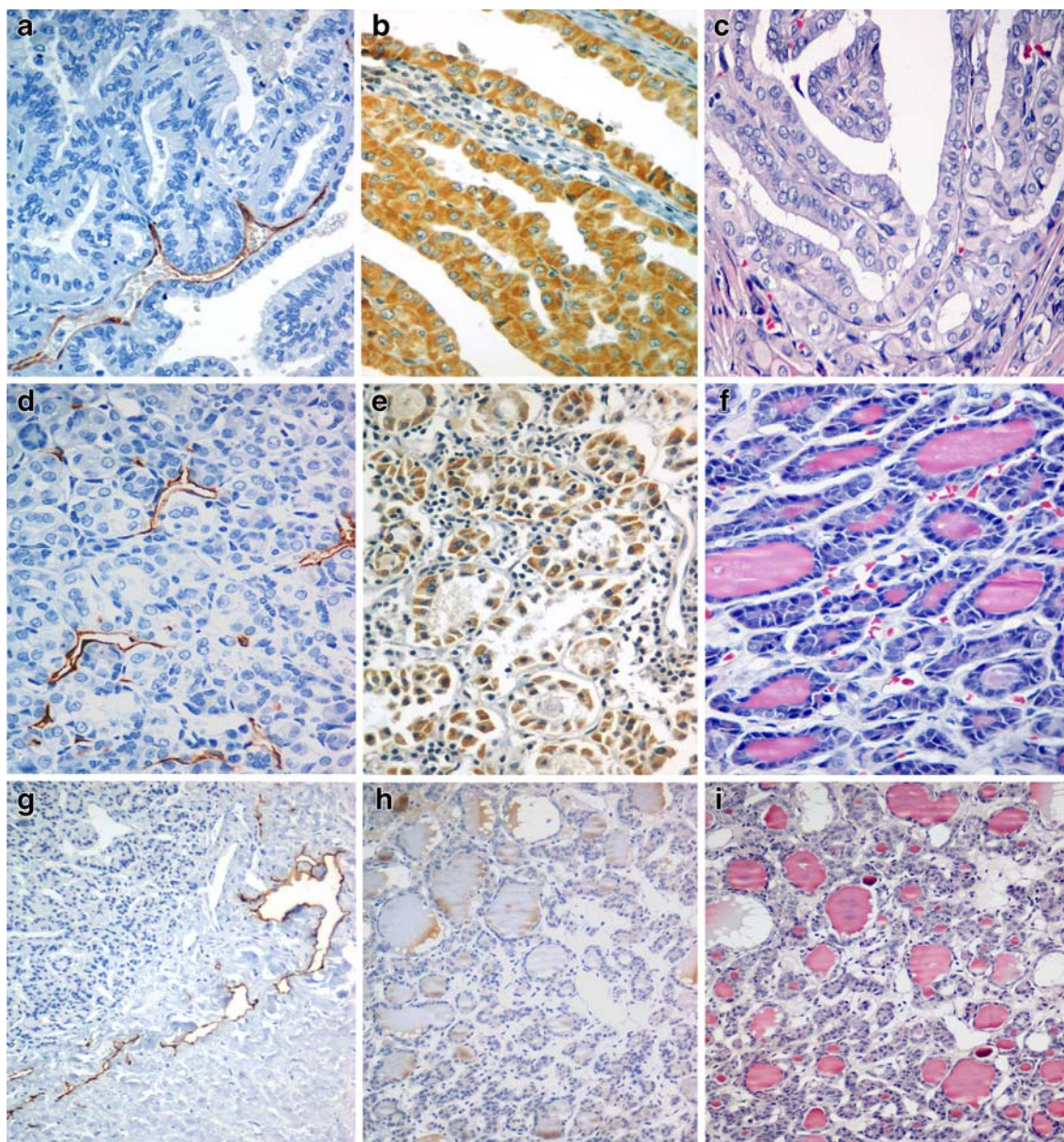


Data were analyzed with Statistical Package of Social Sciences software, version 13.0.

## Results

A total of 110 thyroidectomy specimens were evaluated. Women were 3.7-fold more likely to be affected than men (87 female and 23 male patients) and the mean age was

45.9 years old. Capsular invasion were present in 16 (45.7%) and not detected in 19 (54.3%) samples; vascular invasion were present in 12 (27.3%) and not detected in 32 (72.7%) samples; peri-thyroidal soft tissue compromise were present in six (17.6%) and non-detected in 28 (82.4%) samples; and lymph node status were positive in 14 (40%) and negative 21 (60%) samples (Table 1). The tumors were predominantly found in the right or in the left lobe, each one of them with similar frequencies: 32.7% and 30.8%,



**Fig. 1** The D2-40 antibody stains specifically intratumoral lymphatic endothelial cells in papillary carcinoma (a) 40 $\times$  and follicular carcinoma (d) 40 $\times$ . The D2-40 antibody stains specifically peritumoral lymphatic endothelial cells in follicular carcinoma (g) 20 $\times$ . The VEGF-C antibody strongly (++++), and specifically stains tumoral

cells in papillary carcinoma (b) 40 $\times$  and follicular carcinoma (e) 40 $\times$ . VEGF-C antibody specifically weak (+) stain in goiter (h) 20 $\times$ . Hematoxylin and eosin (HE) staining of papillary carcinoma (c) 40 $\times$ , follicular carcinoma (f) 40 $\times$  and goiter (i) 20 $\times$ . These HE images are from the same samples represented in b, e, and h, respectively

**Table 2** Total, intra- and peritumoral lymphatic vessel density

	Total mean $\pm$ SD	Intratumoral mean $\pm$ SD	Peritumoral mean $\pm$ SD
Goiter	11.2 $\pm$ 4.2	–	–
Thyroiditis	10.3 $\pm$ 5.2	–	–
Follicular adenoma	6.8 $\pm$ 2.8	5.3 $\pm$ 2.1	8.0 $\pm$ 3.5
Papillary thyroid carcinoma	8.4 $\pm$ 2.7	7.0 $\pm$ 3.0	9.0 $\pm$ 3.8
Follicular carcinoma	12.2 $\pm$ 4.1	11.3 $\pm$ 5.9	12.3 $\pm$ 1.9

respectively. The other cases affected right lobe, isthmus, and left lobe in different combinations, but none of these combinations were greater than 10%.

The immunohistochemical expression of D2-40 and VEGF-C in thyroid lesions was analyzed in all samples as exemplified in Fig. 1.

Different lesions showed different LVD patterns as summarized in Table 2. Among neoplasm's, peritumoral LVDs mean were always higher when compared with the intratumoral LVD in the same group. When analyzed in peers, LVD showed significant differences between papillary and follicular carcinomas in total ( $p=0.045$ ) and peritumoral LVD ( $p=0.042$ ). Follicular adenoma and follicular carcinoma showed an important difference of intra ( $p=0.019$ ) and peritumoral ( $p=0.033$ ) LVD (Table 3).

Total, intratumoral or peritumoral LVDs did not differ according to the gender, age, vascular invasion, or lymph node status of patients, although the capsular invasion correlate with peritumoral LVD ( $p=0.045$ ).

The VEGF-C show different patterns of immunoreactivity in all samples and was markedly expressed in both papillary (80.9% positive) and follicular carcinoma (100% positive) when compared to goiter (41.2% positive), thyroiditis (62.5% positive), and follicular adenoma (16.7% positive,  $p=0.0001$ ; Table 4). The more aggressive tumor subtypes, like the tall cell variant of papillary carcinoma, and almost all samples with high positive VEGF-C expression exhibit increased LVD when compared with the negative cases. However, statistical significance was found only when comparing VEGF-C expression with peritumoral LVD ( $p=0.049$ , Table 5). VEGF-C immunoreactivity did not differ according to the gender, age, vascular invasion, capsular invasion, or lymph node status.

**Table 3** Total, intra- and peritumoral LVD comparison by diagnosis

	LVD total		LVD intra		LVD peri	
	Mean $\pm$ SD	<i>P</i> value	Mean $\pm$ SD	<i>P</i> value	Mean $\pm$ SD	<i>P</i> value
Papillary thyroid carcinoma	8.4 $\pm$ 2.7	0.045*	7.0 $\pm$ 3.0	0.083	9.0 $\pm$ 3.8	0.042*
Follicular carcinoma	12.2 $\pm$ 4.1		11.3 $\pm$ 5.9		12.3 $\pm$ 1.9	
Follicular adenoma	6.8 $\pm$ 2.8	0.054	5.3 $\pm$ 2.1	0.019*	8.0 $\pm$ 3.5	0.033*
Follicular carcinoma	12.2 $\pm$ 4.1		11.3 $\pm$ 5.9		12.3 $\pm$ 1.9	

## Discussion

Thyroid cancer usually follows an indolent clinical course and is associated with good prognosis, with few examples emerging as aggressive tumors as anaplastic or medullary carcinomas [17–19]. Papillary carcinomas generally shows good prognosis whereas follicular and some papillary subtypes have more aggressive behavior [27]. Increased lymphatic vessel density is assumed as one of the most important parameters associated to the aggressive behavior of malignant tumors [22]. Nevertheless, we failed to corroborate this rationale because papillary carcinoma and benign lesions showed similar LVD, comparatively. However, the overall findings clearly demonstrated that thyroid lesions have different LVDs, which opens a new exciting view to explore the differences between them based on specific lymphatic vessels development. Remarkably, we identified a significant difference in LVD between follicular adenoma and follicular carcinoma. This is important because it is well-known that this discrimination is difficult to be assessed by morphological criteria, the carcinoma diagnosis is only confirmed if truly capsular invasion is observed [28, 29]. Our results are confirmed, in part, by those reported by Giorgadze and colleagues that showed that LVD determination can be a valuable tool to discriminate follicular lesions of thyroid gland [21]. Of note, we have to stress that peritumoral and intratumoral LVD were significantly different between these neoplasms ( $p=0.033$  and  $0.019$ , respectively), but not the total LVD ( $p=0.054$ ), probably due to the very limited number of cases.

Additionally, we also investigated the expression of VEGF-C, one of the major players in lymphatic vessel



**Table 4** VEGF-C expression in different thyroid lesions

	VEGF-C		<i>P</i> value
	Negative (<10%)	Positive (>10%)	
Goiter ( <i>n</i> =34)	58.8%	41.2%	0.0001*
Thyroiditis ( <i>n</i> =16)	37.5%	62.5%	
Follicular adenoma ( <i>n</i> =6)	83.3%	16.7%	
Papillary thyroid carcinoma ( <i>n</i> =47)	19.1%	80.9%	
Follicular carcinoma ( <i>n</i> =4)	0.0%	100.0%	

sprout, to verify some particular differences among the thyroid lesions we studied. Of interest, we also hypothesized that the well recognized more aggressive behavior of thyroid tumors in males could be attributed to the enhanced LVD stimulated by augmented VEGF-C expression in these patients; however, this was not supported by our results since LVD and VEGF-C expression for both genders had similar values.

Peritumoral LVDs did not correlate with vascular invasion or other clinicopathological features indicative of aggressiveness such as lymph node metastases, which is believed to be associated to the good prognoses in general imputed for thyroid cancer [23]. However, we observed that capsular invasion was related to higher peritumoral LVD ( $p=0.045$ ). This result endorses previous reports with solid tumors where peritumoral, but not intratumoral LVD is associated with more aggressive cancer behavior [4, 7]. Additionally, intratumoral lymphangiogenesis is presumed to form large, weak, and distorted new vessels, implicating a new formed fragile vascular network. Thus, it is reasonable to assume that peritumoral LVD is more tailored to provide competent routes for malignant cells escape. Functional lymphatics at the tumor margin is believed to be sufficient for promoting metastasis by offering a large area for tumor cell escape [4]. This fact was clearly evidenced by Padera and colleagues which studied the influence of tumoral pressure in intratumoral lymphatic vessels, showing that these vessels were collapsed and unable to support the malignant cells spread [30].

Our results showed that peritumoral LVD was significantly higher in follicular carcinoma than in papillary

carcinoma ( $p=0.042$ ), but no other conjecture could be extract of this limited observation.

The significance of VEGF-C expression in the thyroid neoplasms is contentious [23]. Despite that, we found some interesting correlations which indicate VEGF-C as an important feature contributing to thyroid neoplasm development. Notably, VEGF-C was markedly expressed in both papillary and follicular carcinoma, which significantly separated malignant from benign lesions. Parallel to this statement, it was also important to highlight that follicular carcinoma importantly expressed more VEGF-C than its benign counterpart, follicular adenoma. The majority of malignant cases with increased LVD also exhibited high VEGF-C expression, with statistical significance in peritumoral LVD ( $p=0.049$ ). A higher VEGF-C immunoreactivity at the tumor borders have been demonstrated in several types of cancers [4, 31, 32]. This finding suggests that VEGF-C expressed by thyroid tumor cells at the invasive front can directly drive peritumoral lymphangiogenesis [7]. This assumption, however, was not ratified in the present work. Additional data with whole tumor samples of more robust casuistic of thyroid lesions would clarify this interesting point. The contradicting data about LVD and thyroid carcinomas maybe due to differences samples selection and LVD quantification methodology. Also, these contentious results might be related to the inherent complex mechanisms of lymphangiogenesis and metastatic spread. Further studies evaluating the molecular biology involved with lymphatic vascularization network in thyroid tumors progression should be conducted in order to clarify the differences herein observed.

**Table 5** Comparison between VEGF-C expression and LVD

VEGF-C	LVD total		LVD intra		LVD peri	
	Mean $\pm$ SD	<i>P</i> value	Mean $\pm$ SD	<i>P</i> value	Mean $\pm$ SD	<i>P</i> value
Negative (<10%)	90.9 $\pm$ 439.2 ( <i>n</i> =40)	0.246	61.6 $\pm$ 289.0 ( <i>n</i> =14)	0.303	75.4 $\pm$ 414.6 ( <i>n</i> =14)	0.049*
Positive (>10%)	99.5 $\pm$ 361.5 ( <i>n</i> =67)		73.9 $\pm$ 356.0 ( <i>n</i> =43)		97.2 $\pm$ 297.5 ( <i>n</i> =43)	

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**Conflict of interest** The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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