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Value of confocal laser endomicroscopy in the diagnosis of vocal cord lesions

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Abstract. – OBJECTIVE: The aim of this study was to assess the reliability and limitations of confocal laser endomicroscopy (CLE) for diagnosing lesions of the vocal cords and differentiating malignant from non-malignant lesions.

PATIENTS AND METHODS: During microlaryngoscopy, the vocal cords were scanned by probe-based CLE (pCLE: a GastroFlex probe with the Cellvizio® laser system, Mauna Technologies, Paris, France). The video recordings were analyzed and compared with the histological results. Thirty-one representative images were extracted and presented to four medical professionals (blinded examiners) for assessment.

RESULTS: The accuracy for the category malignant/nonmalignant ranged between 58.1% and 87.1%. Overall interrater reliability was 0.29. Sensitivity ranged between 45.5 and 100%, specificity between 60.0 and 100%, PPV between 38.5% and 100% and NPV between 66.7 and 100%.

CONCLUSIONS: CLE is a promising method for the non-invasive diagnosis of vocal cord lesions *in vivo*, but factors such as small penetration depth, not available contrast media for the nuclei and subjective analyses of the images limit, at the moment, its diagnostic value.

Key Words:
Confocal laser endomicroscopy, Laryngeal cancer, Optical biopsy, Non-invasive histological imaging, Vocal cords.

Abbreviations

ENT = ear, nose and throat; CLE = confocal laser-scanning endomicroscopy; pCLE = probe-based confocal laser-scanning endomicroscopy (pCLE = GastroFlex probe with the Cellvizio laser system, Mauna Technologies, Paris, France); OCT = optical coherence tomography; PPV = positive predictive value; NPV = negative predictive value.

Introduction

The incidence of laryngeal cancer worldwide is estimated to be around 5.1 for men and 0.6 for women per 100 000 of the population[1]. The lesion is found in the vocal cords in almost half of the cases[1]. More than 90% are squamous cell carcinomas and about 75-80% are located primarily in the vocal cords[2]. The main risk factors are alcohol consumption and tobacco use[3], although other factors such as HPV, HSV and gastro-oesophageal reflux have also been discussed as possible risk factors[4,5]. Many of these carcinomas are preceded by precancerous changes such as (severe analyzed) dysplasia, associated with leukoplakia and erythroplakia[6]. In the ideal case, these precursors are recognized by (micro-) laryngoscopy and promptly removed in their entirety. The gold standard for the diagnosis of laryngeal cancer at the present time is tissue biopsy and histology of the specimen obtained. Histological examination requires an adequate amount of material. An extensive biopsy of the vocal cords may lead to functional problems such as dysphonia, dyspnea, and dysphagia[7]. In addition, there are whole ranges of benign mucosal changes in the vocal cords, which do not show any tendency to undergo malignant transformation: these include a hyperplastic epithelium and chronic laryngitis without dysplasia. Better screening methods are, therefore, needed to evaluate whether mucosal changes in the vocal cords require biopsy. Alternative optical diagnostic methods (more accurate than white-light endoscopy) would also be useful in keeping the resection margins as small as possible when removing vocal cord carcinomas. At present, horizontal diagnostic procedures such as narrow band imaging (Olympus, Tokyo, Japan), SPECTRA (Karl Storz GmbH & Co. KG, Tuttlingen, Germany) or fluorescence endoscopy differ from the vertical procedures such as the optical coherence tomography (OCT) or confocal laser-scanning endomicroscopy (CLE)[8]. The horizontal diagnostic methods provide us with a screening proce-

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dure that allows the evaluation of large areas of mucosa and possibly reveals mucosal changes that could not previously be differentiated from normal mucosa with certainty under white light. Once the area of interest has been identified, it has to be assessed for the presence of malignancy and/or depth of infiltration. The main optical procedures in current use for this purpose are CLE and OCT. The aim of the present study is to test the reliability of CLE in the assessment of whether a vocal cord lesion is benign or malignant. CLE is now being increasingly used with very promising results in gastroenterology, especially in the diagnostic investigation of Barret's esophagus, stomach cancer, colorectal cancer and various lesions of the biliary tract[9-11]. With an intravenous injection of fluorescein, probe-based CLE (pCLE: GastroFlex probe with the Cellvizio laser system, Mauna Technologies, Paris, France) allows us to view the interstitial spaces, cells, and small capillaries in a window of 240 µm and to a depth of 60 µm[12]. As this often does not demonstrate breakthrough of the basement membrane, it is often not possible to distinguish between carcinoma *in situ* and invasive lesions. The successful differentiation of healthy cells from severely dysplastic cells means that biopsies can be more targeted, resection margins defined more precisely, and unnecessary biopsies avoided.

Patients and Methods

This prospective study was carried out at the ENT Department of a Tertiary Level University Hospital. The local Ethics Committee approved the study and all patients gave their written informed consent. All procedures involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments. Between July and October 2015, 10 patients (4 women, 6 men: average age 55 ± 6.7 years) underwent microlaryngoscopic examination under general anaesthetic. All patients had a suspected unilateral lesion of unknown nature in the vocal cords, so that they all had an indication for this procedure. Microlaryngoscopy to demonstrate the vocal cords was performed under general anesthetic, using a Kleinsasser scope and chest support. After documentation of the findings under white light microscopy, 5 ml fluorescein (Fluorescein Alcon 10%, Alcon Pharma GmbH, Freiburg, Germany) was administered intravenously and the vocal cords scanned by pCLE (GastroFlex probe with Cellvizio laser system, Mauna Technologies, Paris, France). The Cellvizio GastroFlex probe has a threshold wavelength of 660 nm, a penetration depth of 60 µm, and an imaging frequency of 8 to 12 images/s. The probe tip measures 2.6 mm in diameter. The images were taken within 5 min of the intravenous fluorescein injection, as the image quality can be expected to deteriorate considerably after 8 min[13]. Under direct vision, the probe was placed at a right angle on the vocal cords with double-spoon forceps. To ensure the biopsy was taken directly from the area filmed, the probe was retracted and a biopsy obtained from the area of interest using the double spoon forceps. The video recordings were analyzed and compared with the definitive histological results. Thirty-one representative images were extracted and presented to four medical professionals (blinded examiners) for assessment. The professionals consisted of two ENT specialists with no experience of confocal microscopy, one ENT specialist who was experienced in confocal microscopy and one pathologist who also had experience with this technique. The blinded examiners had to allocate the CLE image into the categories of "healthy", "mild to moderate dysplasia or benign mucosal changes" or "severe dysplasia/carcinoma *in situ* or invasive carcinoma". As has already been mentioned, the maximum pCLE penetration depth is approximately 60 µm. It is, therefore, not possible to distinguish carcinoma *in situ* from invasive carcinoma, since stromal invasion cannot be used as a criterion to differentiate between the two[14].

Statistical Analysis

Statistical analysis was done using SPSS version 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated for each examiner. Assessment of the results was blinded to histological findings. The histological findings were regarded as the reference standard. The inter-rater reliability/agreement was tested statistically using Cohen's kappa (Cohen's kappa coefficient). The κ-values were interpreted according to the widely accepted Landis and Koch classification[15]. If the agreement obtained between the two raters is mathematically equivalent to chance, κ is zero (no agreement). Agreement with values of κ between 0 und 0.20 is slight, between 0.21 and 0.40 is adequate, between 0.41 and 0.60 is moderate, between 0.61 and

Table I. Patients and classification of the lesions.

		Lesion	
	Overall	Benign changes no dysplasia)	Severe dysplasia to invasive carcinoma
Patients, n (%)	7	4 (57.1%)	3 (42.9%)
Age	56,7±5,8	51.5±7.2	61±3.6
Sex	Men: 4		
Women: 3	Men: 1 (25%)		
Women: 3 (75%)	Men: 3 (100%)		
Women: 0 (0%)			

0.8 is substantial, and between 0.81 and 1.0 is almost perfect[15].

Results

All of patients tolerated the pCLE well. There were no complications or adverse effects from the intravenous fluorescein. The investigation prolonged the time under anesthetic from 5 to 10 min. The video recordings from the ten patients were analyzed. Recordings from three patients were not acceptable because of the poor quality of the images: recordings from the remaining seven patients (three women, four men: average age 56.7±5.8 years) were examined (Table I). In each of the seven patients, biopsies were taken from unremarkable areas of mucosa (in non-corresponding sites of the vocal cords) in addition to areas of pathological mucosal change. The definitive histology of the benign lesions included hyperkeratosis, discrete leucoplakia without any associated dysplasia, a granular cell tumor with associated excessive pseudoepitheliomatous hyperplasia, and a retention cyst covered with respiratory epithelium. The granular cell tumor with associated excessive pseudoepitheliomatous hyperplasia was included here under benign changes on pCLE, as this technique does not encompass the stromal characteristics of granular cell tumors. Only the associated excessive pseudoepitheliomatous hyperplasia can be assessed. Squamous cell carcinomas (G2) were found in the remaining three patients (Figure 1). The administration of fluore-

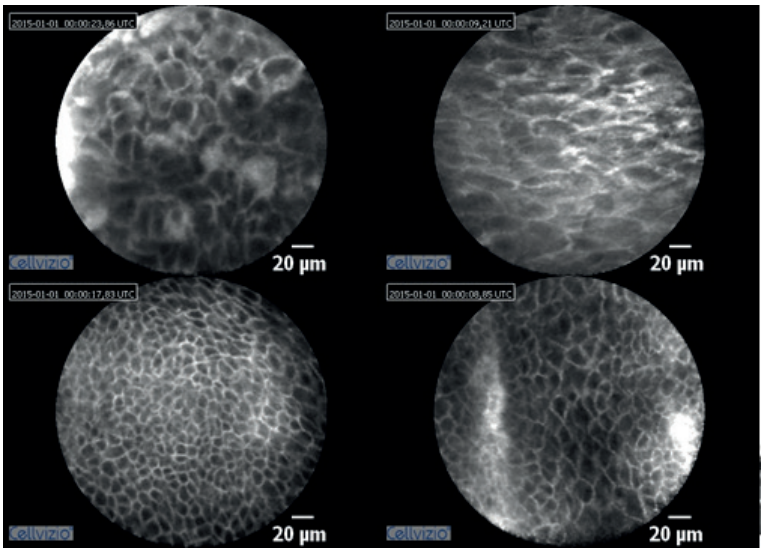

Figure 1. The upper row shows two images of G2 squamous cell carcinoma of the vocal cords. Below left: healthy mucosa. Below right: hyperplastic epithelium without dysplasia. Clear differences can be seen: cells that have undergone malignant change are considerably larger, the interstitial spaces cannot be identified so clearly, and the cell structure differs greatly from one cell to the next. The healthy cells (below left) are regular in size, consisting of small polygonal cells with clear interstitial spaces. Although larger than the healthy cells, the hyperplastic cells (below right) have a regular appearance with benign characteristics.

Table II. Accuracy of the different examiners, D= doctor.

	ENT specialist with CLE experience (D1)	Pathologist with CLE experience (D2)	ENT specialist without CLE experience (D3)	ENT specialist without CLE experience (D4)
Malignant/non-malignant	27/31 (87.1%)	24/31 (77.4%)	24/31 (77.4%)	18/31 (58.1%)
Healthy/benign/malignant	18/31 (58.1%)	15/31 (48.4%)	18/31(51.8 %)	15/31(48.4 %)

scein demonstrates the cells, the interstitial spaces, and the small capillaries with red blood cells. The two-dimensional cell structure and size can be assessed and represent the most important characteristics for classifying the lesion as benign or malignant. Blood, saliva, or roughly raised areas of mucosa lessen the imaging quality, as they reduce the complete adaptation between probe and mucous membrane. Table II summarizes the accuracy of the different examiners in classifying the images. Images were divided into two categories: malignant/not malignant, and into three categories: healthy/benign/malignant. The inter-rater reliability was tested with Cohen's kappa and evaluated according to the Landis and Koch criteria[15] (Table III and Table IV). Table V shows the sensitivity, specificity, PPV and NPV calculated for each examination classified into one of the two categories. Sensitivity ranged between 45.5 and 100%, specificity between 60 and 100%, PPV between 38.5 and 100%, and NPV between 66.7 and 100%. The results were better for the examiners who had previous experience with confocal microscopy, although even here there were large differences in sensitivity and specificity. Sensitivity, specificity, PPV and NPV were calculated for each examiner in the two-category classification (Table V). In general, the results were better for the doctors with previous experience in CLE: sensitivity 63.6%, specificity 100%, PPV 100%, and NPV 83.3% for the ENT specialist with CLE experience; sensitivity 100%, specificity 65.0%, PPV 61.1%, and NPV 100% for the pathologist. The agreement between these two blinded examiners was, however, only "adequate", as confirmed by the kappa coefficients.

Discussion

To date, only a few studies on CLE in head and neck cancer have been published. As far as we know, this is the first study, which specifically addresses the vocal cords. Zheng et al[16] showed that the topical application of a 0.4% solution of 5-ALA allowed malignant cells on the surface of the tongue to be distinguished from healthy cells. In 2007, the same research team used an *in vivo* rat model to show that the i.v. administration of fluorescein together with the topical 5-ALA provided even greater detail[17]. Maitland et al[18] used

Table III. Inter-rater reliability with respect to the classification into malignant *vs*. non-malignant

Malignant vs. non-malignant (Doctor: D)	31 images – κ-value
D1 vs. D2	0.348
D1 vs. D3	0.561
D1 vs. D4	0.114
D2 vs. D3	0.282
D2 vs. D4	0.111
D3 vs. D4	0.348
Overall	**0.29**

The overall agreement was 0.29 (adequate) between the examiners and 0.38 (adequate) between in the subgroup from doctors with experience in CLE.

Table IV. Inter-rater reliability with respect to the classification into healthy *vs*. benign *vs*. malignant changes.

Healthy vs. benign vs. malignant: Doctor (D)	31 images – κ-value
D1 *vs*. D2	0.059
D1 *vs*. D3	0.42
D1 *vs*. D4	0.178
D2 *vs*. D3	-0.01
D2 *vs*. D4	-0.015
D3 *vs*. D4	0.52
Overall	**0.19**

The results here had very low scores, indicating the random allocation of healthy and benign lesions. There seem to be no definable criteria to distinguish between these two entities. Overall agreement was 0.19 (slight), and the agreement between the two CLE experienced examiners was no more than could be expected by chance.

Table V. Sensitivity, specificity, PPV and NPV calculated for each blinded examiner, with respect to allocation of the lesions into two categories.

ENT specialist with CLE experience (D1) N=31 images examined	Malignant changes (requiring biopsy) - HISTOLOGY	Healthy tissue or benign changes (not requiring biopsy) - HISTOLOGY
Suspected malignant or severe dysplastic changes on pCLE	7	0
Suspected healthy mucosa or benign changes on pCLE	4	20
Pathologist with CLE experience (D2) N=31 images examined	Malignant changes (requiring biopsy) - HISTOLOGY	Healthy tissue or benign changes (not requiring biopsy) - HISTOLOGY
Suspected malignant or severe dysplastic changes on pCLE	11	7
Suspected healthy mucosa or benign changes on pCLE	0	13
ENT specialist without CLE experience (D3) N=31 images examined	Malignant changes (requiring biopsy) - HISTOLOGY	Healthy tissue or benign changes (not requiring biopsy) - HISTOLOGY
Suspected malignant or severe dysplastic changes on pCLE	6	2
Suspected healthy mucosa or benign changes on pCLE	5	18
ENT specialist without CLE experience (D4) N=31 images examined	Malignant changes (requiring biopsy) - HISTOLOGY	Healthy tissue or benign changes (not requiring biopsy) - HISTOLOGY
Suspected malignant or severe dysplastic changes on pCLE	5	8
Suspected healthy mucosa or benign changes on pCLE	6	12

ENT specialist with CLE experience (D1): sensitivity 63.6%, specificity: 100%, PPV: 100%, NPV: 83.3%.
Pathologist with CLE experience (D2): sensitivity 100%, specificity: 65.0%%, PPV: 61.1%, NPV: 100%.
ENT specialist without CLE experience (D3): sensitivity 54.5%, specificity: 90.0%, PPV: 75.0%, NPV: 78.3%.
ENT specialist without CLE experience (D4): sensitivity 45.5%, specificity: 60.0%, PPV: 38.5%, NPV: 66.7%.

topical acetic acid immediately before the CLE examination to show that, compared with the regular structure of healthy cells, the superficial cells of a squamous cell carcinoma appear disorganised with overlapping nuclei. Haxel et al[19] demonstrated that the *ex vivo* application of acriflavine provided good nuclear contrast that could be seen particularly well on CLE. This research group from Wiesbaden also presented results in 2012 describing the inter-operative feasibility of CLE and the characteristics of carcinomas that could be distinguished with this technique: irregular cell structure and small interstitial spaces[20]. In 2014, Nathan et al[21] were the first team to present a statistical analysis of the reliability of CLE with the injection of fluorescein, the only contrast medium with regulatory approval for this diagnostic procedure. Twenty-one lesions in the oral cavity were examined by pCLE. Nathan et al[21] reported impressive results in distinguishing healthy cells (without dysplasia) from squamous cell carcinomas: they found the sensitivity, specificity, PPV and NPV of pCLE to be 100%. In distinguishing dysplasia from healthy tissue, they gave a sensitivity and NPV of 80%, with a specificity and PPV of 100%. When differentiating dysplasia from squamous cell carcinomas, the corresponding figures were sensitivity 85.7%, specificity 100%, PPV 100% and NPV 80%. In 2015, the same group tested the inter-rater reliability and, with $\kappa = 0.7$, showed a substantial agreement according to Landis and Koch[22], not far removed

from the almost perfect agreement between different rates that can be assumed with a value of 0.81 or more. Also in 2015, Volgger et al[14], a research team from Munich, combined CLE with another optical diagnostic method, OCT. In this publication, the sensitivity for distinguishing dysplasia/hyperplasia was calculated to be 100%, but with a specificity of only 40% and a PPV of 52.6%. In our study, the sensitivity, specificity, PPV and NPV were calculated for the classification into two categories (healthy or benign changes/malignancy). Examiner D1's results had a sensitivity of 63.6% and a specificity of 100%. On the other hand, examiner D2 had a sensitivity of 100% and a specificity of 65.5%. The doctors with experience in confocal microscopy gave generally better results than those without experience of pCLE, but even between these two experienced doctors, agreement (according to Landis and Koch[15]) was merely "adequate". Although the results indicate a moderate increase in the diagnostic reliability of pCLE with experience of this technique, the sensitivity and specificity are not sufficiently accurate for even experienced examiners to make a decision for or against biopsy. In addition, the two investigators often did not agree. Allocation into three categories (healthy/benign/malignant) was simply not reliable. To obtain the optimal and most representative images from the raw data, the images for evaluation by the blinded examiners were selected by a doctor who already knew the histological findings – selection bias. We can, therefore, expect that the reliability would be even lower with analysis of the raw data (video recordings) with all its characteristic artefacts. Despite similar methodology, we were unable to reproduce the impressive results from Nathan et al[21]. In our view, certain methodical aspects need to be improved in order to improve the reliability of this very promising method. Better contrast media are needed, as fluorescein does not stain the nuclei. Fluorescein remains in the capillaries and interstitial spaces, so it can be used to assess only the cell structure, cell size, and the size of the interstitial spaces. Important histological features such as the nuclei cytoplasm ratio cannot be taken into account. The lack of this crucial criterion could explain the relatively poor results of CLE compared with the gold standard. Unfortunately, at the present time there is no approved contrast medium that stains the nuclei for CLE[2,23]. Acriflavine has been used for nuclear contrast in *ex vivo* studies[19,24,25], but it binds nucleic acids and is potentially mutagenic. For this reason, it has not been approved by the FDA[25]. A widely accepted classification for CLE of the upper respiratory tract does not exist yet. Oetter et al[26] recently proposed one such classification. The research group from Erlangen (oral and maxillofacial surgery) investigated squamous cell carcinomas of the oral cavity and healthy mucous membranes with pCLE using i.v. fluorescein as the contrast medium. Doctors with experience in CLE compared the CLE-images with the corresponding histological pictures and developed a classification that could be used by examiners who had no previous experience with the technique. Using this classification, inexperienced examiners achieved a sensitivity of 97.3%, a specificity of 88.1%, a PPV of 90.1%, and an NPV of 96.7%. Interestingly enough, these values were very similar to those obtained by the experts and all showed very good inter-rater reliability (0.73 for the experts and 0.81 for the inexperienced examiners). Linxweiler et al[27] compared CLE with the gold standard of histology in the assessment of tumor margins *ex vivo*, mainly in formalin-fixed tissues. In addition, topical acriflavine was applied to obtain any further information about the cell nuclei. The treating ENT specialists were able to identify the tumor margins correctly in more than 80% of cases. The authors were reticent about extrapolating these results into the situation *in vivo*. We agree with their conclusions. Formalin can affect the fluorescence of the tissues and contrasting the nuclei with acriflavine cannot be performed *in vivo* because it does not have regulatory approval. As in the present work, there was a selection bias, since the best images from several video recordings were chosen for the blinded examiners to assess. There are also other artefacts *in vivo*, such as blood and saliva, which were not taken into consideration in their study. Even with the current gold standard, tumor grading and the differentiation of benign lesions in the head and neck is already very difficult, and depends on various features to be listed here in order to illustrate and explain the shortcomings of CLE in this respect. These aspects include nuclear pleomorphism with distorted chromatin structure and an increased nucleus:cytoplasm ratio, the mitotic activity, and the stromal reaction, as well as the immunohistochemistry – none of which can be evaluated by means of CLE. A sure assessment of whether the lesion is benign or malignant is still very difficult, even when all these characteristics are included in the histological examination[28]. It is hardly possible to distinguish a well-differentiated squamous cell carcinoma

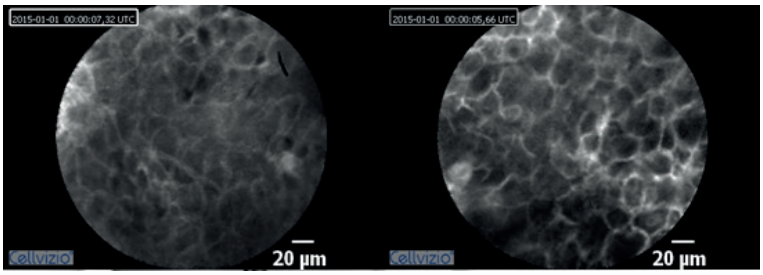

Figure 2. Left: histology showed excessive pseudoepitheliomatous hyperplasia. Below this epithelium was a granular cell tumor that could only be diagnosed by conventional histology and the aid of S100 and SOX-10 immunohistochemical staining to highlight tumor cells in deeper tissue of the biopsy. The lesion had to be resected completely. This example illustrates two problems of CLE: first, it shows that the covering epithelium – although definitively benign – cannot be distinguished from a squamous cell carcinoma. Secondly, the stromal tissue, and the real pathological findings are not included because of the limited CLE penetration depth. Right: a G2 squamous cell carcinoma (moderately differentiated).

from an active inflammatory lesion (e.g. pseudoepitheliomatous hyperplasia) on histology[28]. Figure 2 shows how similar these two entities appear on pCLE. Dittberner et al[29] took an interesting approach by addressing the automated computer-aided classification of CLE images. This research group developed an algorithm that resulted in a mean accuracy of 74%, a specificity of 85% and a sensitivity of 72% in the differentiation of squamous cell carcinomas from tissues without any evidence of malignancy.

Conclusions

This prospective research on the value of pCLE in determining the malignancy of a vocal cord lesion addressed the feasibility, diagnostic value, possibilities and limitations of the technique. CLE is a very promising method for the non-invasive diagnosis of vocal cord lesions *in vivo*. The procedure can be carried out with minimal time and expense, and performed intraoperatively without complications. Cell structure and size of malignant changes in the vocal cords were investigated and diagnosed with adequate reliability. Values between 63% and 100% were calculated for the sensitivity and specificity. The limited penetration depth means that stromal characteristics defining malignancy could often not be recognized, so that the sensitivity could probably be increased by combining this method with other optical procedures such as OCT. Improved contrast media to demonstrate the nuclei, membranes, and cytoplasm, would also improve the diagnostic value. Automated computer-aided analysis of the images, if developed further, could lead to a reliable and reproducible classification of the CLE images.

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

Conflict of Interest
None. Authors don´t have any financial relationship with the organization that sponsored the research.

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