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Sensitivity and specificity of salivary pipecolic acid in head and neck squamous cell carcinoma

Kívia Linhares Ferrazzo[1], Larissa Daiane Willrich de Melo[2], Cristiane Cademartori Danesi[1], Alexander Thomas[3], Laura Izabel Lampert Bonzanini[3], Nilo Zanatta[2]

[1] Department of Pathology, Federal University of Santa Maria, Santa Maria, RS, Brazil.

[2] Department of Chemistry, Federal University of Santa Maria, Santa Maria, RS, Brazil.

[3] Dentistry School, Federal University of Santa Maria, Santa Maria, RS, Brazil.

Aim: The aim of the present preliminary case-control study was to test the sensitivity and specificity of salivary pipecolic acid in predicting head and neck squamous cell carcinoma (HNSCC). **Methods:** High-performance liquid chromatography was used for the analysis of non-stimulated saliva samples from 40 individuals: 20 in the case group (recently diagnosed with untreated HNSCC) and 20 in the control group (individuals without cancer). Both groups included patients taking daily oral hypoglycemic drugs (comorbidity). The case and control groups were matched at a proportion of 1:1 for sex and comorbidity. **Results:** Mean salivary levels of pipecolic acid were 169.38 ng/mL in the case group and 114.66 ng/mL in the control group ($p<0.001$). Individuals who took oral hypoglycemic drugs had higher levels of pipecolic acid in both the case and control groups ($p<0.001$). The receiver operating characteristic curve analysis revealed 90% sensitivity and 65% specificity for head and neck cancer, with an area under the curve of 0.838 between the case and control groups. **Conclusions:** Pipecolic acid had high sensitivity for the diagnosis of HNSCC but low specificity in the sample analyzed. Our findings suggest that salivary pipecolic acid levels are associated with glucose homeostasis. Studies with larger samples are required to evaluate the specificity of this metabolite.

Keywords: Saliva. Biomarkers. Squamous cell carcinoma of head and neck.

Corresponding author:
Kívia Linhares Ferrazzo
Roraima Avenue, 1000,
Building 20; CEP: 97105-900;
Santa Maria/RS – Brazil
Phone: 55 (55) 99131-7537;
Fax: 55 (55) 3225-3353
Email: kivialinhares@uol.com.br

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Introduction

Approximately 744,000 new cases and more than 360,000 deaths from cancer of the oral cavity, pharynx and larynx were estimated worldwide in 2020. In most cases, tumors are diagnosed in an advanced stage[1]. As the early detection of these neoplasms and the rapid institution of treatment are associated with a better prognosis and survival rate[2], the development of fast, efficient, noninvasive diagnostic techniques that can be widely used in populations at risk is of considerable importance.

The malignant transformation of a cell involves complex reactions that ultimately alter the genotype and phenotype of cells, leaving specific markers in the process, which are denominated biomarkers. The study of biomarkers involves proteomic, genomic and, more recently, metabolomic analyses[3-6].

Saliva has attracted attention in the study of biomarkers and has been the target of numerous studies[7-9]. Studies conducted in the last ten years have demonstrated that human saliva can be used for the diagnosis of several diseases, such as cardiovascular diseases[10], prostate cancer,[11] pancreatic cancer[12] and head and neck cancer[4,9,13]. Saliva can reflect systemic as well as local changes, which underscores the importance of this diagnostic method[14].

Pipecolic acid is a product of the metabolism of lysine found in physiological body fluids, such as plasma, urine and cerebrospinal fluid[15]. Pipecolic acidemia is well recognized in peroxisomal diseases, such as Zellweger syndrome[16,17], although its participation in the pathogenesis has not yet been fully clarified. Pipecolic acid has been identified as a potential biomarker with the capacity to detect oral squamous cell carcinoma in the early stages of the disease[3,6,18]. However, this metabolite has been shown to be increased in another systemic condition as well. An experimental model with primates and rats demonstrated an increase in urinary levels of pipecolic acid in diabetic animals[19]. Moreover, pipecolic acid has been identified as a potential biomarker that is overregulated in the corneas of individuals with type 1 diabetes in comparison to healthy controls[20]. A recent study found an association between pipecolic acid and an increased risk of type II diabetes[21].

Therefore, the aim of this preliminary study was to test the sensitivity and specificity of pipecolic acid in predicting head and neck squamous cell carcinoma (HNSCC). The metabolomic strategy was targeted tandem validation[22]. The hypothesis is that pipecolic acid has high sensitivity and specificity in predicting HNSCC and that pipecolic acid levels are not influenced by the presence of comorbidity related to glycemic control.

Methods

Ethical considerations, study design and sample size calculation

The present case-control study was conducted at a university hospital of the public education system in southern Brazil that serves as a reference for the treatment of

head and neck cancer. The study was conducted in accordance with the precepts stipulated in the Declaration of Helsinki and received approval from the local human research ethics committee (certificate number: 63198116.3.0000.5346/1.889.748). Voluntary research participants were recruited from the head and neck surgery clinic between November 2016 and November 2017. Volunteers in the test group were patients who received a diagnosis of HNSCC. Volunteers in the control group were the companions of the patients and were recruited from the waiting room. All volunteers received clarifications regarding the objectives and procedures of the study and those agreeing to participate signed a statement of informed consent.

The sample size was calculated using data from a previous study,[18] which demonstrated that pipecolic acid has more than 90% sensitivity and specificity for predicting whether a tumor is positive or negative for oral squamous cell carcinoma. Considering a population of 100 individuals (approximate number of new cases of HNSCC diagnosed at the hospital per year), a 90% confidence level and 10% sampling error, a minimum of 20 individuals was determined for the case group.

Sample and eligibility criteria

The sample was composed of 40 male and female adults (> 18 years of age): 20 in the case group (patients with histological diagnosis of HNSCC not yet treated) and 20 in the control group (patients with no current or past history of cancer). Each group included three individuals who used oral hypoglycemic drugs (comorbidity). The case and control groups were matched at a proportion of 1:1 for sex and comorbidity.

The eligibility criteria for the case group were a recent diagnosis of HNSCC (oral cavity, oropharynx, hypopharynx or larynx) with no prior treatment (surgical approach, chemotherapy or radiotherapy). The diagnosis of HNSCC was based on clinical criteria and confirmed through histological analysis. Individuals with a past history of a malignant disease, auto-immune disease, psychiatric disease or infectious-contagious disease were excluded from both groups.

Data collection

A questionnaire was created to collect socio-demographic characteristics, clinical data, risk factors (*e.g.*, smoking) and data on the neoplasm. The following data were collected: age, sex, ethnicity, years of formal schooling, type and location of tumor, Tumor-Node-Metastasis (TNM)-based staging system of cancer according to the seventh edition of the American Joint Committee on Cancer (AJC)[23] and comorbidity. With regards to smoking, the individuals were classified as non-smokers (those who never smoked or smoked less than one cigarette per day for less than one year), ex-smokers (those who quit smoking at least one year earlier) or current smokers. Cumulative smoking was evaluated considering 'pack-years', which is defined as the number of cigarette packs smoked per day multiplied by the number of years in which the individual smoked[24].

Collection of saliva

Saliva samples were collected between 1:30 and 3:30 pm. For such, the participant needed to have refrained from consuming solid food or liquids for at least two hours

prior to the collection. Non-stimulated saliva was collected following the method proposed by Navazesh and Kumar[25] (2008). After rinsing the mouth with distilled water, the participant was instructed to remain seated with his/her mouth partially open until saliva accumulated on the floor of the mouth. The participant then deposited the pooled saliva into a sterilized universal collector (duly labeled with the internal control number of the sample) until obtaining approximately 2 mL of saliva. The samples were placed in a thermal bag with ice for transportation to the laboratory. All samples were prepared for analysis within a maximum of three hours after collection.

Preparation of saliva

The saliva samples were centrifuged at 3500 g for 20 min at 4º C for the removal of any insoluble matter, cell remains and food scraps. Each sample was divided into aliquots of 400 µL and frozen at -80° C until analysis, which was performed within a maximum of four weeks.

The saliva samples were thawed at room temperature prior to analysis. To precipitate the proteins, a mixture of acetonitrile/methanol (75:25 v/v, 800 µL) was added to 400 µL of saliva in a 1.5-mL Eppendorf tube, followed by vigorous shaking for 60 seconds. The mixture was left to rest for 10 minutes, after which the samples were centrifuged at 15400 g for 20 min at 4° C. The supernatant was filtered using a syringe filter and the material was analyzed[18,26]. All saliva samples were collected, prepared and analyzed by the same researcher.

Liquid chromatography–mass spectrometry (LC-MS/MS)

The chromatographic analysis was performed using a 1200 series liquid chromatography system (Agilent Technologies) equipped with a hypercarb column (100 mm × 2.1 mm i.d. 5 µm) (Thermo Scientific) and coupled to a 6460 Triple Quad detector (Agilent Technologies). The column temperature was set at 30° C. The autosampler is equipped with a 40 µl loop but operated using 5 µl. The flow rate of the mobile phase was 0.8 ml/min. Isocratic elution was performed using the following solvent system: (A) composed of purified water, 0.5% ammonium formate and 0.01% formic acid; (B) acetonitrile, 4.5% purified water, 0.5% ammonium formate and 0.01% formic acid; 80% A and 20% B for 5 min. LC-MS/MS was operated in the positive ion mode. For the analysis of the raw data, an Agilent Mass Hunter Data Acquisition and Processing was used for the qualitative and quantitative analyses. In this study, independent reference lock-mass ions were obtained in previous validation via an electrospray source interface to ensure mass accuracy during data acquisition. The accuracy of the obtained mass ions is 0.1 atomic mass units. Pipecolic acid (Sigma; St. Louis, MO, USA); [PA + H]+, m/z 130.157) was used as the standard compound. Multiple reaction monitoring was conducted to optimize the fragmentation conditions and identify the best precursor/product transitions for quantitation and confirmation. The analysis was performed in the selected-reaction monitoring mode using two transitions: m/z 130.1-->m/z 84.1, collision energy of 15 eV.

Statistical analysis

The data were analyzed descriptively and expressed as mean, standard deviation and median values. The outcome was pipecolic acid determined in ng/mL. The categorical covariables were sex, age, schooling, smoking status and cumulative tobacco dose (pack-years). For the purposes of statistical analysis, age, schooling and pack-years were dichotomized by the median of the entire sample. A variable denominated comorbidity was created to analyze individual patients using oral hypoglycemic drugs. To determine pipecolic acid levels in relation to the stage of the disease, the case group was subdivided into case group 1 (stages I and II) and case group 2 (stages III and IV). The Shapiro-Wilk test was used to determine the normality of the variables. The Student's t-test and Mann-Whitney test were used for the comparisons of mean pipecolic acid levels between the case and control groups as well as among the clinical and demographic variables. The level of significance was set to 5% ($p < 0.05$). The sensitivity and specificity of pipecolic acid were tested. For such, a receiver operating characteristic (ROC) curve was created and the area under the curve (AUC) was determined. All statistical tests were performed using IBM SPSS Statistics for Windows, Version 21.0 (Armonk, NY: IBM Corp).

Results

The sample consisted of 40 individuals: 20 in the control group (mean age: 52.45 ± 11.30 years) and 20 in the case group (mean age: 62.95 ± 10.33 years). The two groups were matched for sex and comorbidity. Table 1 gives a complete description of the sample.

Table 1. Demographic, behavioral and clinical characteristics of sample

Variables	N (%)	
	Control group (without disease)	Case group (with disease)
Sex		
Male	18 (90%)	18 (90%)
Female	2 (10%)	2 (10%)
Schooling		
< 5 years	4 (20%)	10 (50%)
5-8 years	4 (20%)	6 (30%)
> 8 years	12 (60%)	4 (20%)
Smoking status		
Non-smoker	10 (50%)	2 (10%)
Current smoker	2 (10%)	13 (65%)
Ex-smoker	8 (40%)	5 (25%)
Smoking cessation time		
1-4 years	4 (20%)	2 (10%)

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Continuation		
5-9 years	1 (5%)	-
10-19 years	1 (5%)	2 (10%)
≥ 20 years	2 (10%)	1 (5%)
Comorbidity (oral hypoglycemic drug)		
No	17 (80%)	17 (80%)
Yes	3 (20%)	3 (20%)

No statistically significant differences in pipecolic acid levels were found in relation to sex, age, schooling or smoking/pack-years. Patients with comorbidity (who took oral hypoglycemic drugs) had higher salivary levels of pipecolic acid and significant differences were found between those who took such medication and those who did not in both groups ($p < 0.001$) (Table 2).

Table 2. Mean salivary pipecolic acid levels in case and control groups according to demographic, behavioral and clinical variables

Variable	Control group (without disease)	Case group (with disease)
	Pipecolic acid level (ng/mL) Mean (± standard deviation)	
Sex		
Male	117.65 (± 33.37)	166.40 (± 49.09)
Female	87.68 (± 22.35)	196.26 (± 102.51)
p-value	0.257 [a]	0.801 [a]
Age *		
≤ 58 years	115.58 (± 31.25)	165.97 (± 21.70)
> 58 years	111.89 (± 42.79)	170.52 (± 60.43)
p-value	0.694 [a]	0.694 [a]
Years of schooling *		
≤ 7 years	130.64 (± 40.29)	173.39 (± 56.62)
> 7 years	107.80 (± 28.74)	153.35 (± 35.13)
p-value	0.165 [b]	0.512 [b]
Smoking status normal		
Non-smoker	109.85 (± 30.67)	123.10 (± 0.95)
Ex-, current smoker	119.46 (± 36.70)	174.53 (± 53.31)
p-value	0.533 [b]	0.200 [b]
Pack-Years *		
≤ 23	108.00 (± 28.79)	172.95 (± 57.48)
> 23	134.62 (± 41.18)	167.86 (± 52.95)
p-value	0.206 [a]	0.850 [a]

Continue

Continuation		
Comorbidity (oral hypoglycemic drug)		
No	104.13 (± 23.03)	153.23 (± 32.78)
Yes	174.29 (± 2.20)	260.94 (± 56.12)
p-value	**< 0.001** [b]	**< 0.001** [b]

* variables dichotomized by median; [a] Mann-Whitney; [b] Student's t-test

The case group was composed of 12 individuals with squamous cell carcinoma in the oral cavity and/or oropharynx and eight in the hypopharynx and/or larynx, most of whom (55%) were in the advanced stage (III and IV). Mean pipecolic acid levels were 114.66 ± 33.28 ng/mL in the control group and 169.38 ± 52.85 ng/mL in the case group ($p < 0.001$). Statistically significant differences in pipecolic acid levels were found between the control group and both case group 1 (early phase of the disease) ($p = 0.008$) and case group 2 (advanced stage of the disease) ($p < 0.001$) (Table 3). No significant differences in pipecolic acid levels were found with regards to the clinical characteristics of the tumor (Table 4).

Table 3. Mean pipecolic acid levels in control and case groups

Groups	N	Pipecolic acid level (ng/mL) Mean (± SD)	p-value
Control (without disease)	20	114.66 (± 33.28)	
Case (with disease)	20	169.38 (± 52.85)	< 0.001 [a]
Control (without disease)	20	114.66 (± 33.28)	0.008 [b]
Case 1 (early stage of disease)	9	158.54 (± 48.65)	
Control (without disease)	20	114.66 (± 33.28)	< 0.001 [a]
Case 2 (advanced stage of disease)	11	178.26 (± 56.76)	

[a] Student's t-test; [b] Mann-Whitney test

Table 4. Mean levels of pipecolic acid according to clinical characteristics of case group

Clinical variables	N (%)	Pipecolic acid level (ng/mL) Mean (± SD)	p-value
Location of tumor			
Oral cavity and oropharynx	12 (60%)	177.37 (± 59.73)	0.589 [a]
Hypopharynx and Larynx	8 (40%)	157.40 (± 41.26)	
Stage			
Initial (I and II)	9 (45%)	158.54 (± 48.65)	0.305 [a]
Advanced (III and IV)	11 (55%)	178.26 (± 56.76)	
Tumor size			
T1 and T2	14 (70%)	165.31 (± 44.67)	0.612 [b]
T3 and T4	6 (30%)	178.89 (± 72.61)	
Metastasis to lymph nodes			
No	12 (60%)	157.81 (± 46.32)	0.240 [b]
Yes	8 (40%)	186.75 (± 60.31)	

[a] Mann-Whitney; [b] Student's t-test

Based on the ROC curve, pipecolic acid had 90% sensitivity and 65% specificity for predicting whether a patient has SCC of the head and neck, with an AUC of 0.838 ($p < 0.001$; 95% confidence interval: 0.716 - 0.959) and a cutoff point of 121.77 ng/mL. In the analysis stratified by disease stage, sensitivity was 100% and specificity was 65% (AUC = 0.811; $p = 0.008$; 95% confidence interval: 0.655 - 0.967) for the detection of the early stage of the disease, whereas sensitivity was 91% and specificity was 60% (AUC = 0.859; $p = 0.001$; 95% confidence interval: 0.727 - 0.991) for detection of the advanced stage of the disease. Cutoff points for early and advanced stages were 121.77 ng/mL and 120.79 ng/mL, respectively (Figure 1).

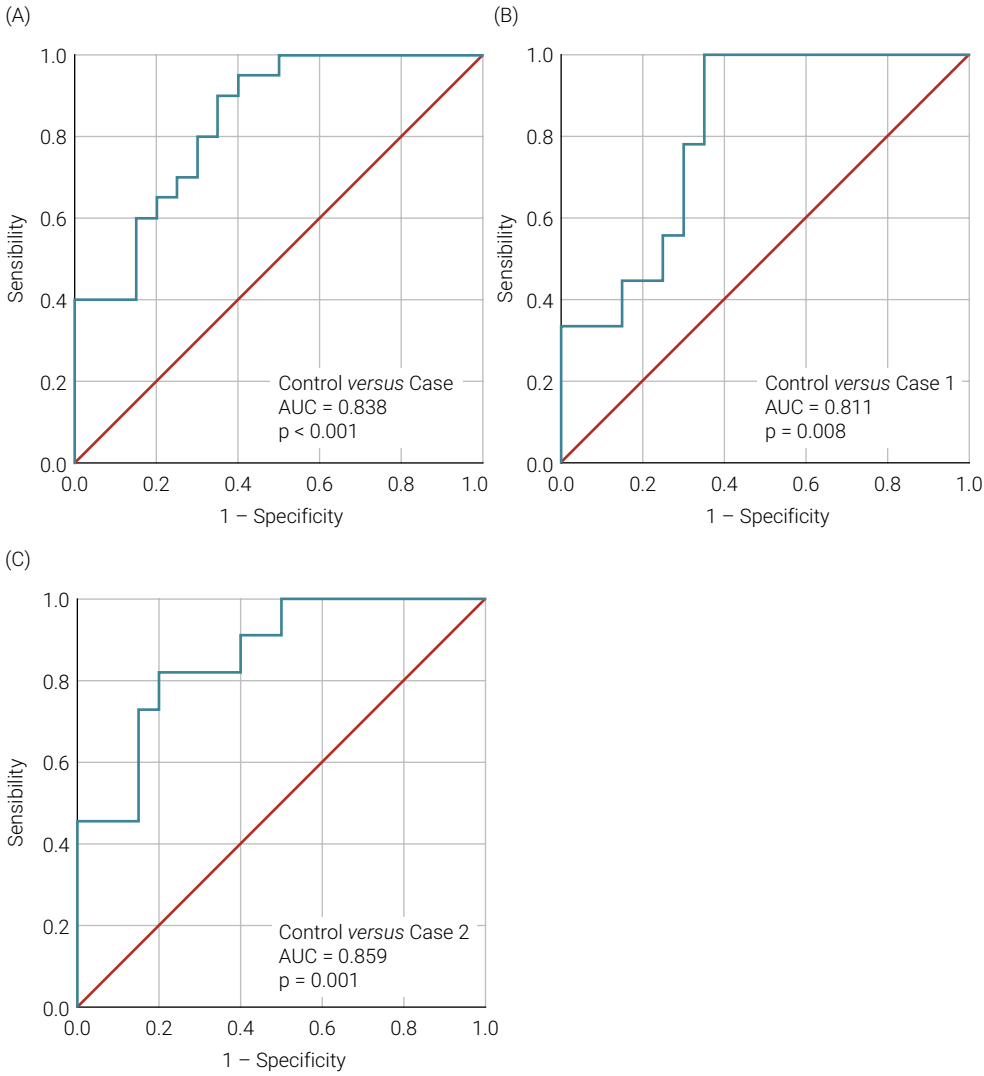

Figure 1. Analysis of ROC curve for determination of sensitivity and specificity of pipecolic acid for prediction of head and neck squamous cell carcinoma. (A) control versus case; (B) control versus case 1 (initial stage of disease); (C) control versus case 2 (advanced stage of disease).

Discussion

In the present study, a significant difference in salivary levels of pipecolic acid was found between individuals without cancer and patients with HNSCC in both the early stages and advanced stages. This finding is important, as well-designed studies that investigate this association are scarce in the literature according to a recent systematic review[27].

An important result found in this paper was that individuals with higher levels of pipecolic acid in both the case and control groups made daily use of some oral hypoglycemic drug. The test and control groups were matched regarding the use of oral hypoglycemic agents to reduce the possibility of a confounding factor.

In a previous study, pipecolic acid exhibited sensitivity and specificity higher than 90% for the early detection of squamous cell carcinoma of the oral cavity[18]. In the present study, sensitivity and specificity were respectively 90% and 65%. The lower specificity in this investigation compared to the study cited may be due to the small sample size. However, pipecolic acid levels appear also to be associated with glucose homeostasis, as demonstrated by other authors[19,20]. Lysine metabolites, including pipecolic acid, have been associated with an increased risk of type II diabetes[21,28]. The development mechanism of this pathway is not yet well understood. Lysine metabolites are believed to positively regulate insulin secretion in order to maintain glucose homeostasis in patients with insulin resistance[28].

Wang et al.[18] (2014) demonstrated that salivary levels of pipecolic acid were increased in patients with oral squamous cell carcinoma compared to healthy individuals and also found higher salivary levels of this biomarker in patients with late-stage tumors, with a statistically significant difference between the stages of the disease. In the present study, although significant differences were found between individuals without cancer and those in both the early (I and II) and advanced (III and IV) stages of the disease, no statistically significant difference in pipecolic acid was found between the stages. This may be explained by the small sample size.

Accumulated evidence has demonstrated an increase in salivary pipecolic acid in cases of HNSCC[3,6,18], but the role of this metabolite in carcinogenesis remains unclear[13]. Wang et al.[18] (2014) suggest that the increase in pipecolic acid in the presence of these tumors is likely due to the fact that the metabolism of lysine is overregulated in the neoplastic cells of the tumor. Lysine is an important constituent of histones, which can undergo alterations, the most widely studied of which are the hypermethylation and acetylation of lysine. When altered, histones exert an influence on the methylation of DNA, which can contribute to the development of tumors[29,30].

The present study has limitations that should be considered. Salivary levels of pipecolic acid in the present study were slightly higher in the control group compared to reports in the literature[18]. This divergence may be explained by the time of the day at which the saliva samples were collected in the present investigation. Due to the office hours of the clinic, it was necessary to collect samples in the afternoon. Components of saliva, especially metabolites, are known to be influenced by diet, the circadian rhythm, etc.[31,32]. To minimize this bias, we standardized the collection in the same time interval and required at least two hours of fasting prior to collection.

The study of salivary biomarkers for different types of systemic diseases has grown considerably in the last decade[8]. Saliva has advantages over other body fluids, such as plasma, as its collection is quick, easy and non-invasive, has a good cost-effectiveness ratio and does not cause stress to patients, as it does not require needles[33]. Another advantage of saliva over plasma is the fact that saliva contains a lower amount of proteins, some of which are unique to saliva, which reduces nonspecific interactions and makes saliva a more sensitive and specific biomarker[8]. Although numerous salivary biomarkers have been discovered, the literature is unanimous in stating that further studies are needed before any biomarker can be consolidated as a diagnostic test with the sensitivity and specificity required for a biomarker[13]. A good biomarker candidate must have diagnostic precision and predictability[34]. Although the present preliminary study showed positive results with the level of pipecolic acid for the prediction of HNSCC, the analysis of salivary biomarkers must be allied with a rigorous oral clinical examination as a strategy for the detection of HNSCC, especially in the early stages of the disease, which will potentially lead to a better quality of life for patients.

Conclusion

The present findings are relevant and add novel information related to salivary levels of pipecolic acid in cases of HNSCC, suggesting the high sensitivity of this metabolite in predicting the disease, even in the early stages. However, the specificity of pipecolic acid for HNSCC needs to be investigated further in larger samples.

Acknowledgement

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Conflict of Interest

None.

Ethics approval

The present study was conducted at a university hospital of the public education system in southern Brazil, in accordance with the precepts stipulated in the Declaration of Helsinki, and received approval from the local human research ethics committee (certificate number: 63198116.3.0000.5346/1.889.748).

Data availability

Datasets related to this article will be available upon request to the corresponding author.

Author Contributions

Kívia Linhares Ferrazzo: Conceptualization and design of the manuscript, data acquisition, formal data analysis and writing the manuscripit – original draft, review

and editing. **Larissa Daiane W. de Melo:** Data acquisition, formal analysis, writing – original draft. **Cristiane Cademartori Danesi:** Formal data analysis, writing – review and editing. **Alexander Thomas:** Formal data analysis and writing – original draft. **Laura Izabel Lampert Bonzanini:** review and editing. **Nilo Zanatta:** Data acquisition, data analysis, writing – original draft and review. All authors gave final approval of the work.

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