CLINICS

CLINICAL SCIENCE

Human papillomavirus types 16 and 18 and the prognosis of patients with stage I cervical cancer

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OBJECTIVE: This study sought to evaluate the prevalence of human papillomavirus (HPV) types 16 and 18 in women with clinical stage IB cervical cancer treated by radical hysterectomy with pelvic lymphadenectomy as well as to establish a correlation between HPV type and cancer prognosis.

METHODS: A single-center cohort study was conducted with 86 patients who had undergone radical hysterectomy for stage I cervical cancer. Prognostic factors and the presence of HPV 16 and 18 were analyzed using a polymerase chain reaction assay. A univariate analysis using Kaplan-Meier curves was conducted to estimate survival.

RESULTS: The prevalence of HPV 16 in the study group was 65.3%, and the prevalence of HPV 18 was 33.3%. The prevalence of infection with both viruses was 26.9%. Overall survival at 5 years was 91% among women with HPV 18 and 96% among those without this virus type ($p = 0.133$). Among the women with HPV 16, the overall survival was 94%, whereas this rate was 96% among those without this virus type ($p = 0.663$). Disease-free survival was unaffected by the presence of HPV type 16 or 18.

CONCLUSION: In the present study, despite the high prevalence of HPV types 16 and 18, the presence of these virus types did not affect the prognosis of patients with stage I cervical cancer who underwent radical hysterectomy.

KEYWORDS: Human Papillomavirus (HPV); Cervical Cancer; Prognosis; Survival.

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INTRODUCTION

Cervical cancer is the second most common type of cancer among women in Brazil (1,2), and in 2012, it was estimated that 17,540 new cases of cervical cancer would occur in Brazil (approximately 17 cases in every 100,000 women) (3). Human papilloma virus (HPV) has been identified as a key factor in the development of cervical cancer (4-6). Among the HPV types classified as high-risk, HPV 16 and HPV 18 are responsible for the largest percentage of cervical cancer cases (6,7).

Many prognostic factors for cervical cancer have been established, including clinical staging, pelvic lymph node involvement, parametrial involvement and lymphovascular space invasion (8-11).

Cervical cancer screening studies have reported that the prevalence of HPV infection in Brazil ranges from 15% to 27%, according to hybrid capture (HC) or polymerase chain reaction (PCR) assays (12,13). In patients with cervical cancer, HPV DNA has been detected in 55.2% to 91% of patients, depending on the type of biological material and the method used (14,15).

For almost 2 decades, studies have indicated the possibility that HPV 18 may negatively affect the prognosis of cervical cancer patients (7,11,16-18). Furthermore, a significant association was found between lymphovascular space invasion and lymph node involvement and the presence of both HPV 16 and 18 (17). Nevertheless, other studies have reported varying results; some have implicated HPV 16 as an unfavorable factor, while others have failed to detect any differences between these 2 virus types (17,19).

No potential conflict of interest was reported.

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Attempts have also been made to correlate the viral load with prognosis, and different studies have produced conflicting reports (20).

Using immunohistochemistry, research has shown that activation of the epidermal growth factor receptor (EGFR) is associated with chemoradiotherapy resistance in cases of advanced cervical cancer. As a result, EGFR activation is also associated with a poor prognosis (21).

Investigation of the EGFR status in early stage tumors has revealed that the lack of expression of the phosphatase and tensin (PTEN) tumor suppressor gene is associated with metastases to pelvic lymph nodes (22). This same line of research demonstrated that PTEN expression decreases progressively from normal cervical tissue to cervical intraepithelial neoplasia to squamous cell carcinoma. On the other hand, the expression of survivin, a protein encoded by an anti-apoptotic gene, was shown to increase as the neoplasia progresses. Thus, PTEN and survivin expression levels may serve as indices for evaluating prognosis (23).

When an invasive tumor is confined to the cervix, i.e., stage IB cervical cancer, it is often treated using the classic surgical technique known as the Wertheim-Meigs hysterectomy, a radical hysterectomy with pelvic lymphadenectomy (11). Nevertheless, radiotherapy also produces results of similar efficacy (24).

The objective of this study was to evaluate the prevalence of HPV types 16 and 18 in women with stage IB cervical cancer who underwent a radical hysterectomy with pelvic lymphadenectomy and to establish a correlation between HPV type and cancer prognosis.

MATERIALS AND METHODS

Sample selection

A cohort study was conducted in the Araújo Jorge Hospital in Goiânia, Goiás, Brazil. The charts of 160 women with stage I invasive cervical cancer who underwent a radical hysterectomy with lymphadenectomy between 1992 and 2003 were reviewed. This study was designed to include only those patients at clinical stage IB who had received a radical hysterectomy with pelvic lymphadenectomy. All of the patients were treated at a single institution in the city of Goiânia, Goiás, Brazil. The clinical and pathological data were analyzed according to HPV type to evaluate their effects on tumor recurrence and overall survival. The study was approved by the institution's internal review board (approval letter number 027/07). To analyze overall survival, an active attempt was made to contact the patients by telephone and telegram with the objective of reducing the rates of censoring due to loss to follow-up.

Samples

A total of 92 biopsies of the cervix (samples fixed in formalin and embedded in paraffin) were selected from the hospital's anatomopathology department and tested for HPV using PCR. The molecular analysis was performed at the Oncology Research Institute (IPON) of the Federal University of Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.

DNA extraction from paraffin-embedded samples

The paraffin-embedded blocks were cut into 5-μm-thick sections and placed in 2-ml Eppendorf tubes. They were then submitted to the following deparaffinization process. Briefly, 1 ml of 97% xylol was added to each microtube, and the mixture was homogenized in a vortex mixer, heated in an oven at 60℃ for 10 minutes and centrifuged at 2,500 rpm for 10 minutes. The excess xylol was removed, and the quality of the DNA was verified using β-actin. Next, 200 μL of chloroform was added for each 1.0 ml of TRIZOL®. The mixture was then vortexed for 15 seconds, incubated at room temperature for 3 minutes and centrifuged at 12,000 g for 15 minutes at 4℃. Next, the pellets were washed twice in 300 μl of 100% ethanol, and then 1 ml of 75% ethanol was added. The material was placed in the refrigerator and allowed to dry for 12 hours before posterior amplification of the sample. At the time of use, this precipitate was again suspended in Tris-acetate-EDTA buffer.

HPV genotyping and DNA amplification cycle

To identify molecular HPV and β-actin, PCR amplification was performed. The reaction mixture contained 5.0 μl 10× buffer, 1.0 μl dNTPs (10 mM), 1.5 μl 50 mM $MgCl_2$, 0.2 μl Taq DNA polymerase and distilled H_2O to a final volume of 50.0 μl. The reaction mixture was then added to a tube containing 1.0 μl primer and 4.0 μl of DNA to reach a final volume of 50.0 μl. Type-specific primers for HPV 16 (5′ = 5′ACCGAAACCGGTTAGTATAAAAGC3′ and 3′ = 5′ ATAACTGTGGTAACTTTCTGGGTC3′) with a product of 477 base pairs (bp) and primers for HPV 18 (5′ = 5′CGG TCGGGACCGAAAACGGTG3′ and 3′ = 5′CGTGTTGGATC CTCAAAGCGCGCC3) with a product of 422 bp were used. β-actin primers (5′ = 5′GTGGGGCGCCCCAGGCACCA3′ and 3′ = 5′CTCCTTAATGTCACGCACGATTTC3′) with a product of 295 bp were used as an internal control. Annealing was performed at 56℃ for each of these 3 primers (25,26). The reaction was initiated at 94℃ for 1 minute for denaturation, followed by 30 cycles of 2 minutes at 50℃ for annealing and 3 minutes at 72℃ for polymerization. The reaction was amplified using an Eppendorf thermal cycler.

Statistical analysis

Various clinical and pathological characteristics were analyzed, including the age of the patient at the time of cancer diagnosis, the number of pregnancies and deliveries she had prior to her cancer diagnosis and the histological type of the tumor (based on the World Health Organization's (WHO) classifications). In addition, the following factors were taken into consideration: the degree of anaplasia according to the WHO classifications (grades I, II, III or undifferentiated), whether there was lymphovascular invasion and whether the pelvic lymph nodes were affected. Because the study objective was to characterize the sample of patients with cervical cancer, descriptive analyses rather than statistical analyses were initially performed on the study variables mentioned above. The differences in parameters between the groups were assessed using the chi-square test and Fisher's exact test, as appropriate. To calculate survival, the Kaplan-Meier method was used, while the log-rank test was applied to compare the mean survival rates associated with different possible prognostic factors for cervical cancer. In calculating the overall survival, all deaths were taken into consideration, regardless of their cause. For cancer-associated survival, the criterion applied was the event (i.e., the recurrence of locoregional or metastatic disease). The patients who were alive at the time of the last medical follow-up visit or who

died after 60 or more months of follow-up were considered censured. P-values <0.05 were considered statistically significant for all tests. The Statistical Package for the Social Sciences (SPSS), version 15.0 for Windows (SPSS®, Chicago, IL, USA), was used for all statistical analyses.

RESULTS

The ages of the patients in this study ranged from 26 to 64 years, with a mean of 40±8.95 years (standard deviation [SD]). The mean duration of follow-up was 67 months (range 4-134 months; median 73 months; SD 44.14 months).

Of the 86 patients studied, 8 suffered a disease recurrence, and 4 of these patients died during the study. Only 1 patient had a diagnosis of vaginal intraepithelial neoplasia (VAIN) III during the clinical follow-up period, and this condition regressed spontaneously. The VAIN III diagnosis occurred 2 years after the patient had been treated for a recurrence of the pelvic tumor. This recurrence was treated with radiotherapy, and there is currently no sign of disease in this patient. The demographic, clinical and pathological characteristics of the patients who tested positive for HPV 16 and 18 are shown in Table 1.

Table 1 - Demographic, clinical and pathological characteristics of the patients with stage I cervical cancer who underwent radical hysterectomy and tested positive for HPV types 16 and 18.

Characteristics	HPV 16*		HPV 18*	
	n	%	n	%
Age (years)				
Median	39		39	
Range	9.27		8.77	
≤30	5	9.3	3	11.5
30–50	39	72.2	19	73.1
>50	10	18.5	4	15.4
Clinical stage (FIGO)				
IB1	46	85.2	22	84.6
IB2	8	14.8	4	15.4
Histological type				
Squamous cell carcinoma	46	85.2	22	84.6
Adenocarcinoma	5	9.3	2	7.7
Adenosquamous cell carcinoma	3	5.6	2	7.7
Others	0	0	0	0
Grade of differentiation				
Grade I	1	2	1	4.2
Grade II	33	66	17	70.8
Grade III	16	32	6	25
Metastases to lymph nodes				
Yes	3	5.6	3	12
No	51	94.4	22	88
Angiolymphatic invasion				
Yes	12	28.6	5	25
No	30	71.4	75	75
Initial treatment				
Surgery alone	42	77.8	20	76.9
Surgery + radiotherapy	9	16.7	4	15.4
Radiotherapy + surgery	3	5.6	2	7.7
Recurrence of the disease				
Yes	5	9.8	3	12
No	46	90.2	22	88
Treatment of recurrence				
Radiotherapy	0	0	0	0
Chemotherapy	1	100	1	100
Radiotherapy + chemotherapy	0	0	0	0

* HPV-positive patients.

With respect to genotype, HPV 16 alone was found in 30 samples, whereas HPV 18 alone was detected in only 5 samples. The concomitant presence of both types of HPV was detected in 21 samples. In 22 samples, neither of these viral types was detected.

With regard to the histological type, HPV 18 was detected in 84.6% of squamous cell carcinomas (22 cases), in 7.7% of adenocarcinomas (2 cases) and in 7.7% of adenosquamous cell carcinomas (2 cases). HPV 18 was not detected in any cases of undifferentiated carcinoma. HPV 16 was detected in 85.2% of squamous cell carcinomas (46 cases), in 9.3% of adenocarcinomas (5 cases) and in 5.6% of adenosquamous carcinomas (3 cases).

Regarding the degree of anaplasia, in tumors that tested positive for HPV 18, the prevalence of grade II (moderately differentiated) was 70.8% (17 cases), while 25% (6 cases) of the tumors were classified as grade III (highly differentiated).

For the tumors that tested positive for HPV 16, the prevalence of grade II anaplasia was 66% (33 cases), while 16 cases (32%) were classified as grade III and 1 case (2%) as grade I.

In this sample, only 5 cases were found in which the pelvic lymph nodes were affected by the neoplasia. HPV 18 was detected in 2 of these cases (7.7%), and HPV 16 was detected in another 2 cases (5.6%).

Lymphovascular invasion associated with HPV 18 was observed in 29.4% of the 63 cases for which this information was available in the patients' charts. With respect to HPV 16, lymphovascular invasion was found in 66.7% of the 66 cases for which this variable was provided.

The majority of recurrences occurred within 30 months after the initial treatment was completed, and the 5-year disease-free survival rate was 91%. The eighth recurrence is not shown on the survival curve because it occurred after 60 months. The 5-year overall survival rate in the study group was 95%. The 5-year disease-free survival rates were 92% and 88% for the patients who tested positive and negative for HPV 16, respectively ($p>0.05$). The 5-year disease-free survival rates were 83% and 93% for those who tested positive and negative for HPV 18, respectively, and there were no statistically significant differences between the groups.

The overall survival rate among women who tested positive for HPV 16 was 94% compared to 96% among those who tested negative ($p>0.05$) (Figure 1A). The women who tested positive for HPV 18 had an overall 5-year survival rate of 91% compared to 96% among those who tested negative ($p>0.05$) (Figure 1B). When disease-free survival was analyzed in relation to the other independent variables, none of the factors evaluated were associated with cervical cancer recurrence (Figure 1). Furthermore, none of the factors evaluated in this study were found to affect overall survival, as shown in Table 2.

DISCUSSION

Of the various prognostic factors for cervical cancer, the clinical staging system proposed by the International Federation of Gynecology and Obstetrics (FIGO) remains the most significant, with a 5-year survival rate of approximately 90% up to clinical stage IB1. Other relevant factors include pelvic lymph node metastases (12-20% at clinical stage IB) and para-aortic lymph node metastases (4-7% at clinical stage I). However, recovery rates may fall

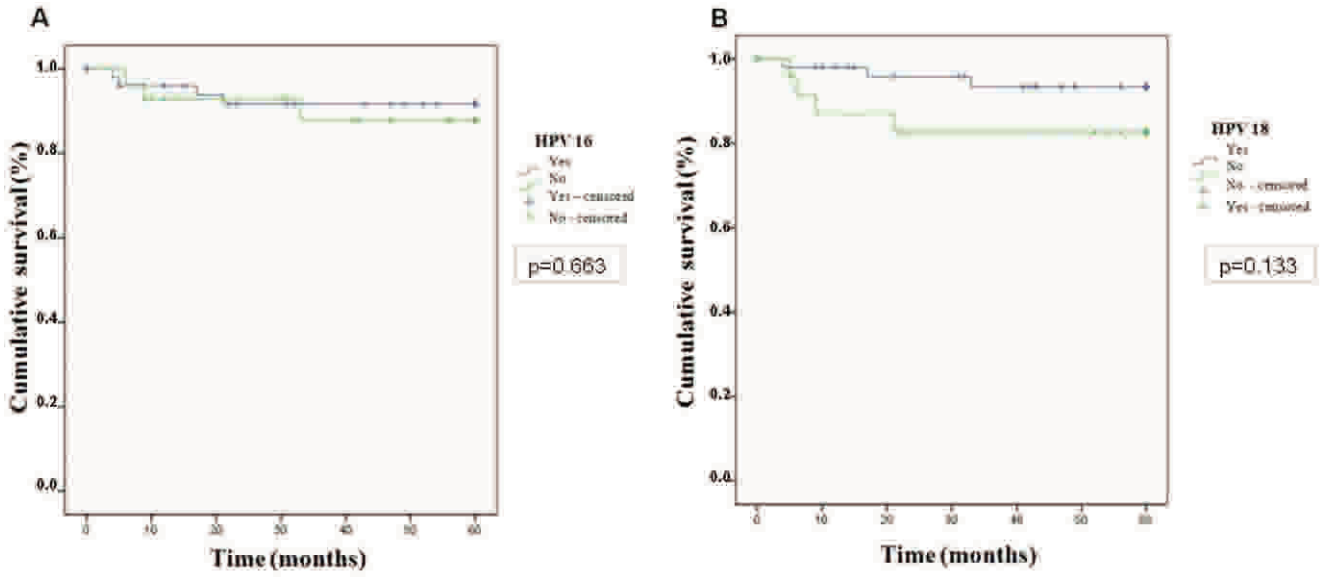

Figure 1 - (A) Disease-free survival according to whether HPV 16 was detected. (B) Disease-free survival according to whether HPV 18 was detected.

considerably when the pelvic lymph nodes are involved, and para-aortic metastases are present principally when the pelvic lymph nodes are also affected. Parametrial invasion and lymphovascular space invasion are also considered relevant findings. As individual factors, age and tumor grade are not as important as other prognostic factors, according to a previous report by Zaino et al. (27).

In the present study, HPV 16 and 18 DNA was found in 62.7% and 30.2% of the women, respectively, which is not surprising because the majority of women between 50-59 years of age have been shown to test positive for these HPV types (28). In this study, HPV 16 and 18 DNA was found in the majority of the older women between 30-50 years of age, and this finding is also in agreement with the results of other studies (29,30).

Previous work has shown that the presence of HPV 18 may be considered an independent prognostic factor for a poor outcome in early stage cervical cancers (7,11,16), and the results of the present study are in agreement with this line of reasoning. In fact, the current study included early stage cervical tumors precisely because the literature indicates that the prognosis is otherwise good in these cases.

The current study evaluated the effect of HPV 16 and 18 on the prognosis of women with early stage invasive cervical cancer. The results demonstrated an overall 5-year survival rate of 95%, which may be because only 10.4% of the women in this study sample were staged as IB2 (i.e., tumors larger than 4 cm) according to the staging classification of the FIGO. In other studies, the overall 5-year survival rate for those with early stage tumors has also been high (1,31). Nevertheless, although 5-year survival rates for small-volume tumors are usually as high as 90%, the survival rates for larger tumors may fall to 70% after 5 years (32).

The most prevalent histological type was squamous cell carcinoma, which was present in 84.4% of the cases evaluated in this study, followed by adenocarcinoma and adenosquamous cell carcinoma, which were found in 12.4% of the cases. These findings are in agreement with the data published in the literature (7,11). However, it remains controversial as to whether adenosquamous carcinoma is associated with a poor prognosis; although some authors advocate this hypothesis (33), others argue that there is no supporting evidence (24).

Adenocarcinomas were not associated with a greater likelihood of tumor recurrence or a poorer prognosis in this study group, and only 1 patient experienced tumor recurrence. HPV 18 was only associated with 1 case of adenocarcinoma and 2 cases of adenosquamous cell carcinoma. Furthermore, 20 cases of adenocarcinoma had to be excluded from the study because it was impossible to recover the corresponding paraffin blocks from the anatomopathology department, and it is possible that this exclusion may have affected the final results.

In the present study, HPV type had no effect on the disease-free or overall survival rates and appeared to have no effect on the likelihood of tumor recurrence.

Numerous prognostic factors for cervical cancer have been documented in the literature, including clinical staging, invasion of the lymph nodes by tumor cells, parametrial involvement and invasion of the lymphovascular space (8,9,10,11). Nonetheless, various investigators have attempted to clarify the precise role of HPV type in tumor progression (7,11,34-37).

In this study, the number of cases in which the tumor had invaded the pelvic lymph nodes was small (5 cases, 5.5%). Of the 8 patients who experienced recurrences, pelvic lymphatic metastasis was present in only 1 patient. Lymphatic metastasis is considered one of the most important prognostic factors and one that may affect survival. In turn, lymphatic metastasis can be affected by angiolymphatic invasion, an increase in tumor size or the depth of the stromal invasion, as shown in a study conducted by the Gynecology Oncology Group (10,32). The small number of recurrences in the present study may also be attributable to the fact that there were few cases in which the lymph nodes were affected.

Table 2 - Univariate analysis of the presence or absence of recurrence according to the clinical and pathological characteristics and the HPV type in women with stage IB cervical cancer.

Variable	Recurrence				*p*-value*	RR (95% CI)
	Yes		No			
	n	%	n	%		
Age group						
≤39 years	5	13.5	32	86.5		1
40-64 years	3	6.5	41	93.5	0.283	2.240 (0.498-0.064)
Clinical staging						
IB1	7	9.3	68	90.7		1
IB2	1	12.5	7	87.5	0.572	0.721 (0.077–6.735)
Pregnancies						
1-3 pregnancies	2	9.1	20	90.9		
>4 pregnancies	6	10	55	90	0.635	0.900 (0.168–4.832)
Deliveries						
1-3 deliveries	1	3.1	31	96.9		1
>4 deliveries	7	14	43	86	0.141	0.198 (0.023–1.694)
Histological type						
Squamous cell carcinoma	7	9.7	65	90.3		1
Non-squamous cell carcinoma	1	9.1	10	90.9	0.947	1.077 (0.120–9.705)
Lymph nodes						
None	7	9	71	91		
>1	1	25	3	75	0.342	3.381 (0.309-6.996)
Treatment						
Hysterectomy	5	8.1	57	91.9		1
Other treatments	3	14.3	18	85.7	0.604	0.526 (0.114–2.422)
HPV 16						
Negative	3	10.7	25	89.3		1
Positive	5	9.8	46	90.2	0.898	1.104 (0.243–5.007)
HPV 18						
Negative	5	9.8	46	90.2		1
Positive	3	12	22	88	0.769	0.797 (0.175–3.640)

*Fischer's exact test.

When a specific analysis was conducted on patients with early stage tumors (IB and IIA) who had undergone radical hysterectomy and pelvic lymphadenectomy, it was found that women with HPV 18 tended to have a poorer prognosis (7). In another study, the presence of HPV 18 in patients with early cervical carcinoma was associated with a significantly poorer prognosis compared to women infected with HPV 16, even after adjusting for other relevant factors, such as clinical stage, lymph node status and histological type (12). Nevertheless, in a study conducted in Russian women is not observed in HPV type influences the prognosis of women, which had staged tumors classified as stage I and stage II (19). However, in that study, no specific analysis was performed with respect to the type of treatment used, unlike the study mentioned previously (19). In the study of van Muyden et al. 1999, HPV was detected in all cases studied, corroborating the hypothesis that HPV-negative cervical cancer does not exist (5,19).

It has also been reported that tumors positive for HPV 16 are more likely to metastasize to the pelvic and parametrial lymph nodes compared to HPV-16-negative tumors (38) and that HPV 16 negatively affects the prognosis of patients who receive a radical hysterectomy with pelvic lymphadenectomy (39). A study conducted by Pilch et al. (17) found a significant association between involvement of the lymph nodes and the lymphovascular space and the presence of HPV types 16 and 18. However, in the present study, the presence of HPV 16, although more prevalent, had no effect on the characteristics mentioned above.

Multiple-type HPV infection has been associated with a poorer response to radiotherapy and a poorer prognosis in patients with local advanced cervical cancer (31,40). Nevertheless, for the patients evaluated in the present study, concomitant infection with HPV 16 and 18 had no detrimental effect on their prognosis.

In recent years, some studies have tried to establish HPV 18 as an indicator of an unfavorable clinical progression. In this respect, the present study sought to select a more homogenous group of women with early cervical cancer (i.e., restricted to clinical stage IB) to evaluate whether HPV 18 had a negative effect on their prognosis. However, it was difficult to obtain an adequate number of cases because an unexpected number of charts (230) could not be located by the Department of Medical Records and Statistics of the Araújo Jorge Hospital. Furthermore, due to a similar situation in the anatomopathology department, it was impossible to recover the paraffin blocks of tumor samples for 68 patients who were eligible for the study, a fact that certainly contributed to the inadequate number of cases for the projected analysis.

Furthermore, it was impossible to identify any factor analyzed that was significantly associated with disease recurrence, which may have been due to the limited size and homogeneity of the sample or the effect of other factors that are still under investigation.

In the present study, the prevalence of HPV 16 in the study group was greater than the prevalence of HPV 18. However, the presence of HPV 16 or 18 was unrelated to the histological type and the degree of anaplasia, vascular invasion or lymph node involvement. Despite the high prevalence of HPV 18 and/or HPV 16, the presence of these HPV types did not affect the prognosis of the study patients with stage I cervical cancer who underwent radical hysterectomy.

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■ AUTHOR CONTRIBUTIONS

Zampronha RA and Freitas-Junior R contributed to the conception and design of the study, acquisition of the data, analysis and interpretation of data, initial draft of the manuscript and final approval of the submitted version. Murta EF and Michelin MA contributed to the conception and design of the study, acquisition of the data, interpretation of the data, critical review of the manuscript and final approval of the submitted version. Barbaresco AA, Adad SJ and Oliveira AM contributed to the conception of the study, analysis and interpretation of the data and approval of the manuscript final version. Rassi AB contributed to the analysis and interpretation of the data, review of the article and approval of the manuscript final version. Oton GJB contributed to the analysis and interpretation of the data and approval of the manuscript final version.

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