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Differences in intraosseous and extraosseous post-chemotherapy regression of Ewing sarcomas and their influence on prognosis

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ABSTRACT

Background: In Ewing sarcomas (ES), histological response to polychemotherapy is the main prognostic factor. We aimed at evaluating the histological response separately for the extraosseous and intraosseous tumor compartment as well as its prognostic influence.

Methods: Thirty-one patients with ES and marked soft tissue expansion, treated at our department between January 2006 and December 2015, were retrospectively included. Data was taken from medical records. Original histologic specimens of the resected tumors were re-evaluated separately for intra- and extraosseous tumor regression according to Salzer-Kuntschik regression grading. Multivariate survival analysis with stepwise backward variable selection was calculated to determine the impact of extraosseous and intraosseous regression on prognosis.

Results: All patients had received chemotherapy, 15 (48.4%) had been administered preoperative radiotherapy. Extraosseous tumor regression was significantly worse than intraosseous regression (Wilcoxon signed-rank test, $p = 0.018$). While neither intraosseous nor extraosseous tumor regression had an impact on overall survival, extraosseous complete remission had a beneficial impact on event-free-survival in the multivariate analysis (Cox-regression; hazard ratio: 0.148, 95% confidence interval 0.031-0.707, $p = 0.017$).

Conclusions: On average, regression of ES seems to be worse in the extraosseous tumor compartment following preoperative chemotherapy. Moreover, extraosseous tumor regression may have a stronger prognostic influence on event-free survival than intraosseous regression.

1. Introduction

Ewing's sarcoma is a rare tumor entity especially arising in children and young adults, with a slight male predominance [12]. Following osteosarcoma, Ewing's sarcoma constitutes the second most common malignancy of bone [8]. The t(11;22) chromosomal translocation leading to the *EWS-FLI1* fusion gene is characteristic for 85% of Ewing's sarcoma [6]. In the remaining 15%, other translocations are observed, involving either the EWS or FUS gene [16]. Patients usually present with pain or a palpable mass. One-quarter of patients may already have developed metastatic disease [4,8].

In patients with localized disease at time of diagnosis, chemotherapy (CTX) significantly improves survival rates from 10% to nearly 80% [4]. The Euro-EWING-99 protocol recommends vincristine, ifosfamide, doxorubicin and etoposide (VIDE) as initial therapy for all patients with Ewing's sarcoma [10]. Neoadjuvant radiotherapy (RTX) in combination with CTX (chemoradiotherapy; CRT) may be administered in order to eliminate micrometastases and reduce the tumor volume when close resection margins are to be expected [15].

However, it is not clearly defined whether the Salzer-Kuntschik regression grade, a histological-based scale predominantly used in German-speaking countries to evaluate tumor response to CTX, should be analyzed from intra- or extraosseous tissues [14].

Therefore, the aim of the present study was to analyze the difference

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in response rates of extraosseous and intraosseous Ewing's sarcoma tissue following CTX or CRT.

2. Material and methods

Thirty-one patients were included, treated between January 2006 and December 2015 for Ewing's sarcoma of the extremity, trunk and thorax (formerly called Askin tumor) with marked extraosseous tumor mass. Eleven patients were female (35.5%) and 20 male (64.5%), with a mean age of 20.1 years (range: 3–55 years). Diagnosis was made on HE stained slide of core biopsy material with additional immunohistochemical stains (particularly for CD99) and molecular testing with ancillary Fluorescence in situ Hybridisation (FISH) examination with dual-color break apart probes for the *EWSR1*-gene. In 22 patients (71.0%), an *EWSR1* break apart could be detected. In the remaining 9 patients, too few or poor-quality material made FISH and/or polymerase-chain-reaction (PCR)-based diagnosis impossible. In these cases, diagnosis of Ewing's sarcoma was based on histology and immunohistochemistry, with corresponding second opinions from independent pathologists.

Demographic, tumor- and treatment-specific data as well as follow-up information were documented in each case using medical, surgical and pathological reports. Patients were followed up until the last clinical consultation or date of death. Specimens were taken from the local Biobank and re-evaluated by separately analyzing the intra- and extraosseous Salzer-Kuntschik regression grade (SK) [14]. Upon receipt of the definite surgical specimens, a cross-section covering the entire tumour had been embedded, together with regions of specific interest, e.g. soft tissue and bony resection margins, biopsy tract and involved joints. More recent samples had been mapped, whilst in those older ones included, the native location of the sample (i.e. intra- or extraosseous) must have been evident based on presence of trabecular and/or cortical bone. According to the amount of histologically visible tumor necrosis, the SK is subdivided into 6 categories; a SK of 1 describes a tumor with 100% necrosis and no vital tumor cells, whilst a SK of 5 involves tumors with more than 50% of vital tumor tissue and a SK of 6 defines completely viable tumors (Supplementary Table 1). The SK was evaluated by 4 pathologists (K.S.; B.L-A.; C.V.; K.B.) and differences in SK category were resolved by consensus at multidiscussion microscope. The resected specimens were mapped macroscopically and numerous paraffin blocks selected from a large medial section of the tumor were embedded (or at least an average of one section per centimeter of the tumor's greatest dimension), for histological evaluation at the time of the operation. Vital and non-vital intra- and extraosseus areas of the tumor were evaluated separately, considering therapy-related morphological changes such as necrosis, reactive fibrosis and inflammatory cells within the original tumor area.

HE slides were scanned with Aperio ScanScope AT in a resolution of 40x and pictures were taken using Aperio ImageScope software (Version 12.0.0.5039, Aperio Technologies).

2.1. Statistical analysis

Statistical analysis was carried out using Stata Version 15.1 (StataCorp, TX, US). χ^2-tests and Spearman's correlation analyses were performed, as well as univariate survival analysis using univariate Cox-regression models (with given hazard ratios [HRs] and 95% confidence intervals [CI], where applicable). Multivariate survival analysis was calculated by using multivariate Cox-regression models with stepwise backward selection. A p-value < 0.05 was considered statistically significant. The given P-values derive from two-tailed tests.

2.2. Ethics

This study has been approved by the institutional review board of the Medical University of Graz (EK 28-099 ex 15/16).

Table 1
Baseline characteristics. Baseline characteristics of patients included in the present study.

Parameter	*n = 31*	*%*
Gender		
Male	20	64.5
Female	11	35.5
Age		
< 18	14	45.2
≥18	17	54.8
Tumour location		
Extremities	15	48.4
Trunk	13	41.9
Askin	3	9.7
Preoperative CTX scheme		
VIDE	24	77.4
VIDE, VAI	3	9.7
VIDE, VI, VAI	1	3.2
VAIA-A/B	1	3.2
VAIA	1	3.2
n.a.	1	3.2
Preoperative RTX		
Yes	15	48.4
No	16	51.6
Primary metastases		
None	22	71.0
Pulmonary	8	25.8
Pulmonary, bone, lymph nodes	1	3.2

3. Results

3.1. Baseline characteristics

Fifteen patients had tumors located in the extremities (48.4%), followed by the trunk in 13 (41.9%) and the thorax in 3 cases (9.7%). Nine patients presented with primary metastases (29%), of whom 8 had pulmonary metastases and one patient multiple metastatic nodules located in bone, lymph nodes and lung. All 31 patients underwent neoadjuvant and adjuvant polychemotherapy (PCT), most commonly according to the VIDE-scheme (Table 1). In one case data on PCT scheme was not available. Preoperative RTX was administered in 15 patients (48.4%). All patients underwent definite surgery with wide resection margins according to Enneking, and in case of metastatic lesions only after complete consolidation of metastatic nodules. Three patients (9.7%) received postoperative radiotherapy (one with Ewing's sarcoma in the metatarsus, one in the paravertebral region, one in the pubic arc).

The median follow-up was 6.2 years (IQR: 3.9 years – 8.9 years). Postoperatively, 9 patients had developed metastatic disease (25.8%) after a median of 19 months (IQR: 16–45 months), and two patients a local recurrence (6.5%), after 13 and 22 months, respectively. Median time to recurrence was 19 months (IQR: 13–45 months). At last follow-up, 16 patients (51.6%) were alive without disease, 5 patients (16.1%) were alive with disease (local recurrence: n = 1, distant metastasis n = 4), 9 patients (29.0%) had died of disease and 1 patient (3.2%) had died due to other causes.

3.2. Response to poly- and radiochemotherapy

A complete intra- and extraosseous remission (SK 1) could be achieved in 13 patients (41.9%) following neoadjuvant therapy, whilst more than 50% of vital tumor cells were found at intra- and extraosseous location in 9 tumors (SK 5 + SK 6; 29%; Table 2). Altogether, differences between the extraosseous and intraosseous SK were found in 13 tumors (41.9%), whilst the same SK regression grade was present in 18 tumors (58.1%). The extraosseous tumor compartment showed significantly higher SK grades than the intraosseous compartment (Wilcoxon signed-rank test; p = 0.018). In 11 cases, a worse extraosseous

Table 2
Salzer-Kutschnig regression grades. Regression grades listed for intraosseous and extraosseous tumour compartments with overlaps.

		Extraosseous Regression						
	SK	**1**	**2**	**3**	**4**	**5**	**6**	**Sum**
	1	13			1	2		16
	2	1			1	0		2
	3	1		1	3	1		6
Intraosseous Regression	**4**				1	2	1	4
	5					3		3
	6							0
	Sum	15	0	1	6	8	1	

Legend: SK – Salzer-Kutschnig regression grade

SK was present as compared with the intraosseous SK (35.5%; Fig. 1). On the other hand, a better extraosseous than intraosseous SK was found in 2 cases (6.5%). The administration of preoperative RTX had no influence on the pattern of intraosseous vs. extraosseous SK (Spearman-correlation; p = 0.186). Moreover, neither the presence of primary metastases (Spearman-correlation; p = 0.333) nor tumor location (χ^2-test; p = 0.53) or gender (Spearman-correlation; p = 0.224) had a significant impact on the relation of the intraosseous and extraosseous SK.

3.3. Influence of SK regression grade on overall- and event-free-survival

Neither a complete remission of intraosseous SK regression grade (p = 0.516), nor an extraosseous complete remission (p = 0.900), patient age (p = 0.290), preoperative RTX (p = 0.945), gender (p = 0.955) or tumour location (p = 0.910) had a significant influence on overall survival (OS) in the univariate analysis (Table 3). During backward stepwise selection for the multivariate model, all factors were removed due to non-significance.

In the univariate setting, intraosseous complete remission had no significant influence on event-free survival (p = 0.726). On the other hand, an extraosseous complete remission was associated with a significantly lower risk of developing local recurrence or distant metastasis (p = 0.046; Table 4, Fig. 2). Moreover, neither gender (p = 0.823) nor age (p = 0.323), preoperative RTX (p = 0.639) or tumour location (p = 0.590) were significantly associated with an altered event-free survival (Table 4). In the multivariate analysis, after exclusion of gender and preoperative RTX during stepwise backward selection, complete remission as seen in the extraosseous part of the tumor remained an independent positive prognostic factor regarding event-free survival (p = 0.017; Table 4), irrespective of the intraosseous complete remission (p = 0.288), age (p = 0.409) and location of the tumor (p = 0.426).

Interestingly, there was no difference in either EFS (HR: 0.374; 95%CI: 0.101–1.383; p = 0.141) or OS (HR: 1.615; 95%CI: 0.457–5.711; p = 0.457) between those 13 patients with full intra- and

Table 3
Univariate Cox-Regression Analysis for Overall-Survival. Results from univariate Cox-regression analysis for overall survival; in stepwise backward selection, none of the factors proofed statistically significant; HR = hazard ratio, 95%CI = 95% confidence interval, EO = extraosseous, IO = intraosseous.

	Univariate			
Parameter	***HR***	***95%CI***		***p-value***
		Lower	*Upper*	
Gender				
Female	1			0.955
Male	1.038	0.209	3.725	
Age				
per year	0.967	0.908	1.029	0.290
Preoperative RTX				
no	1			0.945
yes	0.957	0.274	3.341	
Tumour location				
Trunk/Askin	1			0.910
Extremity	0.931	0.269	3.227	
EO full regression				
No (SK > 1)	1			0.900
Yes (SK = 1)	1.084	0.311	3.775	
IO full regression				
No (SK > 1)	1			0.516
Yes (SK = 1)	1.572	0.426	5.479	

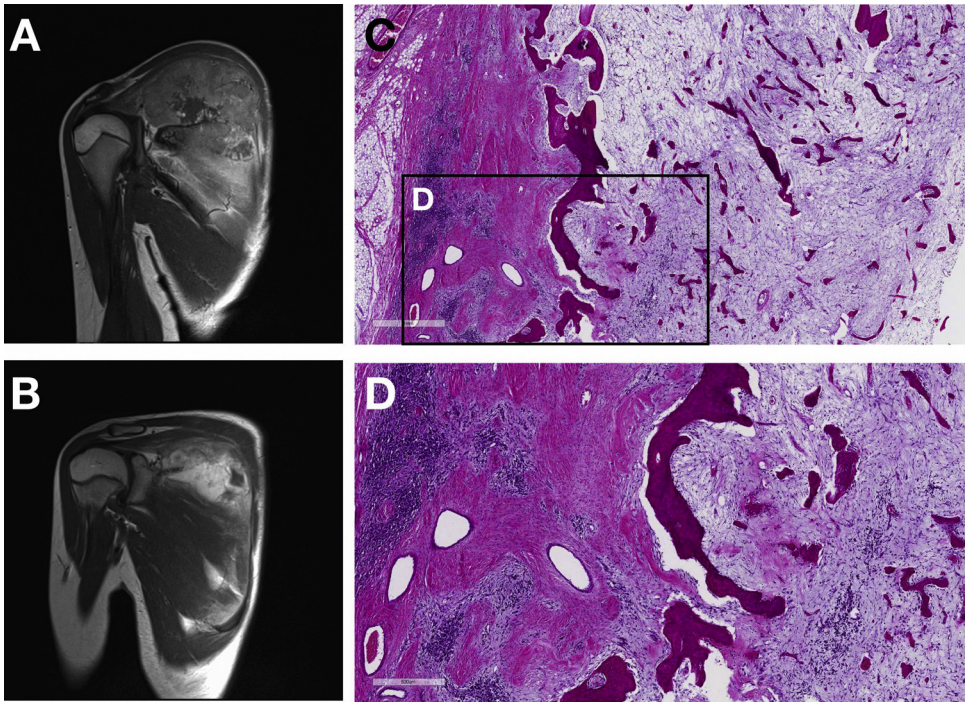

Fig. 1. Macroscopic and microscopic tumour regression. MRI taken prior to (Fig. 1A) and after polychemotherapy (Fig. 1**B**; 4 months in-between). Large tumour emerging from the upper border of the right scapula of a 12-year old girl. Same patient as in Fig. 1**A** and **B**: A typical histopathological example of tumour regression in Ewing's sarcoma following chemotherapy is depicted. Haematoxylin/eosin staining demonstrates a high extraosseous content (left side) of vital, small, round, blue tumour cells as compared to the low intraosseous tumour content (right side) in a fibrous background (former tumour area). Magnification: Fig. 1**C:** 20-fold, Fig. 1**D**: 40-fold.

Table 4
Uni- and multivariate Analysis for Event-Free Survival. Results from multivariate Cox-regression analysis for event free survival; in stepwise backward selection, gender (p = 0.815) and preoperative radiotherapy (p = 0.813) had been removed; HR = hazard ratio, 95%CI = 95% confidence interval, EO = extraosseous, IO = intraosseous.

	Univariate				Multivariate			
Parameter	HR	95%CI		p-value	HR	95%CI		p-value
		Lower	Upper			Lower	Upper	
Gender								
Female	1			0.823				
Male	1.147	0.345	3.816					
Age					0.975	0.918	1.036	0.409
per year	0.973	0.929	1.027	0.323				
Preoperative RTX								
no	1			0.639				
yes	0.759	0.240	2.400					
Tumour location								
Trunk/Askin	1			0.590	1			0.426
Extremity	0.729	0.231	2.300		0.621	0.192	2.006	
EO full regression								
No (SK > 1)	1			**0.046**	1			**0.017**
Yes (SK = 1)	0.265	0.071	0.979		0.148	0.031	0.707	
IO full regression								
No (SK > 1)	1			0.726	1			0.288
Yes (SK = 1)	0.816	0.262	2.540		2.146	0.524	8.786	

extraosseous regression. Also, the overall-SK score (defined as the mean of intra- and extraosseous regression in each patient) was not significantly associated with EFS (HR: 1.340; 95%CI: 0.942–1.908; p = 0.103) and OS (HR: 0.967; 95%CI: 0.630–1.483; p = 0.877).

4. Discussion

In the present retrospective study, the aim was to evaluate differences in intraosseous and extraosseous regression of Ewing's sarcomas after CTX with and without additional RTX. Furthermore, parameters affecting the amount of tumor regression as well as the influence of intraosseous and extraosseous tumor regression on event-free-(EFS) and overall survival (OS) were evaluated. The extraosseous but not the intraosseous response to chemotherapy has a significant influence on EFS. However, the separate analysis of the intra- and extraosseous portions of the tumor had no significant impact on OS.

As proposed by *Albergo et al.*, we only classified those Ewing's sarcomas with 100% tumor necrosis as tumors with complete remission [2]. Of the 31 patients analyzed in the present study, 13 had an intra- and extraosseous complete remission (100% necrosis, SK 1), and 18 patients more than 50% of viable tumor tissue (SK 5 + SK 6). Overall, in 41.9% of patients, tumors of differing extra- and intraosseous regression grades were identified. This is a large number, considering that it is unclear up to today whether intra- or extraosseous parts of Ewing's

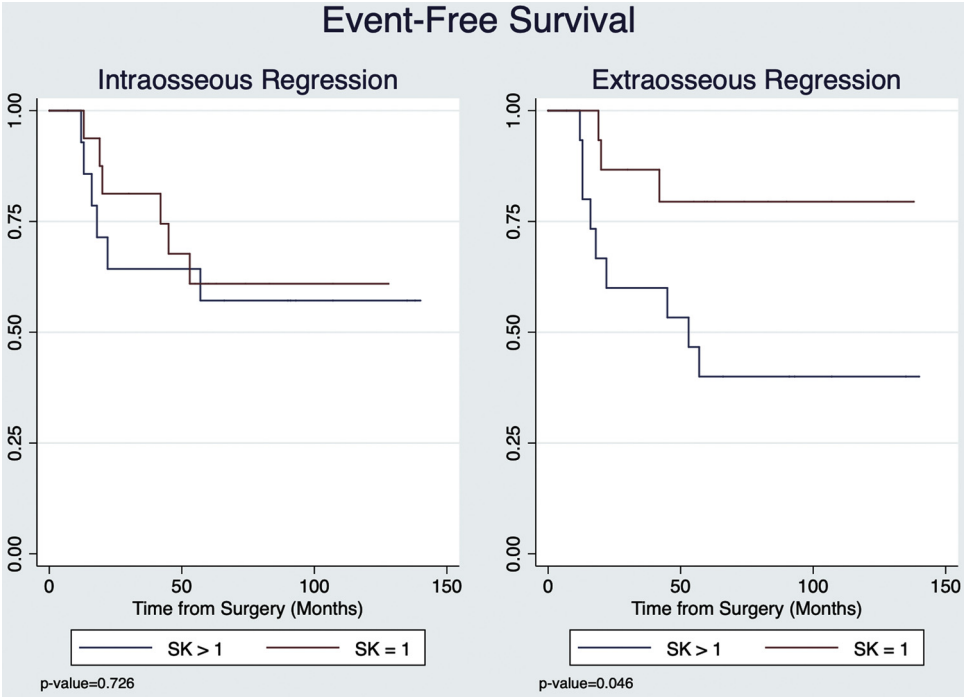

Fig. 2. Kaplan-Meier Curves. Kaplan-Meier curves for the impact of intraosseous (Fig. 2**A**; p = 0.726) and extraosseous (Fig. 2**B**; p = 0.046) SK regression on event-free-survival.

sarcoma should better be analyzed for chemotherapy response.

Hence, and due to usually present extensive soft tissue component of the tumor, most studies in the past have been undertaken by assessing both intra- and extraosseous specimens with subsequent addition and division of the SK regression grade by the specimens analyzed [13]. By using this approach, *Wunder* et al. discovered that the histological response of the tumor is significantly associated with EFS [17]. Of 14 patients with complete remission (i.e. 100% necrosis), none developed recurrent or metastatic disease [17]. In another study by *Lin et al.*, the same approach to histologically evaluate tumor response was chosen [11]. The authors discovered that good response to CTX was associated with improved local recurrence-free survival [11]. Likewise, *Albergo* et al. calculated the mean percentage of necrosis from both intra- and extraosseous parts of the tumor [2]. In their cohort, a complete remission following CTX was associated both with an improved EFS and OS [2]. Interestingly, we were unable to discover an improvement in terms of event-free and overall-survival for patients with complete intra- and extraosseous remission or depending on the overall-SK score.

Considering that intra- and extraosseous regression grades often differ, it is under debate, whether Ewing's sarcomas should be resected according to their original size or only along the remaining macroscopic border following CTX or CRT. The great concern is that single tumor cells may have survived in the tissues that would lead to early local recurrence if spared upon surgery. Logically, the histological tumor response may be analyzed from the central part of the tumor, as chemotherapeutic agents should reach even the innermost tumor parts in order to develop full efficacy. Therefore, in order to approach the issue in which way and from which tumor parts the regression grade may best be analyzed, we retrospectively analyzed the intra- and extraosseous components of the tumor separately. Notably, we discovered that the extraosseous part of the tumor generally showed a higher (worse) SK regression grade than the intraosseous counterpart. This could be related to the fact that central parts of large high-malignant tumors rather develop necrotic areas due to impaired blood supply. Therefore, a potential over-estimation of the overall tumor necrosis may result when analyzing the average percentage of devitalized tumor tissue by combining both extra- and intraosseous tumor parts. Of note, neither the administration of preoperative RTX nor the tumor location had an influence on the pattern of extra- and intraosseous regression grade.

Several studies have shown that the overall response to chemotherapy (i.e. amount of tumor necrosis, SK regression grade) is associated with an improved event-free survival [3,11,17] as well as OS [2]. Therefore, one may argue that the average percentage is reliable enough to predict EFS in Ewing's sarcoma patients. In the present study, though, the extraosseous complete remission only had a significant influence on EFS, irrespective of patient age, intraosseous complete remission, and tumour location. On the other hand, neither the extra- nor the intraosseous regression grades were associated with OS. This could be associated with the fact that distant metastases have a larger negative impact on overall survival than local recurrences [5], and that the former ones may not derive from remaining tumour cells following neoadjuvant RTX and CTX but rather emerge from already present, subclinical micro-metastases not responding well to systemic therapy.

Besides the overall response to chemotherapy, other prognostic markers have been discovered over the years. For example, BIRC5 (Survivin) is overexpressed in Ewing's sarcoma and high levels correlate with poor patient outcome [9]. On the other hand, expression loss of methylthiadenosine phosphorylase (MTAP) is associated with a poor overall survival [1]. Moreover, the methylation of Ras associated domain-containing protein 2 (RASSF2) correlates with a poor OS, especially in young patients [7].

The major limitation of the present study is the small cohort as well as its retrospective design, making accurate data ascertainment and interpretation contingent on integrity of medical and surgical reports. Of note, as the histological specimens were re-evaluated exclusively for this study, the regression grades could be accurately documented. Moreover, we did not address the question whether further molecular markers have a prognostic influence on patient prognosis. Furthermore, information on pre-therapeutic tumour volume could not be ascertained, which may have had an influence on response to neoadjuvant chemotherapy. Another limitation is that we are unable to assess the impact of post-operative patient management dependent on the originally analyzed tumor regression grade. Therefore, the results of the present study have to be judged bearing in mind its retrospective design and the relatively small number of patients, therefore further larger studies may be performed to confirm the herein presented results.

5. Conclusions

In summary, we discovered that extraosseous response of Ewing's sarcoma to chemotherapy is worse than intraosseous response. Furthermore, an extraosseous complete remission seems to be more important than intraosseous complete remission regarding event-free survival. Therefore, in the future, histo-pathologists may analyze both the extra- and intraosseous components rather than combining the regression grades in order to better predict the prognosis of patients with Ewing's sarcoma.

6. Author contributions

Study concepts were created by AL and LH, and study desing by AL. and CS. MAS and CS were responsible for data acquisition. Statistical analysis was performed by AL, CS and MAS. The manuscript was prepared by AL, LH and CS. KS, MB and CV contributed to the histopathological part of the manuscript. AL, LH and MAS were responsible for the orthopaedic-oncological part of the manuscript. Data was controlled, the manuscript edited and reviewed by all authors, namely AL, MAS, CS, KS, MB and CV.

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Declaration of Competing Interest

None.

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