Head and neck rhabdomyosarcoma in pediatric patients: an international collaborative study

Authors:

Karen Patricia Dominguez Gallagher [1]; Keith D. Hunter [2]; Lady Paola Aristizabal Arboleda [3]; Caique Mariano Pedroso[4]; Bruno Augusto Linhares Almeida Mariz [4]; Paulo Victor Mendes Penafort [4]; Lucas Lacerda de Souza [4]; Carla Isabelly Rodrigues-Fernandes [4]; Elena María José Roman Tager [4]; Roman Carlos [5†], Liam Robinson [6]; Ciska-Mari Schouwstra [6]; Francisco Germán Villanueva-Sánchez [7]; Francisco José Paz Gómez [8]; María del Carmen González Galván [9], Allan Vinícius Martins-de-Barros [10], Marianne de Vasconcelos Carvalho [10]; Roberta Barroso Cavalcante [11]; Eveline Turatti [11]; Hélder Antônio Rebelo Pontes [12]; Sheila Aparecida Coelho Siqueira [13]; Regina Maria Holanda de Mendonça [14], Lara Maria Alencar Ramos Innocentini [15]; Leandro Dorigan de Macedo [15]; Alfredo Ribeiro-Silva [15*]; Aline Corrêa Abrahão [16]; Mário José Romañach [16]; Willie van Heerden [6]; Pablo Agustin Vargas [4]; Alan Roger Santos-Silva [4].

Affiliations:

1. Departamento de Diagnóstico Oral, Área de Semiologia e Patologia Oral, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (FOP-UNICAMP), Piracicaba, Brazil. Facultad de Odontología, Universidad Nacional de Asunción, Asunción, Paraguay.
2. Liverpool Head and Neck Centre, Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, United Kingdom.

3. Graduate Program of A.C.Camargo Cancer Center. Group of Epidemiology and Statistics on Cancer. International Research Center, A.C.Camargo Cancer Center, São Paulo, Brazil.
4. Departamento de Diagnóstico Oral, Área de Semiologia e Patologia Oral, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (FOP-UNICAMP), Piracicaba, Brazil.
5. Departamento de Patología, Integra Cancer Center, Ciudad de Guatemala, Guatemala. † In memoriam 05-November-2021.
6. Department of Oral and Maxillofacial Pathology, Faculty of Health Sciences, University of Pretoria, South Africa.
7. Escuela Nacional de Estudios Superiores Unidad León – Universidad Autónoma de México, León – Guanajuato, Mexico.
8. Hospital Regional de Alta Especialidad del Bajío, León – Guanajuato, Mexico.
9. Facultad de Odontología, Universidad Nacional de Asunción, Asunción, Paraguay.
10. Faculdade de Odontologia, Programa de Pós-graduação em Odontologia, Universidade de Pernambuco (UPE), Recife, Pernambuco, Brazil, Centro Integrado de Anatomia Patológica (CIAP), Hospital Universitário Oswaldo Cruz (HUOC/UPE), Recife, Pernambuco, Brazil
11. Departamento de Patologia Oral e Maxilofacial, Faculdade de Odontologia, Universidade de Fortaleza (UNIFOR), Fortaleza, Brazil.
12. Departamento de Patologia Oral, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará (UFPA), Pará, Brazil.
13. Departamento de Patologia, Universidade de São Paulo, São Paulo, Brazil.
14. Centro Infantil Boldrini, Campinas, Brazil.

15. Divisão de Odontologia e Estomatologia do Departamento de Oftalmologia, Otorrinolaringologia e Cirurgia de Cabeça e Pescoço, Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil. *In memoriam 28-July-2021.
16. Departamento de Diagnóstico e Patologia Oral, Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

Corresponding author:

Karen Patricia Dominguez Gallagher

Assistant Professor

School of Dentistry, National University of Asunción (UNA)

Av. España c/ Brasil, Asunción - Paraguay.

Phone number: (+595) 975539705.

E-mail: **karendg89@gmail.com**

Funding statement:

Part of this research was funding by the Pathological Society (UK) through an International Collaborative Grant Scheme (Ref. ICGS 1022 02).

Conflict of interest disclosure:

KPDG is supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), Ministry of Education, Brazil; the Brazilian National Council for Scientific and Technological Development through the PEC-PG (student's postgraduate agreement program, ProcessNo.88881.284222/2018-01; and the National Program of Postgraduate Scholarships Abroad "Don Carlos Antonio Lopez" (BECAL) Ninth Self-managed Call for Applications 2019, Paraguay.

Ethics approval statement:

All procedures performed in this retrospective observational study were in accordance with the ethical standards of the institution and/or national research committee and with the 1964 Helsinki declaration and its later amendments, ensuring patient privacy and data confidentiality. Ethical approval was obtained from the Research Ethics Committees at Piracicaba Dental School, University of Pretoria, and the University of Liverpool. Material Transfer Agreements were established between the participating institutions, formalizing the collaborative framework for this study.

Patient consent statement:

The biological specimens utilized were sourced as routine components of patient care. These tissue samples were retrospectively gathered and pose no additional burden to the individuals involved, aligning with established ethical guidelines and standards.

Statement of the authors:

The work described has not been published previously, it is not under consideration for publication elsewhere and will not be published elsewhere in the same form, or in any other language, including electronically without the written consent of the copyright holder. The final version of this work was approved for publication by all parts included.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Authors contributions

All authors contributed equally and significantly to this work. Lady Paola Aristizabal Arboleda and Caique Mariano Pedroso provided crucial support in the statistical analysis and the interpretation of the results in the final version of this manuscript.

Abstract

Background: Rhabdomyosarcoma, a rare malignant tumor, frequently affects pediatric patients, with 35-40% occurring in the head and neck. This study analyzes the clinicopathologic profile of pediatric head and neck rhabdomyosarcomas from Brazil, Guatemala, Mexico, and South Africa. **Methods:** We reviewed 44 cases from ten Oral and Maxillofacial Pathology services, conducting immunohistochemical analyses of Desmin, Myogenin, Myo-D1, and Ki67, with quantification via QuPath software. Cases with ≥50% Myogenin expression were tested for fusion status using AP2β, NOS-1, and HMGA2. Statistical analyses included the Kruskal-Wallis test for age and marker expression comparisons, Fisher's exact test for categorical variables, Spearman's rank correlation for marker relationships, and multinomial logistic regression to assess fusion status likelihood. **Results:** Cases were predominantly from Brazil (40.9%), followed by South Africa (27.3%), Guatemala (22.7%), and Mexico (9.1%). Two-thirds of patients were diagnosed in their first decade with no gender predilection. Non-parameningeal sites (45.5%) were more affected than parameningeal (40.9%) and orbital sites. Microscopically, embryonal rhabdomyosarcoma (77.3%) was most common, followed by alveolar (18.2%) and spindle cell (2.3%) tumors. Immunohistochemistry revealed positivity for myogenic markers, with significant differences in Myogenin expression between embryonal and alveolar RMS variants ($p<0.05$). Fusion status prediction identified two potential fusion-positive alveolar RMS cases, while all embryonal RMS and one alveolar RMS case appeared fusion-negative. Significant correlation with positive fusion status was found only between AP2β and NOS1 ($p < 0.05$). **Conclusion:** Although there are slight clinical-demographic variations among pediatric head and neck rhabdomyosarcomas in these regions, identifying fusion status through immunohistochemistry remains a diagnostic challenge.

Keywords: Rhabdomyosarcoma; head and neck; pediatric; molecular; oral cavity

1. INTRODUCTION

Rhabdomyosarcoma (RMS), a prevalent pediatric soft tissue sarcoma (STS), originates from primitive mesenchymal cells with myogenic differentiation [1]. It affects ~4.5 individuals per million aged <20 years, constituting about 5% of all pediatric malignancies [2,3].

RMS frequently arises in the head and neck region (HNR), where it can be categorized into orbital, parameningeal (PM), and non-parameningeal (NPM) subtypes [4]. Histopathologically, the WHO classifies RMS into embryonal, alveolar, spindle cell/sclerosing, and pleomorphic types [5], with genetic alterations further categorizing RMS into *PAX3/7–FOXO1* fusion-positive or fusion-negative types, as well as spindle cell/sclerosing RMS with *MYOD1* mutations or rearrangements involving *VGLL2/NCOA2* or *TFCP2/NCOA2* genes [3,5,6].

Decades of basic research and clinical studies developed by European and North American international collaborative groups have contributed to a better understanding of RMS pathophysiology and helped optimize clinical care [2,7]. However, some challenges remain despite improvements in RMS treatment and prognosis [8]. A recent study in Central America highlighted disparities in therapeutic standards between high-income countries (HIC) and low-middle-income countries (LMIC), emphasizing the need for further research into RMS profiles in LMIC [9]. Therefore, this study aimed to describe the clinicopathological and immunohistochemical features of pediatric head and neck rhabdomyosarcomas (HNRMS) from Brazil, Guatemala, Mexico, and South Africa.

2. MATERIALS AND METHODS

This retrospective observational study followed the 1964 Helsinki Declaration principles to protect patient privacy and ensure data confidentiality. Ethical approvals were obtained from the Research Ethics Committees at Piracicaba Dental School (Ref No. 12469119.8.0000.5418), University of Pretoria (Ref No. 483/2020), and the University of Liverpool (Ref No. 12077). Material Transfer Agreements were established to formalize collaboration.

The study included pediatric patients (≤19 years) with confirmed HNRMS diagnoses from Oral and Maxillofacial Pathology (OMFP) archives in ten institutions (seven from Brazil and one each from Guatemala, Mexico, and South Africa). Exclusion criteria comprised cases without available material for analysis, tumors affecting the central nervous system (CNS), or rhabdomyosarcomas diagnosed as metastatic deposits in the HNR.

Demographic and clinical data were collected from histopathological requests or medical charts by designated researchers at each institution. RMS classification followed WHO 2020 criteria [5] using hematoxylin and eosin-stained (HE) slides, supplemented by immunohistochemical markers (Desmin, Myogenin, MyoD1, Ki-67). Additional immunohistochemical reactions were conducted on 3µm tissue sections as needed, adhering to outlined protocols (see **Supplement 1**).

Immunostaining patterns were evaluated using a scale from 0 to 4+: (0) absent, (1+) <10%, (2+) 10-49%, (3+) 50-90%, and (4+) >90% expression [10]. Ki67 proliferation index was categorized as low (<10%), moderate (10–29%), or high (≥30%)[11]. HNRMS with high myogenin expression (3+; 4+) underwent gene fusion status prediction using an algorithm by Rudzinski et al. [10], involving AP2β (1:50, Sigma-Aldrich), NOS-1 (1:100, Sigma-Aldrich), and HMGA2 (1:50, Sigma-Aldrich) immunohistochemical markers. Additionally, some embryonal RMS cases with available tissue sections were evaluated for p53 (ready to use, Agilent Technologies). Immunohistochemical reactions were performed on 3µm tissue sections using a BOND RX automated Stainer (Leica Biosystems).

For fusion status prediction, higher NOS-1 and/or AP2β (3 to 4+) with lower HMGA2 (0 to 2+) indicated 'fusion-positive' RMS (FPRMS), while lower NOS-1 and/or AP2β compared to HMGA2 favored 'fusion-negative' RMS (FNRMS). When NOS-1 and AP2β were discrepant, the higher score was used [10].

For immunohistochemical quantification, slides were scanned at 20× magnification with Aperio Scan Scope CS Slide Scanner (Aperio Technologies Inc., Vista, CA), generating whole slide images (WSI). QuPath Bioimage analysis v0.2.0-m8 (University of Edinburgh, Scotland, UK) [12] was employed for automated cell counting and data extraction, following a protocol proposed by Pai et al. [13] and adapted for this study (**Supplement 2**). Mean positive expression for each marker was calculated using Microsoft Excel.

Statistical analyses used the Kruskal-Wallis test for age and marker expression comparisons. Significant differences led to post-hoc analyses to identify specific group variations. Fisher's exact test was used for categorical variables (sex, location, sublocation, and variant type), and Spearman's rank correlation coefficient was calculated to explore relationships among the expression levels of desmin, myogenin, MyoD1, Ki67, AP2β, NOS1, and HMGA2. Multinomial logistic regression assessed fusion status likelihood based on AP2β, NOS1, and HMGA2 expression levels. All analyses were conducted using R Statistical Software (v4.2.1), with significance set at $p < 0.05$ for all tests.

3. RESULTS

Between 1998 and 2023, 49 pediatric HNRMS cases were identified. After excluding two metastatic HNRMS, two CNS tumors, and one with unavailable tissue, 44 HNRMS were included. Brazil had the highest prevalence (40.9%), followed by South Africa (27.3%), Guatemala (22.7%), and Mexico (9.1%). Most patients (62.8%) were <10 years old with a median age of 8.1 years and no gender predilection noted.

NPM sites were most commonly affected (45.5%) with common locations in the oral cavity, masseter and parotid region. Interestingly, 18.2% of all HNRMS included were oral RMSs predominantly located in the buccal mucosa (3/8) and tongue (3/8). PM sites were also involved in 40.9% of cases being the maxillary sinus and nasal cavity often affected. Orbital RMS were observed in 11.4% of cases, and one case referred as head and neck location without specification.

Clinical manifestations included facial asymmetry in 38.6% (17/44) of cases, often caused by painful (5/17; 29.4%) or asymptomatic (2/17; 11.8%) swelling (**Figure 1A-C**). Other symptoms included snoring, nasal obstruction, bleeding, ulceration, necrosis, and tooth mobility. Tumor sizes was available in 16 (36.4%) HNRMS, ranged from 1.8 to 14 cm (mean: 6 cm), with a majority (56.3%) being ≥5 cm. Symptom duration varied from 2 weeks to 8 months (mean: 3.1 months) in 25% of cases.

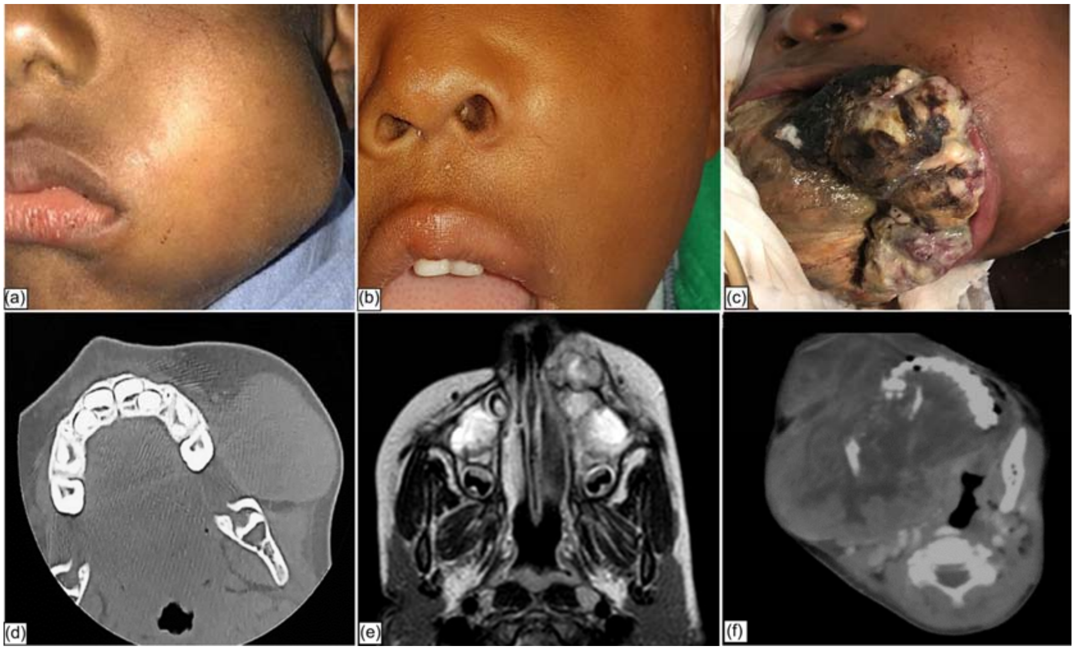

Figure 1. **Clinical and imaging features of HNRMS: a, b.** two male patients presenting facial asymmetry due to marked and discrete swellings, respectively; **c**. extensive mass presenting an ulcer-necrotic surface; **d-f.** imaging findings analysis of computerized tomography in each case, showing an expansive destructive mass invading adjacent tissue.

Imaging studies (CT or MRI) described in some cases showed large lesions causing invasion and destruction of adjacent structures (**Figure 1D-F**). One patient had a history of previous radiotherapy for neuroblastoma before the PM RMS diagnosis.

Microscopic examination classified HNRMS predominantly as embryonal (34; 77.3%) and alveolar (8; 18.2%). Additionally, there was one case each of spindle cell RMS and mixed RMS. Immunohistochemical analysis confirmed positivity for myogenic markers (Desmin, Myogenin, and/or MyoD1) in all cases.

Therapeutic approaches and outcomes were available for 27.9% (12/44) of cases from two hospitals in Brazil. Treatment typically involved combined chemotherapy and radiotherapy (66.7% of cases), with some patients receiving chemotherapy and/or radiotherapy before surgery. One patient received chemotherapy alone. Four patients developed metastases, and one experienced local recurrence two years post-treatment. At the last follow-up (mean: 41.2 months), 66.7% of patients were alive (8/12), while 33.3% had passed away (4/12).

Histopathologic variants comparison and statistical analysis results

Table 1 presents demographic and clinicopathological data for HNRMS by histopathological variant. The median age for embryonal RMS was 8.2 years (range 1-19), with a higher prevalence in females and most tumors (45.5%) located in NPM tissues, particularly the oral cavity (7 cases). Orbital tumors were exclusive to embryonal RMS. Alveolar RMS had a median age of 7.6 years (range 1-13), were more common in males, and 62.5% were located in PM tissues, including the nasal cavity, mastoid, maxillary sinus, and infratemporal fossa. A single spindle cell RMS case involved a 2-year-old female with an NPM location, while a mixed RMS case was a 13-year-old male with PM RMS. Statistical analysis revealed no significant differences in age ($p = 0.40$), sex distribution ($p = 0.57$), tumor location ($p = 0.63$), or sub-location ($p = 0.12$) among the histopathologic variants.

Table 1. Demographic and clinicopathologic features of 44 HNRMS in pediatric patients.

	Histopathological variants				
	Embryonal RMS n= 34 (%)	**Alveolar RMS n= 8 (%)**	**Spindle cell RMS n= 1 (%)**	**Mixed RMS* n= 1 (%)**	**p-value**
Demographic variables					
Median age (range)	8.2 (1 – 19)	7.6 (1 – 13)	2.0	13.0	p=0.40[a]
Sex					p=0.57[b]
Male	16 (47.1)	5 (62.5)	0	1 (100)	
Female	18 (52.9)	3 (37.5)	1 (100)	0	
Clinicopathologic variables					
Tumor location					p=0.63[b]
Non - parameningeal	16 (45.5)	3 (37.5)	1 (100)	-	
Parameningeal	12 (36.4)	5 (62.5)	-	1 (100)	
Orbit	5 (15.1)	-	-	-	
H&N without specification	1 (3)	-	-	-	
Sub location					p=0.12[b]
Masseter	4 (11.8)	1 (12.5)			
Face	0	1 (12.5)			
Pterygopalatine fossa	2 (5.8)	0		-	
Mastoid	1 (2.9)	1 (12.5)			
Maxillary sinus	5 (14.7)	1 (12.5)	-		
Nasal cavity	1 (2.9)	1 (12.5)		1 (100)	
Nasopharyngeal	2 (5.8)	0			
Neck	1 (2.9)	1 (12.5)			
Oral cavity	8 (23.5)		1 (100)		
Orbit	5 (14.7)				
Parotid	2 (5.8)	-		-	
Head and neck	1 (2.9)		-		
Pharyngeal	1 (2.9)				
Infratemporal fossa	1 (2.9)	2 (25.0)			
Histopathologic morphology					p=2.14[b]
Anaplastic	5 (14.7)	-			
Botryoid	3 (8.8)	-			
Classic	-	6 (75.0)			
Solid	-	2 (25.0)	-	-	
Dense	7 (20.6)	-			
Hypocellular and hypercellular areas in mixed stroma	19 (55.9)	-			
Mixed	-	-		1 (100)	
Spindle cell	-	-	1 (100)		

[a]*Kruskall-Wallis;* [b]*Fisher test*
Notes: *RMS showing mixed alveolar and embryonal features
Abbreviations: RMS= rhabdomyosarcoma

Microscopically, 55.8% (19/34) of embryonal RMS cases displayed hypercellular and hypocellular areas with primitive stellate cells or muscle differentiation mixed with undifferentiated round cells. Additionally, 20.6% (7/34) exhibited dense patterns of undifferentiated round-to-oval cells, 14.7% (5/34) showed anaplastic features, and 8.8% (3/34) had botryoid morphology. Rhabdomyoblastic differentiation was present in 61.8% (21/34) of cases, and necrotic areas were observed in 55.8% (19/34) (**Figures 2A-F**).

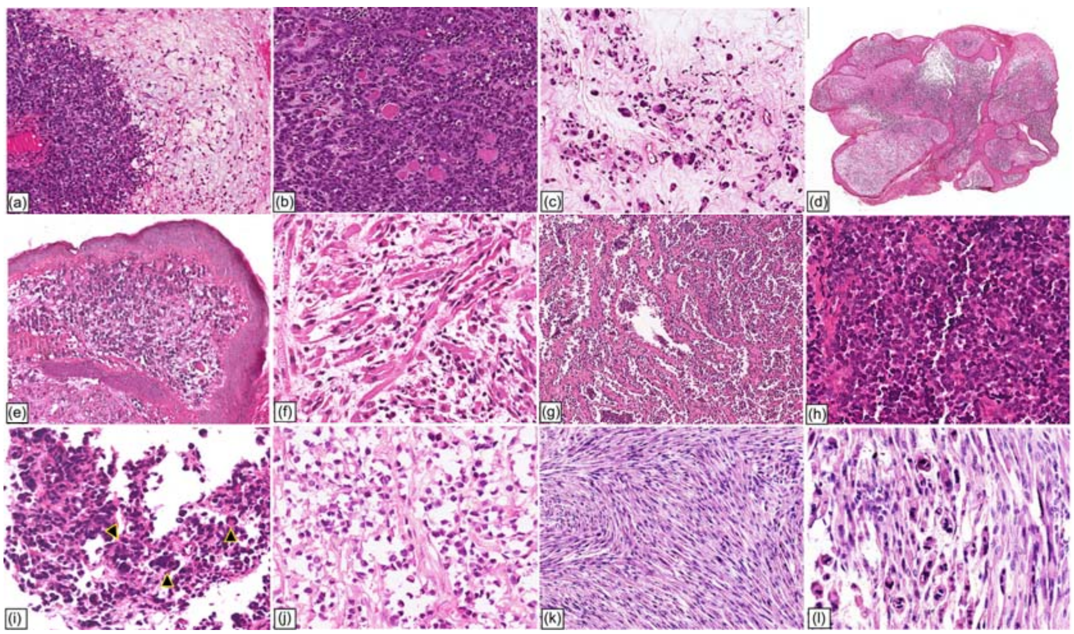

Figure 2. **Microscopic features of HNRMS:** *Embryonal RMS* **a.** hypercellular and hypocellular areas of primitive stellate and small round cells arranged within a fibro-myxoid stroma (HE 5X); **b.** dense pattern of primitive undifferentiated round-to-oval cells (HE 10X); **c**. tumor cells showing anaplastic features (HE 10X); **d.** botryoid morphology in a low-power view (HE); **e.** "*cambium layer"* – a hypercellular zone immediately beneath the epithelial surface (HE 10X); **f.** rhabdomyoblasts with variable grades of differentiation exhibiting eosinophilic cytoplasm (HE 20X). *Alveolar RMS* **g.** classic (HE 5X) and solid (**h;** HE 10X) patterns, **i**. Multinucleated tumor cells signalized with head arrows (HE 20X); **j.** rhabdomyoblasts with dark hyperchromatic nuclei and scant eosinophilic cytoplasm (HE 20X). *Spindle cell RMS* **k.** spindle

cells arranging in fascicles (HE 10X); **l.** some tumor cells with rhabdomyoblastic differentiation exhibiting pleomorphic hyperchromatic nuclei (HE 20X).

In contrast, alveolar RMS cases (6/8) primarily displayed the classic alveolar pattern with small round cells arranged around fibrovascular septa, while two cases showed a solid variant. Notable features included tumor-giant cells and plump rhabdomyoblasts with hyperchromatic nuclei and scant cytoplasm (**Figures 2G-J**). All alveolar RMS cases exhibited varying degrees of necrosis. The spindle cell RMS was characterized by partial encapsulation with fascicles of elongated spindle cells surrounded by fibrous stroma (**Figure 2K**). Scattered rhabdomyoblasts, some pleomorphic cells (**Figure 2L**), and focal necrosis were also observed. Statistically, no significant difference in histopathologic patterns was found among the variants ($p = 2.14$).

Table 2 and **Figure 3** detail the immunophenotype of HNRMS. Desmin showed positive cytoplasmic staining in nearly all cases. Myogenin expression was moderate and heterogeneous in embryonal tumors but consistently high in alveolar RMS. MyoD1 expression varied in both types. The Ki67 proliferation index was significantly higher in alveolar RMS (62.7%) compared to embryonal RMS (47%). The spindle cell tumor was positive for Desmin, SMA, vimentin, HHF-35, myogenin, and Myo-D1, and negative for S100, AE1-AE3, ALK, and H-Caldesmon, with a Ki67 index of 3%. Statistical analysis showed a significant difference in Myogenin expression between alveolar and embryonal variants ($p = 0.01589$), though differences in Desmin, MyoD1, and Ki67 expressions were not statistically significant (**Figure 4A**).

Table 2 -Immunohistochemical features of 44 HNRMS in pediatric patients

Histopathologic variant	**Embryonal (n=34)**	**Alveolar (n=8)**	**Spindle cell (n=1)**	**Mixed (=1)**	**p-value** [a]*Kruskall-Wallis;* [b]*Fisher*
Immunohistochemical features					
Desmin					
Mean of expression	52.5	59.6	71.6	64.9	p=0.80[a]
Negative	3 (8.8)	1 (11.1)	-	-	
Low (1+)	1 (2.9)	-	-	1 (100)	
Moderate (2+)	13 (38.2)	1 (11.1)	-	-	
High (3+); (4+)	17 (50.0)	6 (75.0)	1 (100)	-	
Myogenin	**n= 33 (%)**				
Mean of expression	37.1	72.2	49.3	92.0	**p=0.007**[a]
Negative	4 (12.1)	-	-	-	
Low (1+)	2 (6.1)	-	-	-	
Moderate (2+)	17 (51.5)	-	1 (100)	1 (100)	
High (3+); (4+)	10 (30.3)	8 (100)	-	-	
MyoD1	**n= 33 (%)**	**n= 7 (%)**			
Mean of expression	47.6	31.1	0.0	91.4	p=0.10[a]
Negative	3 (9.1)	1 (12.5)	-	-	
Low (1+)	3 (9.1)	2 (25)	-	-	
Moderate (2+)	13 (39.3)	3 (37.5)	-	1 (100)	
High (3+); (4+)	14 (42.4)	1 (25)	1 (100)	-	
Ki67		**n= 7 (%)**			
Mean of expression	46.2	54.8	3.2	52.5	p=0.39[a]
Negative	1 (2.9)	-	-	-	
Low <10%	3 (8.8)	-	1 (100)	-	
Moderate 10 – 29%	9 (26.5)	-	-	1 (100)	
High ≥ 30%	21 (61.7)	7 (100)	-	-	
AP2β					
Mean of expression	2.0	18.4	-	16.9	p=0.43
NOS-1					
Mean of expression	3.6	17.9	-	78.3	p=0.34
HMGA2					
Mean of expression	50.5	11.0	-	98.0	p=0.08

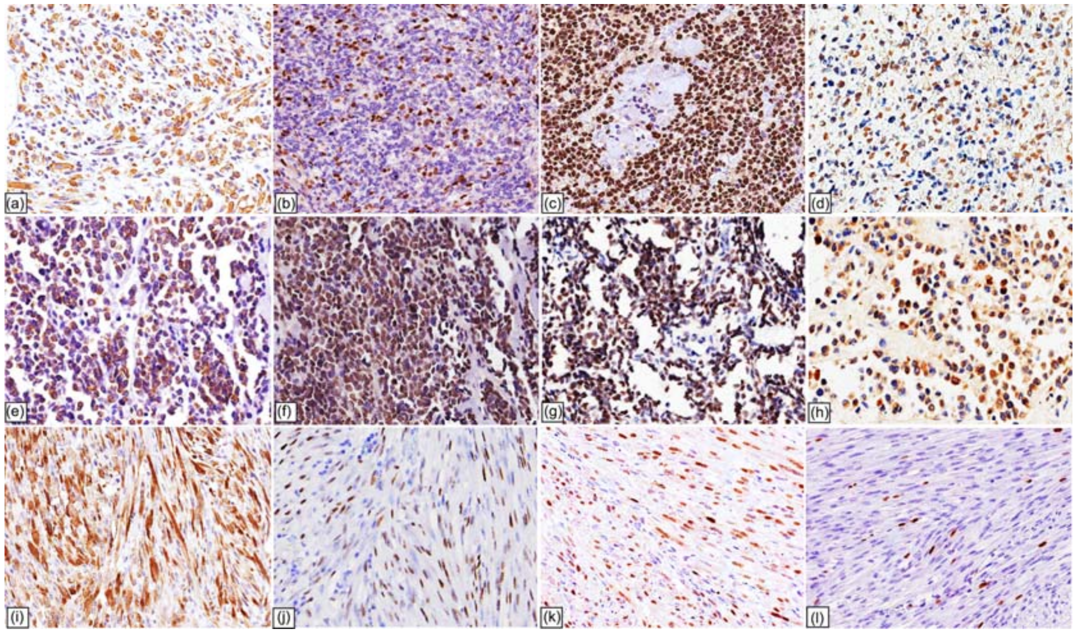

Figure 3. Immunohistochemical features of HNRMS: *Embryonal RMS* **a.** diffuse cytoplasmatic stain for desmin (100X magnification); **b.** positivity for myogenin (100X magnification) and Myo-D1 (**c**; 100X magnification) with nuclear staining patterns; **d.** HMGA2 showing nuclear expression. *Alveolar RMS* **e.** tumor cells showing cytoplasmatic positivity for desmin (100X magnification); **f.** strong and diffuse nuclear myogenin expression (100X magnification); **g**. FPRMS immunophenotype determined by Ap2β (100X magnification) and NOS-1 (**h**; 100X magnification) expressions. *Spindle cell RMS* showing strong and diffuse cytoplasmatic expression for desmin (**i**; 100X magnification); **j.** nuclear expression of MyoD1 and myogenin (**k**; 100X magnification); **l.** low Ki67 expression (100X magnification).

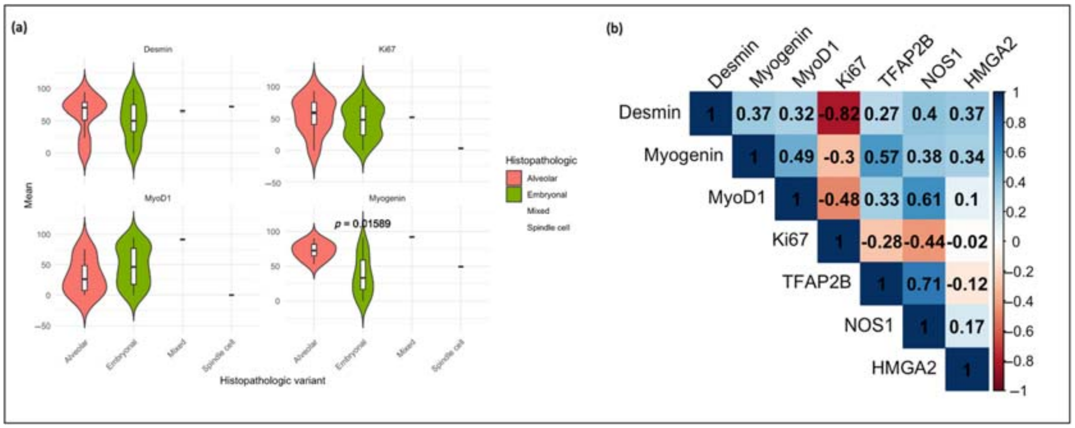

Figure 4. Statistical analysis correlations: a. Violin Plot illustrating the distribution of markers expression across the histopathological variants; **b. Spearman correlation matrix** exhibiting correlations between immunohistochemical expressions of different markers, with coefficients shown through color intensity.

Additionally, immunohistochemical expression of p53 was assessed in 18/34 embryonal RMS cases. Tumors with dense patterns of small, round cells had a mean p53 expression of 47.1%, while those with more rhabdomyoblastic cells showed 29.5%. Botryoid RMS had a mean expression of 15.1%, and anaplastic RMS had 85.8%. Four cases, including one botryoid and one anaplastic subtype, were negative.

To predict gene fusion status, 10 HNRMS with high Myogenin expression were evaluated for Ap2β, NOS-1, and HMGA2; including five alveolar RMS, four embryonal RMS, and one mixed RMS. Two alveolar RMS cases showed higher Ap2β/NOS-1 expression compared to HMGA2, suggesting potential FPRMS. Conversely, all embryonal RMS cases and one alveolar RMS case had profiles compatible with FNRMS. The mixed RMS case displayed variable expressions of Ap2β (2+), NOS-1 (3+), and HMGA2 (3+), and 2/5 alveolar RMS cases were negative for these markers, leading to inconclusive results.

Spearman correlation analysis revealed notable correlations, as shown in **Figure 4B**. AP2β exhibited a moderate positive correlation with Myogenin ($r = 0.57$) and NOS1 ($r = 0.71$). A statistically significant correlation was observed specifically between AP2β and NOS1 ($p < 0.05$).

Multinomial logistic regression analysis revealed that higher AP2β expression significantly increased the likelihood of a positive fusion status (Coefficient = 1.092, Probability = 0.40) and decreased the likelihood of a negative status (Coefficient = -1.27, Probability = 0.28). Elevated NOS-1 expression similarly correlated with a positive fusion status (Coefficient = 1.142, Probability = 0.41) and a negative status (Coefficient = -0.962, Probability = 0.32). The model demonstrated a strong fit with a residual deviance of 0.0001873208 and an AIC of 28.00019, highlighting its effectiveness in explaining variation in fusion status based on immunohistochemical expression levels.

4. DISCUSSION

RMS represents ~50% of pediatric STS, with HNR affected in ~40% of cases [2,3]. Due to RMS's relative rarity, single institutional experience is usually limited [7,14] emphasizing the need for multicenter collaborative research to comprehensively understand this neoplasm [7,9]. To our knowledge, our series of 44 HNRMS represents the first international collaboration analyzing pediatric HNRMS profile in LMICs, including Latin America (Brazil, Guatemala, Mexico) and South Africa, where healthcare resources and access to advanced diagnostic tools are limited.

The age distribution, with a higher prevalence of HNRMS in children <10 years old, is consistent with existing literature [3,4]. However, the lack of gender predilection contrasts with some studies that have reported a slight male predominance [4,15,16]. While previous reports from both LMICs and HICs indicate a predominance of PM locations

[4,9,14,17], making NPM RMS account for only 16-19% of localized tumors[18], our cohort exhibited a predominance of NPM location. Notably, our study identified a higher proportion of oral RMS cases (18.2%) compared to previous studies, which typically find approximately 10%–12% of HNRMS affecting the oral cavity [16,19,20]. Hence, it is important to highlight the need for increased awareness among clinicians and pathologists in LMICs to avoid diagnostic delays and ensure timely treatment.

Most HNRMS in this study presented as painful swellings measuring ≥5 cm, causing facial asymmetry, with a mean symptom duration of 3 months. These findings vary across studies [3,16,17,21], likely due to differences in sample size and data availability. The predominance of tumor size ≥5 cm may suggest an advanced stage at diagnosis. Early-stage RMS signs, such as facial pain, sinonasal congestion, ear pain, and asymptomatic swellings, can mimic benign conditions, leading to misdiagnosis and delayed treatment [1-3,17]. Given the prevalence of benign, inflammatory, and infectious diseases in the pediatric population, healthcare professionals should consider RMS in their differential diagnosis, especially when children present with these symptoms [17].

Embryonal RMS is the predominant subtype in pediatric patients, followed by alveolar RMS [1,2,4]. The first, typically shows two peaks in incidence—early childhood and adolescence—while alveolar RMS primarily affects adolescents [2-6]. Our findings, consistent with LMICs and HICs studies [4,14-18], confirm that embryonal RMS predominates in HNRMS cases, with >70% of tumors being embryonal subtype. Specifically, 41.2% of our cases occurred in children aged 1–5 years, and 32.4% in adolescents. Conversely, the limited number of alveolar RMS cases in this series likely explains the absence of a higher adolescent prevalence for this variant.

Besides embryonal and alveolar RMS, the spindle cell/sclerosing RMS, which predominantly affects young children, particularly those ≤1 year old, represents a small percentage (5-10%) of cases and is commonly found in the H&N region [3,22,23]. In our study, this subtype accounted for 2% of all HNRMS analyzed. Diagnosis can be challenging due to its similarity to other spindle cell neoplasms, necessitating immunohistochemistry for accurate identification [22,23].

Microscopically, all variants described in this series exhibited classic morphological features outlined in the latest WHO classification[5]. The immunohistochemical profile of our cases closely adheres to established diagnostic criteria, with Desmin, Myogenin, and MyoD1 serving as reliable markers for RMS [3,24,25]. Statistical analysis highlighted significant differences in Myogenin expression, confirming its utility in distinguishing between alveolar and embryonal RMS [6].

Incorporating molecular features into risk assessment and treatment decisions remains a significant challenge in managing rare tumors like RMS [26]. About 70-80% of alveolar RMS feature *PAX3/7–FOXO1* fusion genes, which distinguish between fusion-FPRMS and FNRMS subtypes. FPRMS, marked by this fusion, tends to exhibit more aggressive behavior [2,6,10,27]. Therefore, the Children's Oncology Group (COG) and European Pediatric Soft Tissue Sarcoma Study Group (EpSSG) have integrated *FOXO1* fusion status into their protocols, enhancing risk evaluation and guiding tailored treatment approaches [26,27].

Previous research has established AP2β and/or NOS-1 and HMGA2 as reliable markers for differentiating between FPRMS and FNRMS with high specificity (>90%) and moderate sensitivity (>60%).[10,25,28,29]. In our study, we applied these markers in immunohistochemical assays to predict the gene fusion status of 10 HNRMS cases. Our analysis identified two potential FPRMS cases among the alveolar tumors, based on high AP2β and/or NOS-1 expression. In contrast, all embryonal RMS cases and one alveolar RMS case exhibited elevated HMGA2 levels, consistent with FNRMS. Multinomial logistic regression further confirmed that increased AP2β and NOS-1 expression was significantly associated with a positive fusion status, while a negative status was less likely. However, the small sample size limits the study's generalizability, and results should be interpreted with caution.

It is well-established that most embryonal tumors are FNRMS [2,3,6]. Hence, HMGA2 emerges as a candidate marker for identifying FNRMS[10,29], given its consistent high expression in all embryonal RMS tested in this study. Furthermore, HMGA2 has also has been proposed as a potential therapeutic target due to its oncogenic role in previous studies [29].

Identifying gene fusions in RMS typically involves RT-PCR and FISH, which are considered gold standards but can be limited by factors like tissue quality/quantity and high costs, particularly in most OMFP laboratories in LMICs[10]. Hence, immunohistochemical assays using surrogate markers for identifying FPRMS and FNRMS offer a practical, cost-effective alternative [10,28]. However, our reliance on non-molecular tests to confirm findings underscores a study limitation. Validation of these markers across various studies, including ours, is essential before widespread adoption as reliable predictors of fusion status [10,28,29]. Despite limitations, using surrogate markers in immunohistochemical assays provides a feasible option for diagnosing RMS in LMICs lacking routine access to molecular testing. Further research into these markers is crucial to improve diagnostic accuracy and refine treatment strategies based on molecular subtypes of RMS.

Regarding RMS treatment and prognosis, a combination of chemotherapy and radiotherapy was the prevalent treatment modality for HNRMS in the current series, with the majority of patients being alive at the last follow-up. It has been described that anatomical limitations in HNRMS could prevent complete surgical resection, especially in advanced-stage tumors [17,30], potentially explaining the treatment strategy adopted for these patients.

While our study provides valuable insights, its retrospective design and use of archival materials limit the completeness of clinical data, particularly concerning treatment outcomes and long-term follow-up. The small sample size also impacts the statistical analysis and generalizability of our findings. Future research with larger, prospective cohorts is necessary to validate these results and better understand how fusion status affects treatment response and prognosis in pediatric HNRMS, especially in LMICs. Enhancing diagnostic accuracy, improving access to molecular testing, and fostering research collaborations are essential steps for addressing current limitations and improving clinical management of pediatric HNRMS.

In summary, our study analyzes pediatric HNRMS from Brazil, Guatemala, Mexico, and South Africa, emphasizing the prevalence of embryonal RMS and the potential of immunohistochemistry for fusion status identification as a valuable tool in the diagnostic armamentarium. Currently, AP2β, NOS-1 and HMGA2 appear to play predictive roles in recognizing RMS fusion status, but further studies are required to validate them as surrogate markers.

Acknowledgments

We extend our gratitude to Roman Carlos †, a brilliant clinician and prestigious oral pathologist from Guatemala, who made valuable contributions to this study. His dedication is remembered with appreciation.

5. REFERENCES

1- Dasgupta R, Fuchs J, Rodeberg D. Rhabdomyosarcoma. Semin Pediatr Surg. 2016; 25:276-83.

2- Skapek SX, Ferrari A, Gupta AA, Lupo PJ, Butler E, Shipley J, et al. Rhabdomyosarcoma. Nat Rev Dis Primers. 2019; 5:1-19.

3- Leiner J, Le Loarer F. The current landscape of rhabdomyosarcomas: an update. Virchows Arch. 2020; 476:97-108.

4- Turner JH, Richmon JD. Head and neck rhabdomyosarcoma: a critical analysis of population-based incidence and survival data. Otolaryngol Head Neck Surg. 2011; 145:967–73

5- World Health Organization (WHO). WHO Classification of Tumours. World Health Organization classification of tumours: soft tissue and bone tumours. 5th ed. vol. 3. Lyon, France: IARC,2020.

6- Agaram NP. Evolving classification of rhabdomyosarcoma. Histopathology. 2022; 80:98-108.

7- Newton WA Jr, Webber B, Hamoudi AB, Gehan EA, Maurer HM. Early history of pathology studies by the Intergroup Rhabdomyosarcoma Study Group. Pediatr Dev Pathol. 1999; 2:275-85.

8- Curry SD, Jiang ZY, Jain KS. Population-based survival of pediatric rhabdomyosarcoma of the head and neck over four decades. Int J Pediatr Otorhinolaryngol. 2021; 142:110599

9- Garrido C, Letona T, Godoy S, Antillón F, Valverde P, Luna-Finemann S, et al. Rhabdomyosarcoma in low- and middle-income countries: A report from the Asociacion de Hemato-oncología Pediatrica de Centro América (AHOPCA). Pediatr Blood Cancer. 2023; 70:e29669.

10- Rudzinski ER, Anderson JR, Lyden ER, Bridge JA, Barr FG, Gastier-Foster JM, et al. Myogenin, AP2β, NOS-1, and HMGA2 are surrogate markers of fusion status in rhabdomyosarcoma: a report from the soft tissue sarcoma committee of the children's oncology group. Am J Surg Pathol. 2014; 38:654-9.

11- Tanaka K, Hasegawa T, Nojima T, Oda Y, Mizusawa J, Fukuda H, et al. Prospective evaluation of Ki-67 system in histological grading of soft tissue sarcomas in the Japan Clinical Oncology Group Study JCOG0304. World J Surg Oncol. 2016; 14:110

12- Bankhead P, Loughrey MB, Fernández JA, et al. Qupath: open sources software for digital pathology image analysis. Sci Rep. 2017; 7:16878.

13- Pai R, Karki S, Agarwal R, Sieber S, Barasch S. Optimal settings and clinical validation for automated Ki67 calculation in neuroendocrine tumors with open-source informatics (QuPath). J Pathol Inform. 2022; 13:100141.

14- Zhang Y, Zhang WL, Huang DS, Wang YZ, Hu HM, Mei YY, et al. Prognostic factors in children with head and neck rhabdomyosarcoma: A 12-year retrospective study. Brain Behav. 2020;10:e01697.

15- Hicks J, Flaitz C. Rhabdomyosarcoma of the head and neck in children. Oral Oncol. 2002; 38:450-9.

16- Andrade CR, Takahama Junior A, Nishimoto IN, Kowalski LP, Lopes MA. Rhabdomyosarcoma of the head and neck: a clinicopathological and immunohistochemical analysis of 29 cases. Braz Dent J. 2010; 21:68-73.

17- Reilly BK, Kim A, Peña MT, Dong TA, Rossi C, Murnick JG, et al. Rhabdomyosarcoma of the head and neck in children: review and update. Int J Pediatr Otorhinolaryngol. 2015; 79:1477-83.

18- Orbach D, Mosseri V, Gallego S, Kelsey A, Devalck C, Brenann B, et al. Nonparameningeal head and neck rhabdomyosarcoma in children and adolescents: Lessons from the consecutive International Society of Pediatric Oncology Malignant Mesenchymal Tumor studies. Head Neck. 2017;39(1):24-31.

19- Tandon A, Sethi K, Pratap Singh A. Oral rhabdomyosarcoma: A review. J Clin Exp Dent. 2012; 4:e302-8.

20- Nunes MM, da Costa AAS, Tavares TS, Aguiar MCF, Martins CC, Caldeira PC. Rhabdomyosarcoma of the oral cavity in children aged 0-2 years: A scoping review. J Oral Pathol Med. 2023; 52:468-75.

21- Simon JH, Paulino AC, Smith RB, Buatti JM. Prognostic factors in head and neck rhabdomyosarcoma. Head Neck. 2002; 24:468-73.

22- Carroll SJ, Nodit L. Spindle cell rhabdomyosarcoma: a brief diagnostic review and differential diagnosis. Arch Pathol Lab Med. 2013; 137:1155-8.

23- 23- Wang Y, Li J, Tian Z, Zhu Y. Clinicopathologic features and molecular spectrum of spindle cell and sclerosing rhabdomyosarcomas in the head and neck region. Int J Clin Exp Pathol. 2018; 11:3436-44.

24- Dziuba I, Kurzawa P, Dopierała M, Larque AB, Januszkiewicz-Lewandowska D. Rhabdomyosarcoma in children - current pathologic and molecular classification. Pol J Pathol. 2018; 69:20-32.

25- Fan R, Parham DM, Wang LL. An Integrative Morphologic and Molecular Approach for Diagnosis and Subclassification of Rhabdomyosarcoma. Arch Pathol Lab Med. 2022; 146:953-9.

26- Shern JF, Selfe J, Izquierdo E, Patidar R, Chou HC, Song YK, et al. Genomic Classification and Clinical Outcome in Rhabdomyosarcoma: A Report From an International Consortium. J Clin Oncol. 2021;39(26):2859-71.

27- Owosho AA B Ch D, Huang SC Md, Chen S Mbbs, Kashikar S Dds, Estilo CL Dmd, Wolden SL Md, et al. A clinicopathologic study of head and neck rhabdomyosarcomas showing FOXO1 fusion-positive alveolar and MYOD1-mutant sclerosing are associated with unfavorable outcome. Oral Oncol. 2016; 61:89-97.

28- Machado I, Mayordomo-Aranda E, Giner F, Llombart-Bosch A. The Role of Immunohistochemistry in Rhabdomyosarcoma Diagnosis Using Tissue Microarray Technology and a Xenograft Model. Fetal Pediatr Pathol. 2015; 34:271-81.

29- Ouchi K, Miyachi M, Yagyu S, Kikuchi K, Kuwahara Y, Tsuchiya K, et al. Oncogenic role of HMGA2 in fusion-negative rhabdomyosarcoma cells. Cancer Cell Int. 2020; 20:192.

30- Dombrowski ND, Wolter NE, Robson CD, Kawai K, Irace AL, Vargas SO, et al. Role of Surgery in Rhabdomyosarcoma of the Head and Neck in Children. Laryngoscope. 2021; 131:E984-92.