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Microvessel density and cell proliferation in juvenile ossifying fibroma: A comparative study with central ossifying fibroma

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ABSTRACT

Considered as an aggressive counterpart of central ossifying fibroma (OF), juvenile ossifying fibroma (JOF) is a benign fibro-osseous neoplasm characterized by an unpredictable destructive behavior, elevated morbidity, mutilating treatment and high potential for local recurrences. The aim of this study is to compare the analysis for cell proliferation and vascular markers between JOF and OF. Cell proliferation index was measured by Ki-67 and Mcm-2 expression and microvessel density (MVD) was obtained by the immunoexpression of CD34/CD105. We observed a reduced expression of vascular markers, where MVD for CD34 was significantly higher in JOF than in OF ($p = 0.009$), but no statistical difference was found for CD105. JOF and OF showed low expression for Ki-67 and Mcm-2 and no difference was noted between both, suggesting that other mechanisms such as anti-apoptotic and/or pro-autophagic pathways or even increased expression of matrix metalloproteinases may be responsible for the aggressiveness of JOF.

1. Introduction

Juvenile ossifying fibroma (JOF) is an uncommon benign neoplasm characterized by progressive and destructive growing mainly in maxilla and paranasal bones of young patients and is considered as an aggressive counterpart of central ossifying fibroma (OF) [1-3]. OF is an expansive, well-circumscribed and slow growing osseous neoplasia found mainly in posterior areas of mandible in adults between the third and fourth decade of life [4,5].

In 2017, World Health Organization (WHO) [6] defined JOF as progressive and rapid expansive benign fibro-osseous neoplasm that rises in children and adolescents with age between 8.5 and 12 years and has a potential for local recurrence in 20 to 90% of cases. This destructive effect over cortical bone may cause severe morphological and functional defects in nasal cavity, orbits and eventually in the brain [7-11].

In some cases, these features may lead to a concern due clinical similarity with some malignant neoplasms as osteosarcoma [12-14].

JOFs are unencapsulated, well limited and produces variable amounts of calcified tissue as bone and/or cementum-like material that presents two distinct histological patterns: trabecular juvenile ossifying fibroma (TrJOF) and psammomatoid juvenile ossifying fibroma (PsJOF) [6,10,15].

TrJOF is frequently diagnosed in the maxilla of 8 to 12 years-old individuals while PsJOF generally affects sinonasal and orbital bones of 16 to 33 years-old patients [1,16].

Fibro-osseous lesions differential diagnosis may represent a true challenge, justifying the search for useful biomarkers in conflicting cases [17]. Despite studies aimed at clinical, radiographic and histopathological features, there are still few studies about biomarkers in JOF.

In order to understand the differences between JOF and OF, the present study performs a comparative analysis of cell proliferation and microvessel density (MVD) as biomarkers in JOF and OF, considering its clinic and radiographic features.

2. Materials and methods

Research Ethics Committee of the Sao Leopoldo Mandic Dental School and Research Institute approved this study. Our sample was composed by 11 JOF and 11 OF cases. All cases were retrieved from the files of the Department of Oral Pathology of São Leopoldo Mandic Research Center (Campinas/SP, Brazil). All diagnoses were reviewed by two pathologists (JAVGF and VCA) using 5-μm sections obtained from

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formalin-fixed, paraffin-embedded samples and routinely stained with hematoxylin-eosin. Data about age, sex, anatomic site of occurrence and radiographic features were obtained from these same files.

All documentation was strictly revised in each case to exclude the presence of other fibro-osseous lesions as fibrous dysplasia, focal and florid cemento-osseous dysplasia, syndrome associated lesions and metabolic diseases as Paget's bone disease.

To be included in this study, the lesions must be under the WHO's histopathological criteria [6] for JOF, that is a hypercellular fibroblastic stroma containing osteoid bone trabeculae (TrJOF) or presenting calcified psammoma-like material in a fusiform cell background (PsJOF).

2.1. Clinic and image study

From de image exams, we classified the cases according to the anatomic site of occurrence. For the mandible, we considered 3 regions: anterior (from right canine to left canine), posterior (from first premolar to third molar) and region of angle, ramus and condyle. Maxilla was divided in 2 regions: anterior (from right canine to left canine) and posterior (from first premolar to maxillary tuberosity).

Features as limits, density (radiolucent, radiopaque or mixed), uni or multilocular aspect, presence of “grounded glass” appearance and dental resorption were also registered. In cases were tri-dimensional reconstruction by computerized tomography was available, cortical bone destruction was evaluated.

2.2. Morphologic study

Besides histological classification in trabecular (TrJOF) or psammomatoid (PsJOF) subtype, the morphologic study considered the following features:

a. Nature of supporting stroma: highly cellular, cellular or fibrous.
b. Nature of osseous material: mature, immature or osteoid.
c. Nature of calcification: cementicles, ossicles or psammoma-like bodies.
d. Secondary alterations: associations with other lesions, as aneurysmal bone cyst (ABC), or presence of myxoid changes, hemorrhage or necrosis.

2.3. Immunohistochemistry

One paraffin block from each case was chosen for immunohistochemical study and antibodies against Ki-67 and Mcm-2 for cell proliferation, and CD34 and CD105 for blood vessel detection were used. Briefly, 3 μm sections were deparaffinized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide. For all antibodies, except for CD105, antigen retrieval was achieved by immersing the slides in boiling citrate buffer (pH: 6.0). Antigen retrieval for CD105 was performed using 0.4% pepsin for 30 min. Only the sections for CD105 were incubated at 37 °C with serum-free protein block (code x0909, Dako, AS, Denmark) for 30 min. Subsequently, for all antibodies the sections were incubated overnight at 4 °C with the primary antibody and then with the EnVision polymer HRP and EnVision+ (code K1491; DAKO, SA, Denmark) for 1 h at 37 °C. the sections were stained for 5 min at 37 °C with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. A negative control was achieved by omitting the primary antibody. No staining was observed in this section. Detailed information about the antibodies used in the study is compiled in the Table 1.

2.4. Microvessel density

The quantitation of tumor vascularity followed the method used by previous studies [18,19]. Immunohistochemical reactions for CD34 and CD105 were analyzed in the most vascularized areas (*hotspots*) selected at low power magnification. To access vessel number, images were obtained from 5 high power fields (×400 magnification, 0.44 field diameter) using a CCD camera adapted to an Olympus CX30 microscope. MVD was considered the mean number of intra-tumoral microvessel counted. There was no restriction on the size or presence of vascular lumen of a countable microvessel. Vessels with muscular walls were not counted.

Table 1
Details of the antibodies used for immunohistochemistry.

Specificity	Clone	Dilution	Source	Buffer (AR)
Ki-67	MIB-1	1:400	Dako[a]	Citrate
Mcm-2	CRCT2.1	1:15	Novocastra[b]	Citrate
CD34	QBEnd 10	1:50	Dako[a]	Citrate
CD105	SNG	1:10	Dako[a]	Pepsin

AR: antigen retrieval.
[a] Dako Corporation, Glostrup, Denmark.
[b] Novocastra, Newcastle upon Tyne, UK.

2.5. Cell proliferation

Immunohistochemical reactions for Ki-67 and Mcm-2 were interpreted by 2 authors (JAVGF and VAMM) using a double-headed microscope. Ki-67 and Mcm-2 immunoreactivity was semi-quantitatively evaluated in at least 500 cells examined in 5 higher power fields randomly selected under a microscope at 400× magnification and recorded as a percentage of Ki-67 or Mcm-2 positive tumor cells in the total number of cells examined in the same area.

2.6. Statistical analysis

Statistical differences for CD34, CD105, Ki-67 and Mcm-2 immunoexpression between JOF and OF were analyzed using the *Mann-Whitney* test at 5% level and performed on GraphPad Prism 6.

3. Results

3.1. Clinical and radiographic features

Among all cases of JOF, the age of impairment varied from 6 to 24 years-old, with a range of 6 to 16 years for TrJOF and 9 to 24 years for PsJOF. There was a male predilection in all cases (7 of 11 cases) and maxilla was the most affected site (8 of 11 cases), with 5 cases for TrJOF and 3 cases for PsJOF.

At image bank assessment, 4 cases unfortunately did not have available documentation. From the 7 remaining, all cases were present as unilocular lesions situated at posterior region of the jaws, being 5 cases in maxilla (1 of PsJOF and 6 of TrJOF) (Fig. 1A) and 2 cases in mandible (Fig. 1B).

Five of 4 cases were associated with a mixed radiolucent-radiopaque pattern, while 2 cases showed strictly radiolucent images. Destruction of the cortical bone was detected in 6 (Fig. 1C) cases, where 5 already involved maxillary sinus at radiographic or tomographic scans (Fig. 1D).

Although no one of the cases presented root resorption, absence of teeth was verified in 3 cases and dental dislocation of non-erupted teeth in 2 cases.

The age of impairment ranged from 17 to 53 years in OF series, with clear predilection for females and mandible as anatomic site (10 of 11 cases).

From the 8 OF cases with available image exams, 5 were located in posterior and 3 cases involved the anterior region of the jaws. Although all cases showed unilocular and well-defined limits, four cases

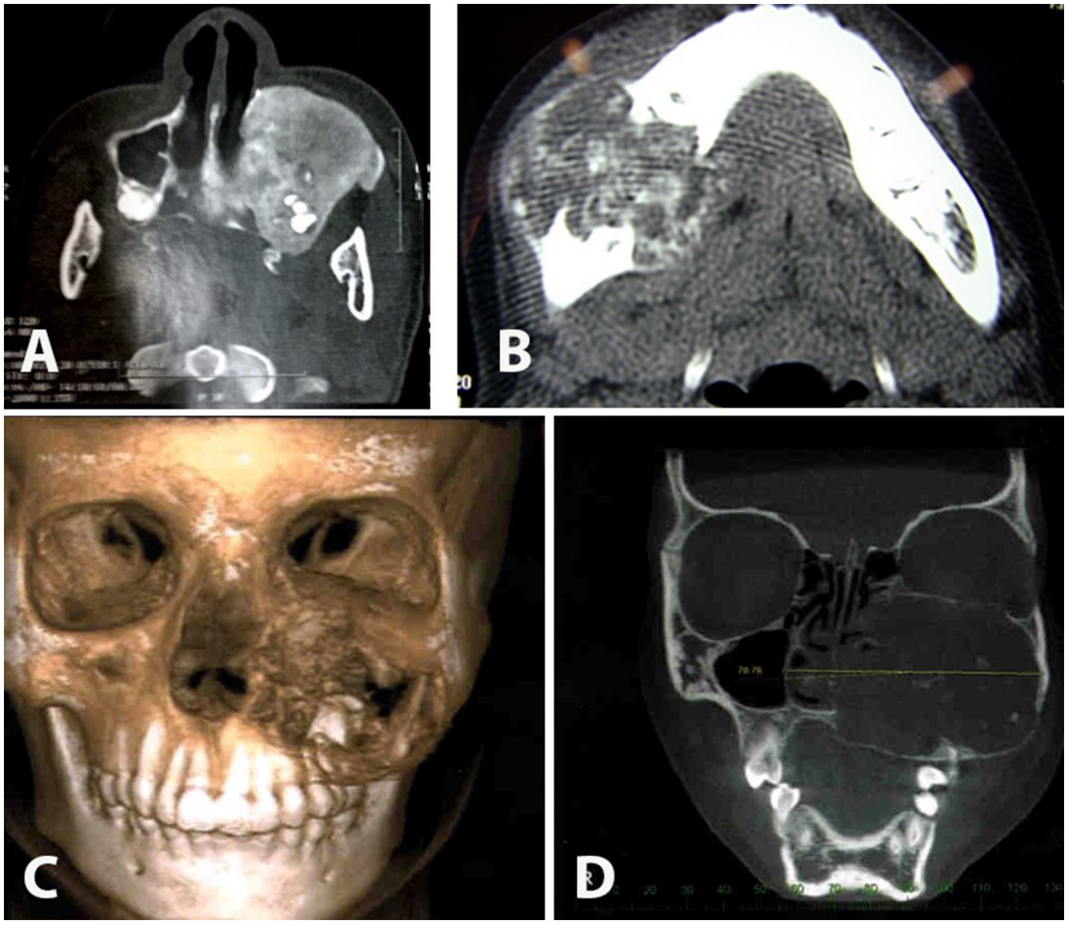

Fig. 1. Computerized tomography (CT) features of juvenile ossifying fibroma (JOF): mixed radiolucent-radiopaque pattern with bone cortical disruption in (A) maxilla and (B) mandible. Tri-dimensional reconstruction shows (C) extensive bone destruction in maxilla, while CT-scan evidence (D) wide involvement of sinonasal region.

presented mixed density and 4 showed radiolucent pattern. Association with root resorption was present in 2 cases and cortical disruption was observed in just one case.

3.2. Morphologic features – juvenile ossifying fibroma (JOF)

Microscopic analysis of the 11 JOF specimens revealed 8 TrJOF (Fig. 2A and C) and 3 PsJOF (Fig. 2B and D). Seven from 8 TrJOF cases presented a cellular stroma composed of cells of ellipsoid to fusiform nuclei with minimal collagen deposition, and 1 case exhibited high cellularity. All PsJOF cases exhibited high cellularity.

Immature pattern of mineralization was predominant and detected in 7 cases (4 of TrJOF and 3 of PsJOF) even with some sparse areas with osteoid pattern in 3 of these cases (2 of PsJOF and 1 of TrJOF). Osteoid pattern was present in 3 cases and mature pattern in just one case of TrJOF.

The nature of calcified material was very distinct, being present as ossicles in all TrJOF cases and psammoma-like bodies in the 3 cases of PsJOF showed. Mixed psammomatoid and ossicles areas were noted in one case of PsJOF.

Secondary alterations included hemorrhagic areas in 3 cases (2 of TrJOF, 1 of PsJOF), myxoid areas in 2 cases of TrJOF, presence of giant multinucleated cells in a cellular background in 4 cases (3 of TrJOF, 1 of PsJOF), microcystic degeneration of stroma fibroblasts in 2 cases of TrJOF and association with aneurysmal bone cyst in 2 cases (1 of TrJOF, 1 of PsJOF).

3.3. Immunohistochemistry

Immunohistochemical analysis was possible in 9 of 11 cases of JOF and in all 11 OF cases. There was a reduced expression for the vascular and proliferative markers. However, MVD for CD34 was significantly higher in JOF than in OF ($p = 0.009$) (Fig. 3A and B). There was no statistical difference for CD105 (Fig. 3C and D) ($p = 0.86$).

JOF and OF showed no difference for Ki-67 (Fig. 3E and F) and ($p = 0.12$) or Mcm-2 immunoexpression (Fig. 3G and H) ($p = 0.58$). Detailed data about immunoexpression in JOF and OF are compiled in Tables 2 and 3, respectively.

4. Discussion

JOF is a benign fibro-osseous neoplasia considered as a locally aggressive and high potential recurrence variant of OF that affects craniofacial skeleton of young patients with less than 15 years-old in most cases [3,21,22]. Our data showed a slight more advanced age of impairment for PsJOF (9 to 24 years) when compared to TrJOF (6 to 16 years), corroborating previous studies [3,5,15,20].

JOFs appeared as unilocular lesions with mixed radiographic density located in posterior region of gnathic bones associated to cortical bone destruction with clear predilection for maxilla and confirmed the tendency of PsJOF to involve paranasal sinuses and facial skeleton mentioned in the literature [11,16,23,24]. OF cases manifested mainly as unilocular radiolucent lesions in the posterior region of the mandible

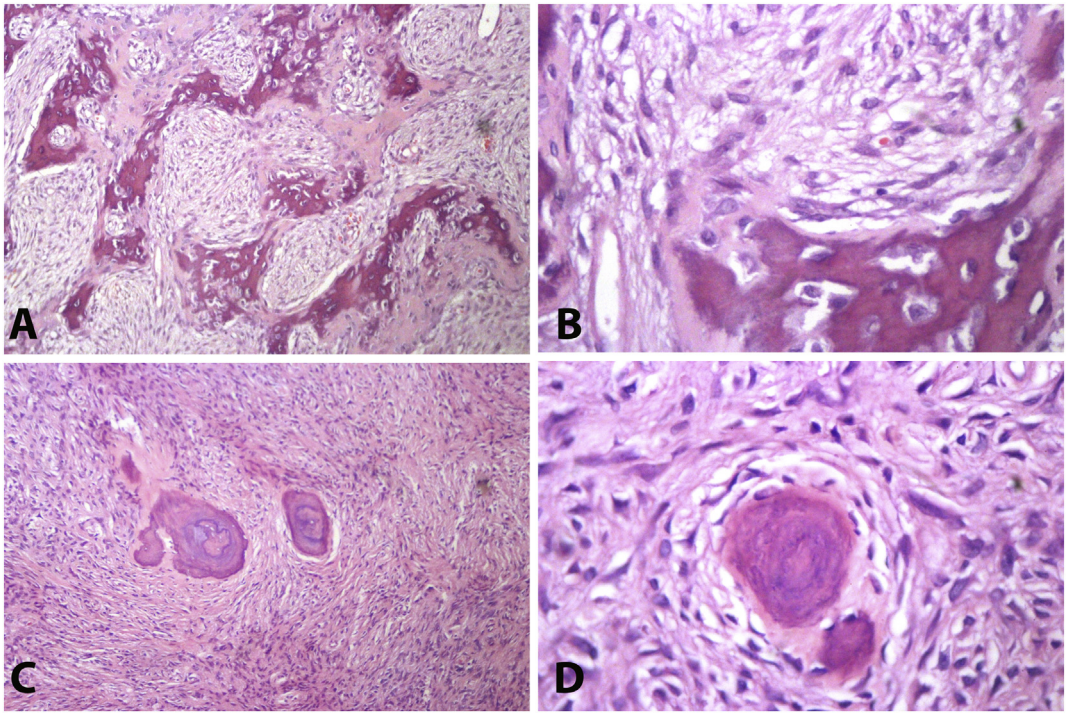

Fig. 2. Morphological features of trabecular juvenile ossifying fibroma (TrJOF) and psammomatoid juvenile ossifying fibroma (PsJOF): osteoid trabeculae deposition in a cellular fibroblastic background in TrJOF at (A) 200× and (B) 400× magnification. PsJOF showed concentric calcifications basophilic in the central portion and eosinophilic and poor cellular at its margins in (C) 200× and (D) 400× magnification.

without cortical disruption. JOFs in mandible are unusual [1,6] and were observed in three of our TrJOF cases.

Several studies show that radiographic findings of JOF may include uni or multiloculated radiolucent, mixed or predominantly radiopaque appearance depending on stage of maturation of the disease [21,25].

JOF and OF has a poorly known pathogenesis. The term "cementum-ossifying fibroma" previously applied to OF has fallen into disuse since the basophilic material primarily described as cementum was also found in OFs of extracranial bones, discarding a possible dental origin for the tumor [21,23]. Immunohistochemical studies in PsJOF with osteonectin, a specific bone protein that binds mineral to collagen, shows positive relation to fusiform cells and osteoid also indicating an osseous origin for PsJOF and excluding periodontal ligament derivation [25].

In our series, both TrJOF and PsJOF subtypes presented proliferation of round to fusiform cells forming a relatively considerable cellular stroma with deposition of a mineralized material predominantly of immature and osteoid nature. The spherical osteoid material deposited as small rounded or ovoid ossicles lined by osteoblasts identifies PsJOF. This calcified tissue of PsJOF exhibits concentric form, generally basophilic in the central portion and eosinophilic with poor cellularity in its margins, resembling psammoma bodies [26,27]. Some cases can present alterations in the fibroblastic background, like myxoid changes, microcystic degeneration, occasional spaces filled with hemorrhage surrounded by osteoclastic multinucleated giant cells and presence of aneurysmal bone cyst (ABC) [8,9,20]. Several reports show that the association of ABC with JOF is more common in PsJOF [14,28,29]. TrJOF cases shows osteoid material lined by osteoblasts organized in the form of anastomosing trabeculae [16,30]. A stromal myxoid component observed in a few of our TrJOF cases has been described as focal alterations that posteriorly give rise to hemorrhagic areas with osteoclastic giant cells [25]. The presence of multinucleated giant cells is more often in JOF than in OF [24].

Despite the aggressive growth and the diagnostic challenge eventually associated with JOF, there are still few studies on biomarkers for this disease. In attempt to understand the peculiar biology of this tumor, we analyzed the expression of Ki-67, Mcm-2, CD34 and CD105, considering OF as comparison standard.

The presence of blood vessels is essential for oxygen and nutrients support in benign and malignant tumors. Microenvironment studies in tumors bring importance of microvascularization in neoplastic lesions metabolism, growing and behavior [19].

Our study showed a low MVD for CD34 (a pan-endothelial marker) in JOFs and OFs, with a slightly higher expression in the first. A practically absent expression of CD105, a marker for angiogenesis, was noted in most of our cases. These evidences suggest that tumoral growth in both JOF and OF does not occur at the expense of increased vascularization.

We found a low proliferative index for Ki-67 expression in JOF and no significant difference when compared to OF. Ki-67 is a nuclear marker widely used to identify proliferating cells and is expressed in all cell cycle stages, except in G_0 [29].

Some papers suggest the positivity for Ki-67 as a marker for aggressiveness for JOF [7]. However, our results strongly disagree with this premise since Ki-67 immunostaining was very weak in our sample. In this context, our results are in accordance with those performed by previous studies that also showed low Ki-67 expression in JOFs and discarded the predictive value of this marker for biologic behavior of this neoplasm [28,29].

In order to confirm this result, we evaluated cell proliferation through Mcm-2 expression in JOFs and OFs. The positivity was higher than for Ki-67 without significant differences between both, confirming the low cell proliferation in JOF despite its aggressive growth.

Minichromosome maintenance proteins (Mcm) represent a group of 10 polypeptides that has a critic role in initial synthesis and replication of DNA preceding cell division, being expressed in the four stages of cell cycle and degraded after mitosis, pointing Mcm-2 as a more specific marker than other proliferation markers, like Ki-67 [22].

Even though there were no reports of malignant transformation, JOF is related to high recurrences rates of 30 to 56%, justifying *en*

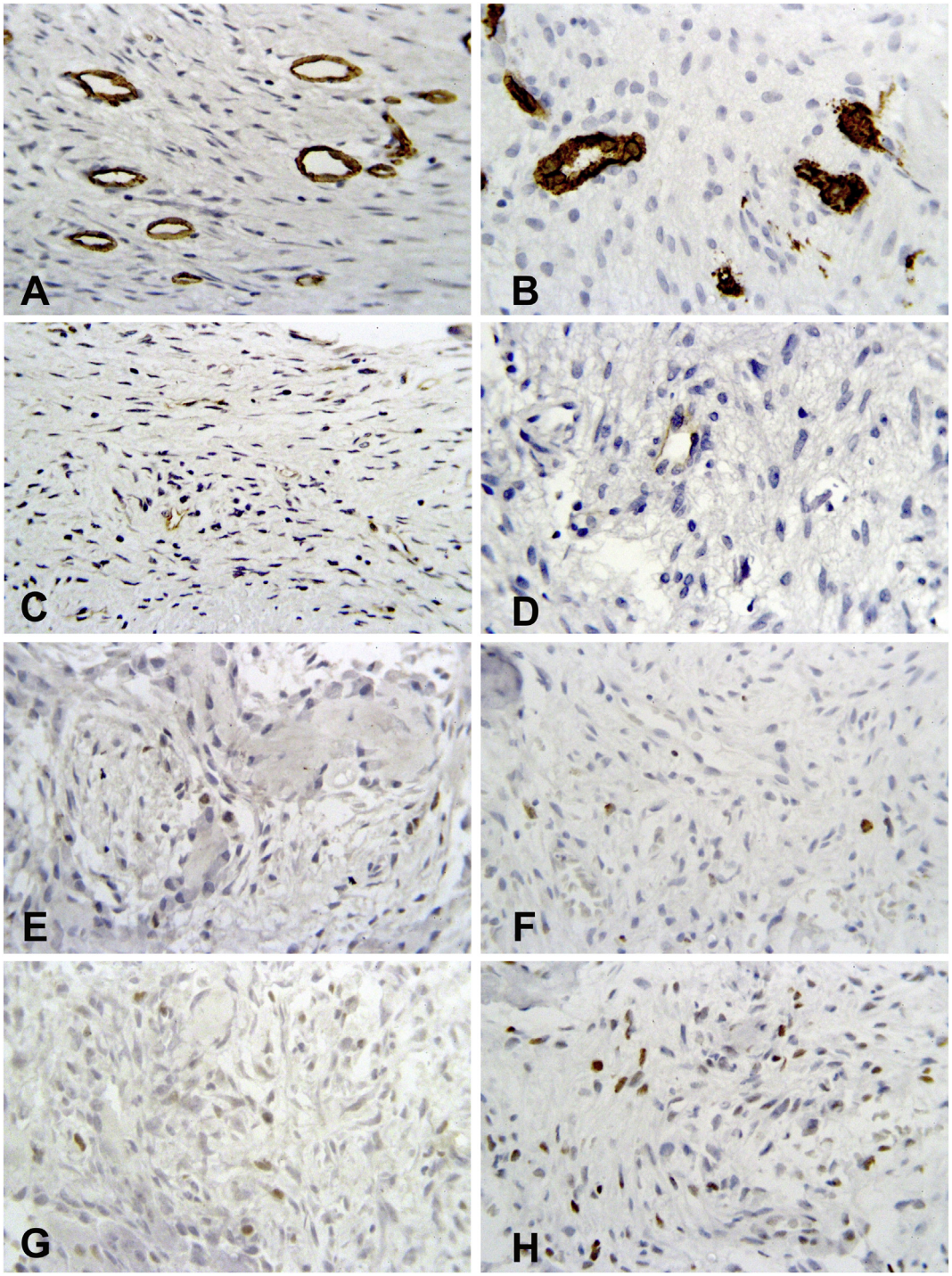

Fig. 3. Immunohistochemical panel used in juvenile ossifying fibroma (JOF) and ossifying fibroma (OF): CD34 expression in (A) JOF and OF (B), scarce CD105 positivity in (C) JOF and FO (D), weak Ki-67 positivity in JOF (E) and OF (F), and Mcm-2 expression in JOF (G) and OF (H).

bloc resection to ensure margins free of tumor [1,11,14,21]. The lack of specific clinical and radiographic parameters, morphologic or biomarkers able to predict an aggressive evolution or recurrence risk for JOF, makes clinical management and prognostic remaining uncertain for this disease [17]. This unpredictable behavior explains the absence of standardized follow-up protocol in the literature, making careful surgical planning and long-term clinical and radiographic follow-up mandatory [21]. Immediate reconstructions should be postponed for aggressive lesions with less than 1 year of evolution [2,23].

Overall, JOF diagnosis requires an intersection of clinical, radiographic and microscopic findings. As it does not express significant levels of cell proliferation and vascularization, subsequent studies are required to investigate other possible pathways and mechanisms, such as anti-apoptotic, pro-autophagic or even increased expression of matrix matalloproteinases, responsible for the aggressiveness of JOF.

Table 2
Percentage index (%) for immunohistochemical staining for Ki-67, Mcm-2 and medium values (MV) corresponding to blood microvessel density (MVD) for CD34 and CD105 in juvenile ossifying fibroma (JOF).

Case	Ki-67 (%)	Mcm-2 (%)	CD34 (MV)	CD105 (MV)
1	0	8,8	21,4	7
2	0,9	10,2	21,8	6,6
3	0	13,3	88	0
4	1,6	37,6	23,2	0
5	1,1	41,2	18,2	0
7	0	9,8	27	4,8
8	0,4	10,1	18,2	0
10	0	0	12,4	0
11	0	2,7	14,2	0

Table 3
Percentage index (%) for immunohistochemical staining for Ki-67, Mcm-2 and medium values (MV) corresponding to blood microvessel density (MVD) for CD34 and CD105 in ossifying fibroma (OF).

Case	Ki-67 (%)	Mcm-2 (%)	CD34 (MV)	CD105 (MV)
1	0	10,0	13,6	0,2
2	0	0	10,2	0,4
3	0	3,20	8,1	0
4	0	3,70	11,2	0
5	4,20	24,40	19,8	0
6	0	9,60	25,6	7
7	0	2,4	11,8	0
8	0	14,8	–	0
9	0	8,8	17,2	1,6
10	0	12,2	10,2	0
11	0	13,4	10	0

Declarations of interests

None.

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