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Hepatic involvement in paediatric patients with paracoccidioidomycosis: a histological study

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Hepatic involvement in paediatric patients with paracoccidioidomycosis: a histological study

Aim: Paracoccidioidomycosis is a systemic mycosis that is endemic to certain countries in Latin America. This study aimed to describe the histological features of liver involvement in patients with paracoccidioidomycosis aged <16 years of age who were treated between 1980 and 2010, with a diagnosis that was confirmed by detection of the fungus by pathological examination.
Methods and results: Liver tissue was obtained from one necropsy and 12 biopsies. Throughout 2007, biopsies were taken from patients with persistent jaundice or portal hypertension, after which biopsies became indicated due to elevated aminotransferase and low albumin levels. Using haematoxylin and eosin (H&E), Masson's trichrome and immunohistochemical (CK7 and CK19) staining, we noted degenerative alterations in bile duct cells and inflammatory injury to the bile ducts in 10 biopsies. Using immunohistochemistry for CK7 and CK19, we observed ductal proliferation in all 12 samples.
Conclusions: Bile duct injuries by inflammatory cells might explain the predominant increase in canalicular enzymes; immunohistochemistry is more sensitive in demonstrating ductular reactions and might show changes that are not apparent on H&E staining.

Keywords: bile ducts, immunohistochemistry, liver, paracoccidioidomycosis, paediatrics

Introduction

Paracoccidioidomycosis (PCM) is a chronic granulomatous disease that is caused by *Paracoccidiodes brasiliensis*, and has the highest mortality rate among systemic mycoses in Brazil (1.45 per million inhabitants). According to the Brazilian Ministry of Health's Mortality Information System, the mean mortality rate per million inhabitants for the other systemic mycoses are 0.33 for cryptococcosis, 0.13 for pneumocystosis and 0.04 for histoplasmosis.[1–3] The acute form of this disease primarily affects children and teenagers, who frequently experience involvement of the abdominal organs.

The liver is one of the organs that are affected most by PCM.[4–7] Necropsy studies of patients with PCM, not restricted to paediatric patients, have demonstrated that liver involvement is frequent (21–57%), although clinical manifestations are not always evident. Although liver involvement is more common in children and young adults than other age groups,[4,8,9] to our knowledge no study to date has detailed the types of lesions that develop in the livers of these patients.[10]

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This study aimed to describe the histological changes in paediatric PCM patients with liver involvement.

Material and Methods

We performed a histological study of liver samples from 10 PCM patients (11 percutaneous biopsies, one surgical biopsy and one autopsy). These cases belonged to a cohort of 102 paediatric patients who were followed between 1980 and 2010, 41 of whom had liver involvement.

The diagnosis of PCM was based on the detection of fungus by pathological examination (lymph node, liver, bone, skin or bone marrow) or culture of pulmonary secretions or lymph node fistulization. The exclusion criterion was an association with other diseases that could have led to liver involvement or any immunodeficiency.

Hepatic involvement was defined as increased levels of one or more liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP) or gamma-glutamyltransferase (GGT)]. Elevation of AP was considered only if associated with an increased level of either of the other three enzymes, due to the possibility of bone involvement.

During the retrospective study (1980–2007), the patients were selected for histological study when jaundice had persisted after 3 months of treatment or if there were ultrasonographic signs of portal hypertension. During the prospective study (2007–10), a biopsy was performed when there were increased levels of liver enzymes at hospital admission (AST, ALT, AP or GGT) and after receiving written informed consent.

The biopsies were performed percutaneously under local anaesthesia and sedation, after a minimum 6-h fast and with a normal prothrombin time, haemoglobin >10 g/dl and platelet count above 50 000/mm^3. We used the technique as per Mowat[11] and abdominal ultrasonography to guide the biopsy. A biopsy that was performed during splenectomy and material that was obtained in an autopsy were included in the study.

The samples were stained with haematoxylin and eosin (H&E), Masson's trichrome, Grocott, silver impregnation for reticulin fibres and Perls's Prussian blue. Immunohistochemistry was also performed with antibodies against CK7 and CK 19, cytokeratins that are expressed strongly by interlobular bile ducts, intralobular and intraportal bile ductules, and the biliary epithelial cells that partly line the canals of Hering, which represent hepatic progenitor cells.[12]

The morphological analysis of liver tissue comprised the following: (i) semiquantification of granuloma density (ii) description of the cellularity of inflammatory infiltrates (iii) characterization of alterations in bile duct cells (iv) counting of original bile ducts and portal spaces for bile duct-to-portal space ratio (evaluation of ductopenia) (v) assessment of ductal proliferation by immunohistochemistry for CK7 and CK19 (vi) semiquantification of the amount of fungi; and (vii) semiquantification and topographic analysis of liver fibrosis.

Results

During the retrospective study (patients 1, 2 and 3) there was an autopsy (the death occurred without treatment), and biopsies were performed in patients with a bad response even after the introduction of therapy. During the prospective study, the biopsies were performed during the first days of therapy. The clinical and laboratory data of the patients are shown in Table 1.

Based on evaluation of the histological sections of the 13 samples [12 from biopsies (four from the same patient) and one necropsy], the cell density of the granulomas ranged from mild to marked (few cells or many cells), with multinucleated giant cells, epithelioid cells, plasma cells, lymphocytes and primarily eosinophils (Tables 2 and 3; Figures 1A,B and 2B). Eleven samples showed varying degrees of fibrosis, with formation of septa and incipient nodules, predominantly in the periportal regions (Table 2; Figure 2A). Biopsies performed during the first several

Table 1. Clinical and laboratory characteristics

Patient	Age	Gender	AST	ALT	FALC	GGT
1	7	Male	**172**	**125**	**4128**	**591**
2*	15	Female	NP	NP	NP	NP
3	6	Male	7	27	492	**116**
4	9	Male	**94**	54	**2895**	**297**
5	9	Male	**95**	31	**233**	**89**
6	12	Male	**65**	**71**	**493**	**489**
7	12	Male	**87**	**93**	**999**	**259**
8	13	Male	26	25	**684**	**352**
9	7	Female	29	23	**497**	**173**
10	5	Male	27	11	**460**	**661**

2*, autopsy; NP, not performed.
The bold type indicates raised values of these enzymes.

Table 2. Histological characteristics

Patient	Cellular density of granuloma	Amount of fungi	Fibrosis			
			PP	z3	S	IN
1	1+	3+	0			
1	2+	2+	2+	x		
1*	2+	1+	3+		x	
1	2+	0	2+	x	x	
2	2+	2+	2+		x	
3	2+	3+	2+			
4	3+	3+	3+		x	
5	3+	3+	2+		x	
6	3+	Rare	3+		x	x
7	3+	3+	3+	x	x	
8	1+	1+	1+			
9	3+	1+	2+		x	
10	2+	1+	0			

0, Absent; 1+, Mild; 2+, Moderate; 3, Intense; x, Present; PP, Periportal; z3, Zone 3 acinar; S, Septa; IN, Incipient nodule; 1*, Surgical biopsy.

Table 3. Characteristics of inflammatory infiltrate

Patient	P	L	E	EC	GC	N
1	x	x	x	x	x	
1		x	xxx	x	x	
1*		x	x	x	f	
1	x	x		x		
2	x	x	x	x	x	
3	xxx	x	x	x	x	
4	x	x	x	x	x	
5		x	xxx	x	x	
6	x	f	xxx	x	x	
7		x	x	x	x	x
8		f	xxx	x	x	
9	x	x	xxx	x	x	
10	x		x	x	x	

P, Plasma cells; L, Lymphocytes; E, Eosinophils; EC, Epithelioid cells; GC, Giant cells; N, Neutrophils; x, Present; xxx, Many cells; f, Few cells; 1*, Surgical biopsy.

days of treatment and after approximately 1.5 months with symptoms demonstrated extensive fibrosis.

Notably, bile duct injuries were characterized by degenerative alterations to bile duct cells that were associated with inflammatory injury caused by lymphocytes or neutrophils (such injury occurring in 11 samples), without characteristic ductopenia (Table 4; Figure 2C,D). The bile duct injuries were also confirmed by immunostaining for CK7 and CK19, demonstrating ductal proliferation in 12 samples, even in cases without histological changes on H&E staining (Figure 1C,D).

Because one patient underwent four consecutive biopsies, we could follow the morphological evolution of his liver. During the first biopsy, performed after 18 months of disease, we noted large amounts of fungus and degenerative alterations to bile duct cells without fibrosis. During treatment, liver enzyme levels rose and hypersplenism and portal hypertension occurred. The patient underwent two additional biopsies –percutaneous biopsy and a surgical biopsy soon after – during splenectomy. The progression of fibrosis was noted in these samples, although the amount of fungi decreased as a result of treatment. The last biopsy was performed due to persistent elevation of liver enzymes, and although *P. brasiliensis* was eradicated, there was portal fibrosis with septa, which correlates with the clinical manifestation of portal hypertension. In this biopsy, we observed ductal proliferation, as evidenced by immunostaining for CK7 and CK19, despite the other stains failing to demonstrate bile duct injuries or ductopenia.

Discussion

We highlight the extensive fibrosis in biopsies from patients with a recent clinical history of PCM during the first several days of treatment and after approximately 1.5 months with symptoms. Teixeira *et al.*[6] found greater fibrosis in biopsies from the early stages of disease (normal or almost-normal liver, with only non-specific chronic inflammatory infiltration in the portal tracts, or liver with extensive areas of necrosis with neutrophilic infiltration in the portal tracts) than in patients without granulomas or necrosis but an enlargement of portal tracts due to fibrosis. This group believes that treatment can decrease the severity of fibrosis.

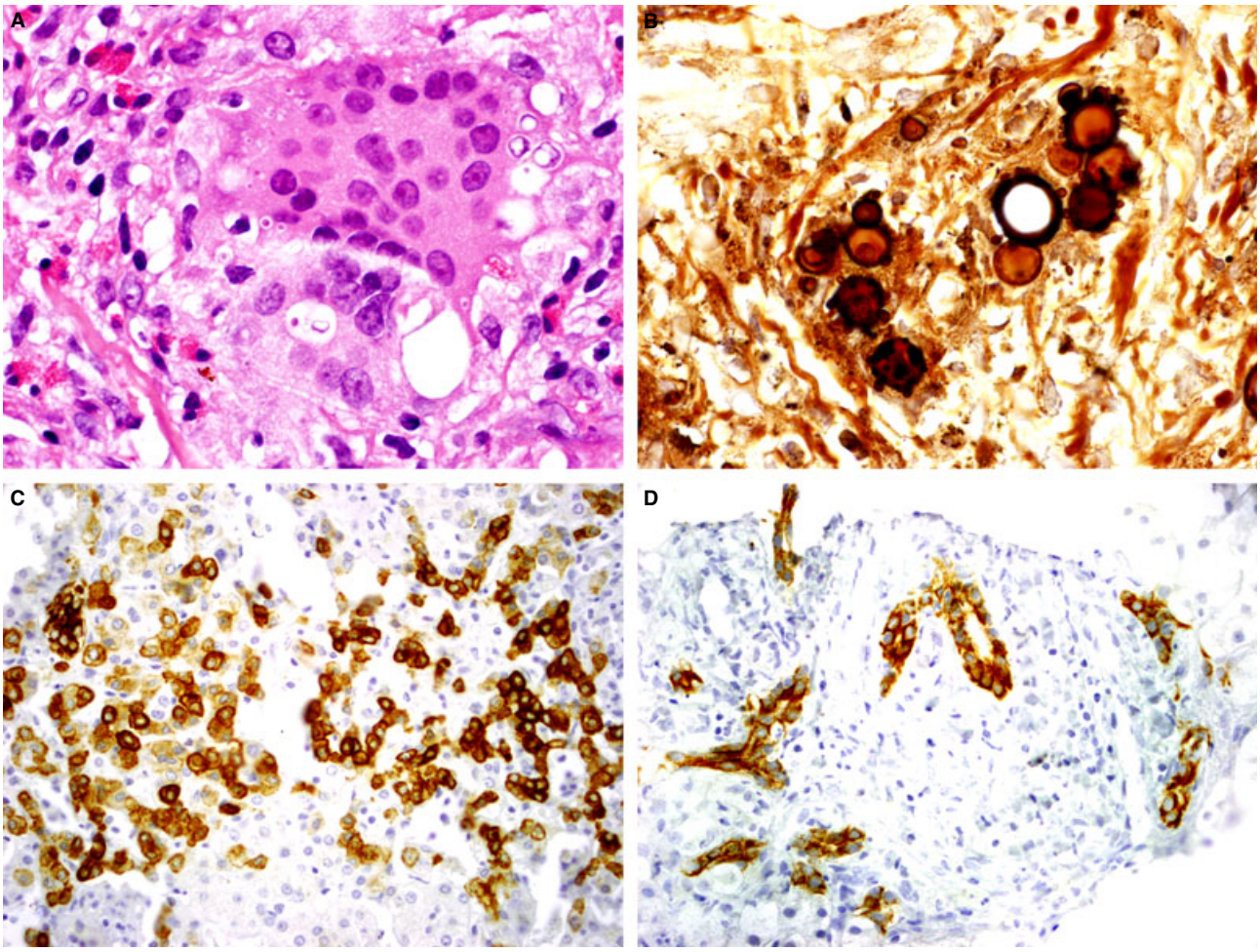

Figure 1. **A**, Portal inflammatory mixed reaction (periductal) with eosinophils and giant cells phagocytosing spores of the fungus (H&E); **B**, budding yeast (Grocott); **C,D**, immunohistochemistry: **C**, immature cells stained for CK7, outlining small ductular structures; and **D**, ductal proliferation demonstrated by CK19 immunostaining.

We observed a relationship between the amount of fungus and the severity of portal/periportal fibrosis in eight samples; the fibrosis was more extensive in cases with more fungi. Periportal fibrosis is associated frequently with a ductular reaction, with production of matrix proteins by (i) biliary epithelial cells (ii) indirect stimulation of fibrogenesis by the production of factors that stimulate portal tract fibroblasts and myofibroblasts or (iii) epithelial–mesenchymal transition, in which biliary epithelial cells undergo transition to fibroblasts. The fibrogenesis and epithelial–mesenchymal transition are mediated by TGF-β from damaged bile ducts or infiltrating T cells.[12]The hepatic fibrosis that occurs in PCM cannot be attributed to any mechanism of hepatotoxicity by drugs in the treatment regimen.[13]

With regard to the tissue hypereosinophilia in five of our 13 samples, Brito[9] described an inflammatory infiltrate in the liver in PCM in a study of patients aged between 17 and 90 years that comprised histiocytes, lymphocytes, plasma cells and a variable number of eosinophils. The acute form of PCM is associated with Th2-mediated immunity, with high levels of IL-4, IL-5, IL-10, specific IgE and IgG4 antibodies, and peripheral and *in-situ* eosinophilia.[14,15]

Peripheral eosinophilia occurs in 50–80% of cases of PCM, primarily in younger patients, with disseminated disease and bone marrow involvement.[16–18] Eosinophilia and hypereosinophilia are transient, sometimes oscillatory, and disappear spontaneously or with antifungal treatment. Eosinophils protect the host against multicellular parasites that are not phagocytosed (multicellular organisms and helminths). Eosinophil granule major basic protein is toxic to helminths and mammalian cells *in vitro*, and its

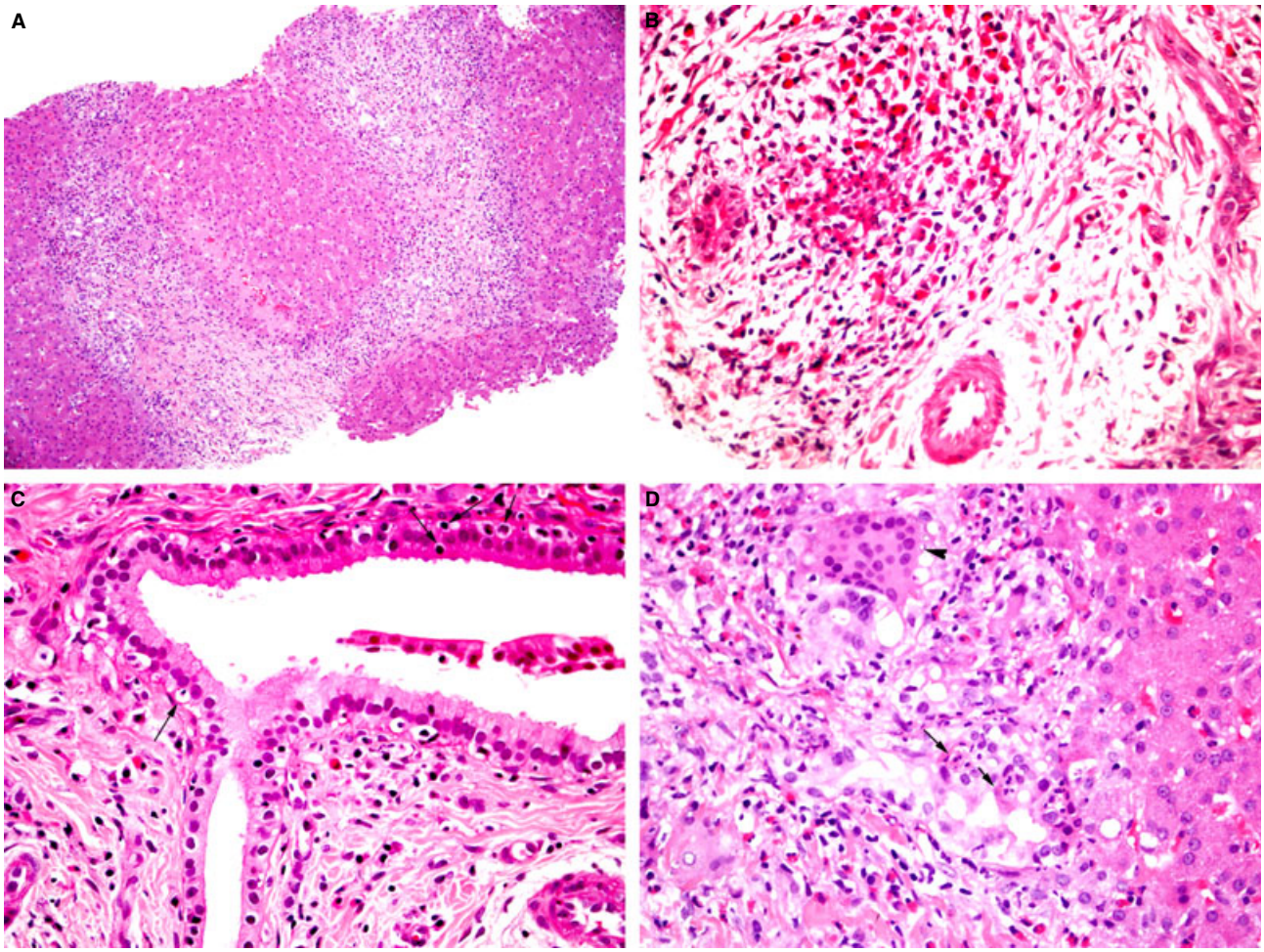

Figure 2. **A**, Severe inflammatory reaction progressing to fibrosis, septa, and incipient nodule (architectural change); **B**, portal inflammatory reaction with abundant eosinophils (note degenerative alterations to biliary epithelial cells; **C**, inflammatory damage to a major bile duct, with predominance of lymphocytes (arrows); **D**, marked inflammatory injury of epithelia of interlobular bile duct, with abundant polymorphonuclear cells(arrows) (arrowhead: giant multinucleated cell). **A**–**D**, all H&E.

release has been used as a marker of eosinophil localization and degranulation. Wagner[19] observed the deposition of extracellular major basic protein in PCM lesions, supporting the hypothesis that eosinophils mediate the disease pathophysiology.

In a biopsy from patient 1, ductal proliferation was apparent only on immunostaining for CK7 and CK19. This pattern occurs because $CK7^+$ cells with an intermediate hepatobiliary phenotype might be present in periportal areas before a well-defined ductular reaction is evident.[12] Thus, immunohistochemistry can show ductular reactions earlier than other stains. In this biopsy, CK19 expression was moderate and that of CK7 was intense, wherein certain $CK7^+$ cells had robust staining while others showed slight expression (Figure 1). Considering that CK7 immunohistochemistry stain the biliary epithelial cells that partly line the canals of Hering, which are probably hepatic progenitor cells, the weakly stained cells in this section might represent cells that are beginning to differentiate towards a biliary phenotype.

There were small amounts of yeast, absence of fibrosis and of inflammatory injury to biliary epithelial cells in the biopsy from patient 10. This patient presented with increased levels of canalicular enzymes that could not be explained by an abundance of fungi that injure the bile duct, or by obstruction of biliary flow due to fibrosis or granulomas. Thus, bile duct injuries caused by inflammatory cells are significant in the pathogenesis of hepatic involvement, as demonstrated by increased levels of alkaline phosphatase and gamma-glutamyltransferase.

Table 4. Bile duct alterations

Patient	Degenerative alterations	Inflammatory injuries	Bile duct/Portal space	Ductal proliferation (CK 7/CK19)
1	x	0	NP	1+
1	x	1+	1/2	1+
1*		1+	9/11	3+
1		0	NP	1+
2		1+	NP	NP
3	x	1+	2/2	1+
4		3+	5/4	2+
5		2+	2/3	2+
6		2+	4/5	1+
7	x	1+	2/4	2+
8		1+	2/2	1+
9		1+	5/5	2+
10		1+	1/1	1+

0, Absent; 1+, Mild; 2+, Moderate; 3+, Strong; NP, Not performed; x, Present; 1*, Surgical biopsy.

There are two possible mechanisms regarding the pathogenesis of this type of inflammatory bile duct injury: (i) expression of a common antigen by the biliary duct cell, leading to cross-reaction with *P. brasiliensis*; and (ii) association with a bacterial infection. Teixeira *et al.*[6] have reported bile duct injuries in PCM, noting that ductal proliferation is more frequent in patients without granulomas but with necrosis and neutrophilic infiltrate. Ductal proliferation was also observed in patients with fibrosis and non-specific inflammatory infiltrate without granulomas or parasites. However, it was not possible to establish the mechanism of the injury. The group proposed the existence of intraepithelial parasitism (although it was not observed in their study), and ruled out extrahepatic cholestasis and the drugs that were used to treat PCM as the cause of injury.

There is no report of ductal proliferation associated with such drugs, other than a description of cholestatic injury by itraconazole.[13] Thus, in PCM, injury to biliary epithelial cells by inflammatory cells may constitute the mechanism by which bile ducts are damaged. We could not find any report of another mycosis with such inflammatory damage to the bile duct. Hepatosplenic candidosis is associated with granulomas, necrosis with minimal inflammatory reaction, and microabscesses with severe inflammatory reaction.[20] In other granulomatous liver disease, the granulomas may be accompanied by a severe inflammatory reaction within or surrounding the granulomatous structures. In other instances, the granulomas may have little or no accompanying inflammation.[21] Sarcoidosis presents, in association with granulomas, parenchymal necroinflammatory foci, portal inflammation, interface hepatitis, loss of bile ducts, and acute or chronic cholangitis.[22] In schistosomiasis, in addition to the presence of periovular granulomas, the fibrotic portal spaces frequently show diffuse mononuclear leucocyte infiltration, sometimes with differentiation of lymphoid follicles and signs of interface activity ('piecemeal necrosis'). Schistosomiasis itself can cause a certain degree of chronic hepatitis, probably of a reactive nature.[23] Also, in the personal experience of the pathologist who examined the specimens, it is sometimes possible to see focal injury to epithelial duct cells, and in particular concentric fibrosis surrounding the duct (as in primary sclerosing cholangitis).

Conclusion

Bile duct injury caused by inflammation, accompanied by defined or incipient ductular reactions (as con-

firmed by CK7 and CK19 immunostaining), occurred in 12 of our 13 samples. This finding should be correlated with elevated canalicular enzymes, which are frequently observed in patients with PCM, as well as progressive fibrosis, which can also develop even with a paucity of fungus, as we observed in one case.

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