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**DISEASE IN WILDLIFE OR EXOTIC SPECIES**

**Short Title: Histiocytosis in Red Squirrels**

**Atypical Histiocytosis in Red Squirrels (*Sciurus vulgaris*)**

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## Summary

Four red squirrels (*Sciurus vulgaris*) were subjected to necropsy examination over a 3-year period as part of a broader surveillance study. The squirrels presented with cutaneous, subcutaneous and/or internal swellings and nodules that consisted microscopically of sheets of atypical round cells and multinucleated giant cells. There was moderate anisokaryosis with rare mitoses. Nuclei ranged from oval to indented or C-shaped and some were bizarre, twisted or multilobulated. Many giant cells also had a bizarre morphology, with anisokaryosis within individual cells. Giant cell nuclei were also often multilobulated, ring-shaped or segmented. Affected internal organs varied depending on the squirrel, but included lymph node, kidney, intestinal tract and lungs. Representative lesions from each of the four squirrels were negative for acid-fast organisms. Formalin-fixed tissues from all four squirrels and ethanol-fixed tissue from one animal were negative for *Mycobacterium* by polymerase chain reaction. Immunohistochemically, the majority of mononuclear and multinucleated giant cells in all four squirrels strongly expressed vimentin and class II molecules of the major histocompatibility complex. Otherwise, the atypical mononuclear and multinucleated cells were negative for CD3, Pax-5, Mac387, CD18 and E-cadherin. Based on the combination of cellular morphology, arrangement and immunophenotype, a novel form of atypical histiocytosis is considered most likely in these squirrels, although the exact origin and triggering factors remain uncertain.

*Keywords:* histiocytosis; immunohistochemistry; red squirrel; *Sciurus vulgaris*

Two decades ago, Harris *et al.* (1995) described the extreme vulnerability of the red squirrel (*Sciurus vulgaris*) in the UK, concluding that the species was virtually extinct in most of England and Wales. A number of reports have since confirmed this decline, linking it to the

introduction of the American grey squirrel (*Sciurus carolinensis*) (Rushton *et al.*, 2006). While direct competition for food and habitat is believed to play a role, much blame has been placed on squirrelpox virus, a member of the subfamily Chordopoxvirinae that was seemingly introduced by the American grey squirrel (Thomas *et al.*, 2003; McInnes *et al.*, 2006). Native red squirrels are more vulnerable to infection than their grey counterparts and, since Scottish red squirrels account for three quarters of the UK population, it is acknowledged that their conservation and a full understanding of their stressors are important (LaRose *et al.*, 2010). To that end, a necropsy examination-based survey has been underway at the University of Edinburgh for several years, focusing partly on the impact of squirrelpox infection on the red squirrel population, but also exploring the role of factors such as trauma, predation and starvation (LaRose *et al.*, 2010). During this larger study, an unusual presentation of skin disease was identified sporadically, characterized by proliferative lesions that also involved some internal organs.

Four female red squirrels were subjected to necropsy examination over a 3-year period (2012–2015) as part of the aforementioned surveillance study (LaRose *et al.*, 2010). All four originated from southwest Scotland and each was found dead with no prior clinical history. The squirrels presented with multiple soft to firm swellings and discrete nodules up to 15 mm diameter. The skin and subcutis of the head were predominantly affected in three squirrels, particularly around the eyes, mandibular area, bridge of the nose, lips and chin (Fig. 1). On cut section, the nodules ranged from pink to brown and many were ulcerated or cavitated. The lungs of two squirrels contained multiple 1–2 mm diameter, tan to white nodules. Other more variably affected areas and internal organs are summarized in Table 1. A selection of organs was collected from each squirrel by the project's principal investigator (AM). Tissues were fixed in 10% neutral buffered formalin and processed routinely. Selected sections were stained with Gram and/or Ziehl Neelsen stains and sections from all

four squirrels were further characterized by immunohistochemistry (IHC). Briefly, serial sections were dried at 37°C, incubated at 60°C, de-waxed, dehydrated and washed in Tris buffer. Endogenous peroxidase was blocked using Dako REAL peroxidase blocker (Dako, Ely, UK). All antibody incubations were performed for 30 min at room temperature using mouse monoclonal antibodies against vimentin (dilution 1 in 400; Novocastra, Milton Keynes, UK), CD3 (1 in 200; Leica, Milton Keynes, UK), anti-feline CD18 (1 in 20; University of California at Davis, California, USA), Pax-5 (1 in 50; Becton Dickinson, Oxford, UK), E-cadherin (1 in 200; Becton Dickinson), Mac387 (1 in 600; Dako) and class II molecules of the major histocompatibility complex (MHCII; 1 in 60; Dako). Pre-treatments comprised incubation for 15 min in high pH buffer (Vector Laboratories, Peterborough, UK) at 110°C for vimentin; 15 min in 0.01M citrate buffer at 110°C for CD3, Pax-5, E-cadherin and MHCII; and 10 min in proteinase K for CD18 and Mac387. For all but CD18 and Mac387, 'visualization' was achieved using Envision anti-Mouse HRP (Dako) for 40 min followed by 3, 3'-diaminobenzidine for 10 min. Visualization of CD18 and Mac387 was achieved using secondary Immpress anti-mouse (Vector Laboratories) for 15 min. Squirrel lymph node served as positive control for all antibodies except for that against E-cadherin. Epidermis in the skin nodules served as a positive internal control for E-cadherin, but additional controls included canine colon and squirrel salivary gland. Negative controls comprised antibody diluent only (Dako).

Possible mycobacterial infection was investigated by polymerase chain reaction (PCR). DNA was extracted from formalin-fixed and paraffin wax-embedded (FFPE) skin from three squirrels, ethanol-fixed skin from one squirrel and FFPE lung from another. The extraction method used for the FFPE tissues was described by Simpson *et al.* (2016). The same method was used for the ethanol-fixed tissue omitting xylene and ethanol washes. A pan-*Mycobacterium* spp. PCR directed against the heat shock protein (hsp) 65 gene was

performed (Telenti *et al.*, 1993). Sections of ileum from a sheep with multibacillary paratuberculosis were used as extraction and positive PCR controls and sterile distilled water as a negative PCR control. Amplified DNA was excised from a 2% agarose gel, extracted using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) and sequenced by MWG BioTech (Eurofins MWG Operon, Ebersberg, Germany).

Microscopically, the skin and subcutaneous nodules consisted of sheets of round cells with a moderate amount of smooth, pale eosinophilic cytoplasm and densely basophilic, generally eccentrically located, oval, indented or C-shaped nuclei. Some nuclei were bizarre, twisted or multilobulated. Nucleoli were variably distinct, anisokaryosis was moderate and mitotic figures were rare. Admixed throughout were small numbers of multinucleated giant cells with nuclei that were either multilobulated, segmented or ring-shaped (Figs. 2 and 3; Supplementary Figs. 4 and 5). Affected internal organs varied with squirrel, but included lymph nodes, lungs, renal cortex, duodenum and spleen. In the lungs atypical cells surrounded airways, effaced bronchial submucosa and expanded the perivascular interstitium. All four squirrels were negative for acid-fast organisms. Most, though not all, mononuclear and multinucleated cells labelled strongly for vimentin and MHCII in lesions tested from all four squirrels (Supplementary Figs. 4–7). The atypical cells were negative for all other antibodies. Scattered individual CD3, Pax-5, Mac387 and CD18-positive cells were compatible with inflammatory lymphocytes, macrophages and neutrophils. Control tissues were labelled appropriately.

All formalin-fixed tissues subjected to PCR were negative for *Mycobacterium* spp., while the positive and negative controls were all reported accurately. PCR analysis of DNA from the ethanol-fixed skin nodule amplified product that was subsequently sequenced. The sequence was compared with nucleotide sequences in the public nucleotide database (nr/nt)

using BLASTn (<http://blast.ncbi.nlm.nih.gov>) and the highest score match obtained was to *Nocardia cerradoensis* strain HUD797051 (98% coverage, 95% identity).

This series of four squirrels describes multi-organ infiltration by a population of atypical round cells, most notably involving the skin and subcutis of the head and, internally, the lungs. Main differentials for nodular skin lesions in squirrels are squirrelpox virus infection and granulomatous inflammation. The lesions were not compatible histologically with squirrelpox (Bangari *et al.*, 2009). Two forms of granulomatous inflammation have been described in squirrel skin, one comprising a lepromatous pattern of granulomatous dermatitis associated with a *Mycobacterium* spp. virtually identical to *M. lepromatosis* (Meredith *et al.*, 2014). The other was a single case report of granulomatous inflammation due to *M. avium* subsp. *avium* in a pet Korean squirrel (*Sciuris vulgaris coreae*). The giant cells in this case were ‘bizarre’ in that they often had irregular shapes or up to 100 nuclei, but the macrophages were not described as atypical (Moreno *et al.*, 2007). In these four squirrels, the morphology of the atypical cells suggested histiocytic origin and a proliferative disorder was suspected due to tissue effacement by sheets of highly atypical cells, pronounced perivascular cuffing in the lungs and the lack of distinct granulomas. As far as it was possible to determine, there was no indication of *Mycobacterium* spp. infection. The significance of the *N. cerradoensis* is uncertain. It is a gram-positive, slightly acid-fast actinomycete originally isolated from soil. Recently, disseminated infection has been described in immunosuppressed people (Piau *et al.*, 2015, Ercibengoa *et al.*, 2016). It was suspected to be opportunistic in this case since the same probes previously failed to detect this organism in four otherwise healthy squirrels that had died due to trauma (personal communication, K. Stevenson).

Histiocytic proliferative disorders may be of macrophage or dendritic cell origin and, in the veterinary context, are best described in dogs and cats. In dogs the main potentially

disseminating forms are cutaneous Langerhans cell histiocytosis; histiocytic sarcoma (HS); and cutaneous or systemic histiocytosis. In cats, pulmonary Langerhans cell histiocytosis and feline progressive histiocytosis are the main types although HS also occurs. Most can arise in or affect the skin, with the exception of pulmonary Langerhans cell histiocytosis, and all may spread to internal organs, apart from cutaneous histiocytosis, though this can involve draining lymph nodes (Affolter and Moore, 2006; Busch, 2008; Moore 2014). The cells in these disorders share some molecular markers, including CD18, but there is also variation. For example, in Langerhans cell histiocytosis, cells also express E-cadherin; in cutaneous and systemic histiocytosis they express CD4 and CD90; and in feline progressive histiocytosis they also express MHCII (Affolter and Moore, 2006; Moore, 2014). Vimentin and MHCII expression were common to all four squirrels, confirming mesenchymal derivation and indicating likely antigen presenting cell origin (i.e. dendritic cell, B cell or macrophage). The lack of Pax-5 reactivity eliminated a B-cell origin. The lack of CD18 expression was more difficult to interpret since dendritic cells and macrophages are generally CD18 positive. While the anti-feline CD18 antibody used is species-specific, this does not adequately explain the lack of reaction in these squirrels since small numbers of CD18-positive cells were observed in control squirrel lymph node. Cross-species variation in the immunophenotype of various dendritic cell subpopulations may offer partial explanation, but it is also possible that some phenotypic factors were lost, particularly in neoplastic tissue (Affolter and Moore, 2006). To conclude, based on several features, an unusual form of histiocytosis is considered most likely in these squirrels, although it remains unclear as to whether this is truly neoplastic. Further characterization would require application of a more specialized panel of molecular markers that would cross react reliably with this more unusual species.

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## Figure Legends

Fig. 1. Red squirrel (*Sciurus vulgaris*) with centrally ulcerated skin nodules around the left eye (white and black arrowheads). Correlates with squirrel 3 in Table 1.

Fig. 2. Skin nodule, squirrel 3 in Table 1. Sheets of atypical round cells and multinucleated giant cells infiltrate the dermis and extend to the dermo-epidermal junction, effacing normal dermal architecture. Example of a bizarre multinucleated cell (inset). HE. Bar, 500  $\mu\text{m}$ ; inset, 20  $\mu\text{m}$ .

Fig. 3. Skin nodule, squirrel 3 in Table 1. Atypical round cells with features reminiscent of histiocytes. Admixed multinucleated giant cells are often bizarre with coarse chromatin. Abnormal mitotic figures are highlighted by arrowheads. HE. Bar, 20  $\mu\text{m}$ .

## Supplementary Figure Legends

Supplementary Fig. 4. Skin nodule, squirrel 1 in Table 1. Atypical round cells with indented or C-shaped nuclei and coarse chromatin. HE with vimentin inset. Bar, 50  $\mu\text{m}$ .

Supplementary Fig. 5. Skin nodule, squirrel 4 in Table 1. Atypical round cells with features reminiscent of histiocytes admixed with pleomorphic multinucleated giant cells. HE with vimentin inset. Bar, 100  $\mu\text{m}$ .

Supplementary Fig. 6. Lung lesion, squirrel 2 in Table 1. The cells expanding the perivascular spaces are strongly positive for MHCII. Many multinucleated cells are also positive (inset). IHC. Bar, 200  $\mu\text{m}$ .

Supplementary Fig. 7. Skin nodule, squirrel 4 in Table 1. Atypical round cells within the superficial dermis are strongly MHCII positive. Epidermis (\*). IHC. Bar, 100  $\mu\text{m}$ .

**Table 1- Summary of pathological, immunohistochemical and Mycobacterial testing of four red squirrels**

<i>Squirrel</i>	<i>Gross lesion distribution</i>	<i>Histopathology</i>	<i>Immunophenotype</i>							<i>Additional testing</i>
			<i>Vimentin</i>	<i>MHCII</i>	<i>E-cadherin</i>	<i>CD18</i>	<i>Mac387</i>	<i>CD3</i>	<i>Pax5</i>	
1	Swellings on head, feet, stifles, thoracic wall; extra-intestinal mass; tan lung nodules	Atypical round cells forming sheets and nodules in dermis, lymph node, kidney, lung, duodenum and extra-intestinal mass (suspected lymph node)	+++/-	+++/-	-	-	-	-	-	<i>Mycobacterium</i> negative by ZN and PCR
2	Enlarged peripheral lymph nodes, including axillary; subcutaneous swelling cervical neck; white/tan lung nodules	Atypical round cells forming sheets and nodules in soft tissue of cervical neck and lungs	+++	+++/-	-	-	-	-	-	<i>Mycobacterium</i> negative by ZN and PCR
3	Subcutaneous swellings on head, ventral neck, axilla, flank and stifle	Atypical round cells forming sheets and nodules in skin and submucosa of lip	+++	+++/-	-	-	-	-	-	<i>Mycobacterium</i> negative by ZN and PCR
4	Firm nodules on head and ventral neck	Atypical round cells forming sheets and nodules in dermis, submucosa of lip and spleen; extensive effacement of lung (grossly normal)	+++/-	+++/-	-	-	-	-	-	<i>Mycobacterium</i> negative by ZN and PCR

+++ , strongly positive; +++/-, strongly positive with a small number of negative cells; -, negative

Figure 1  
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Figure

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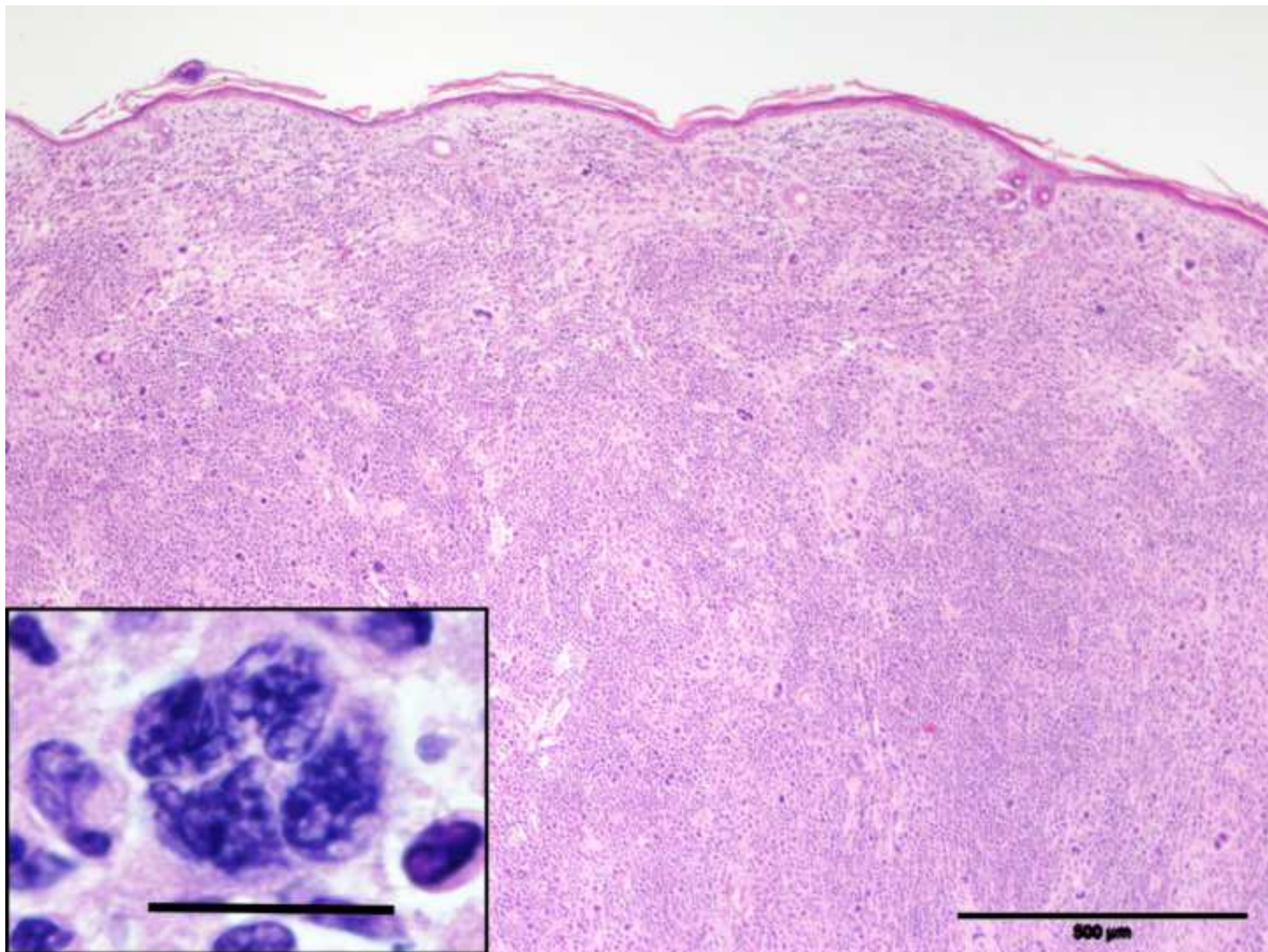




Figure 3  
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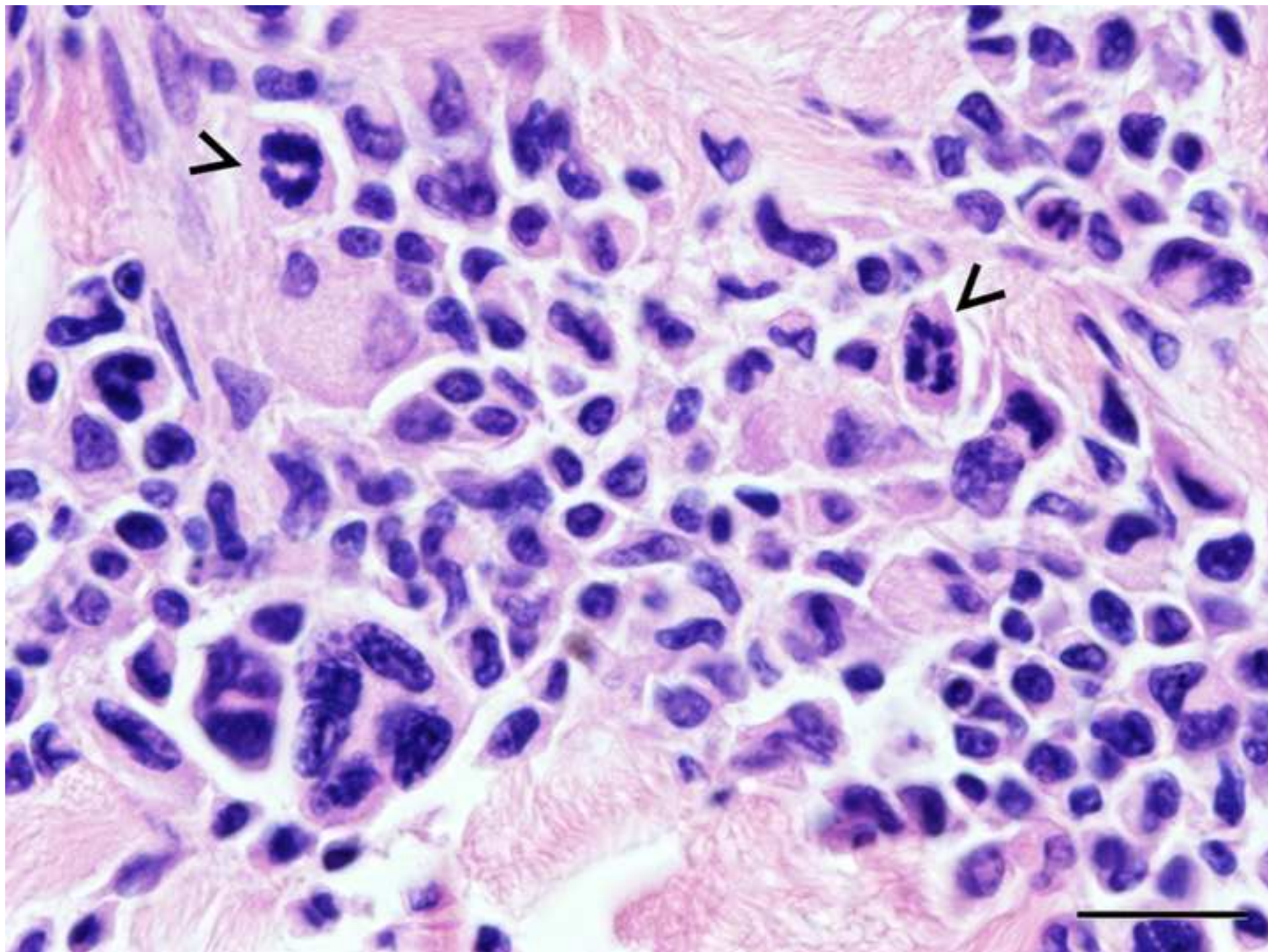


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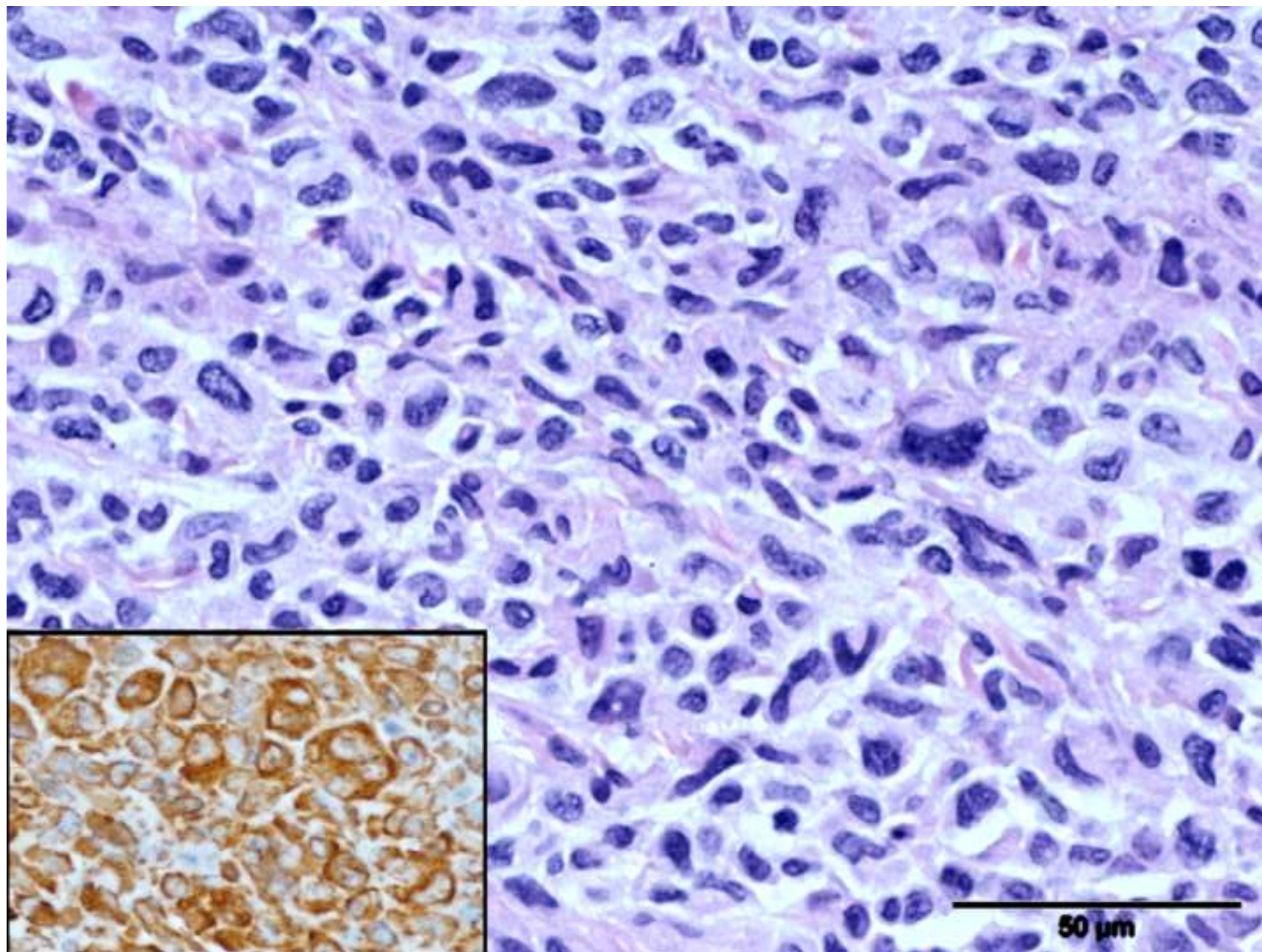


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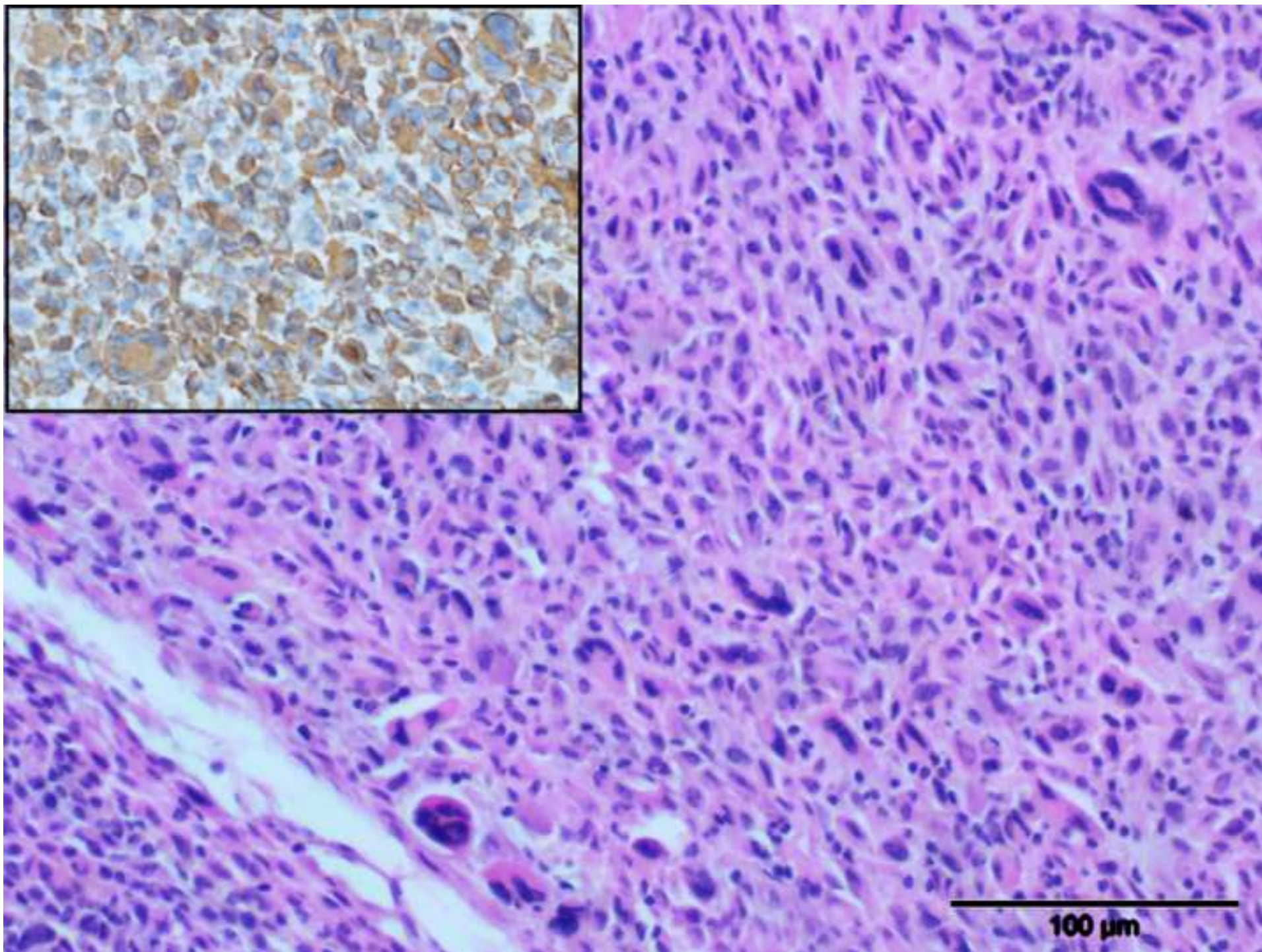


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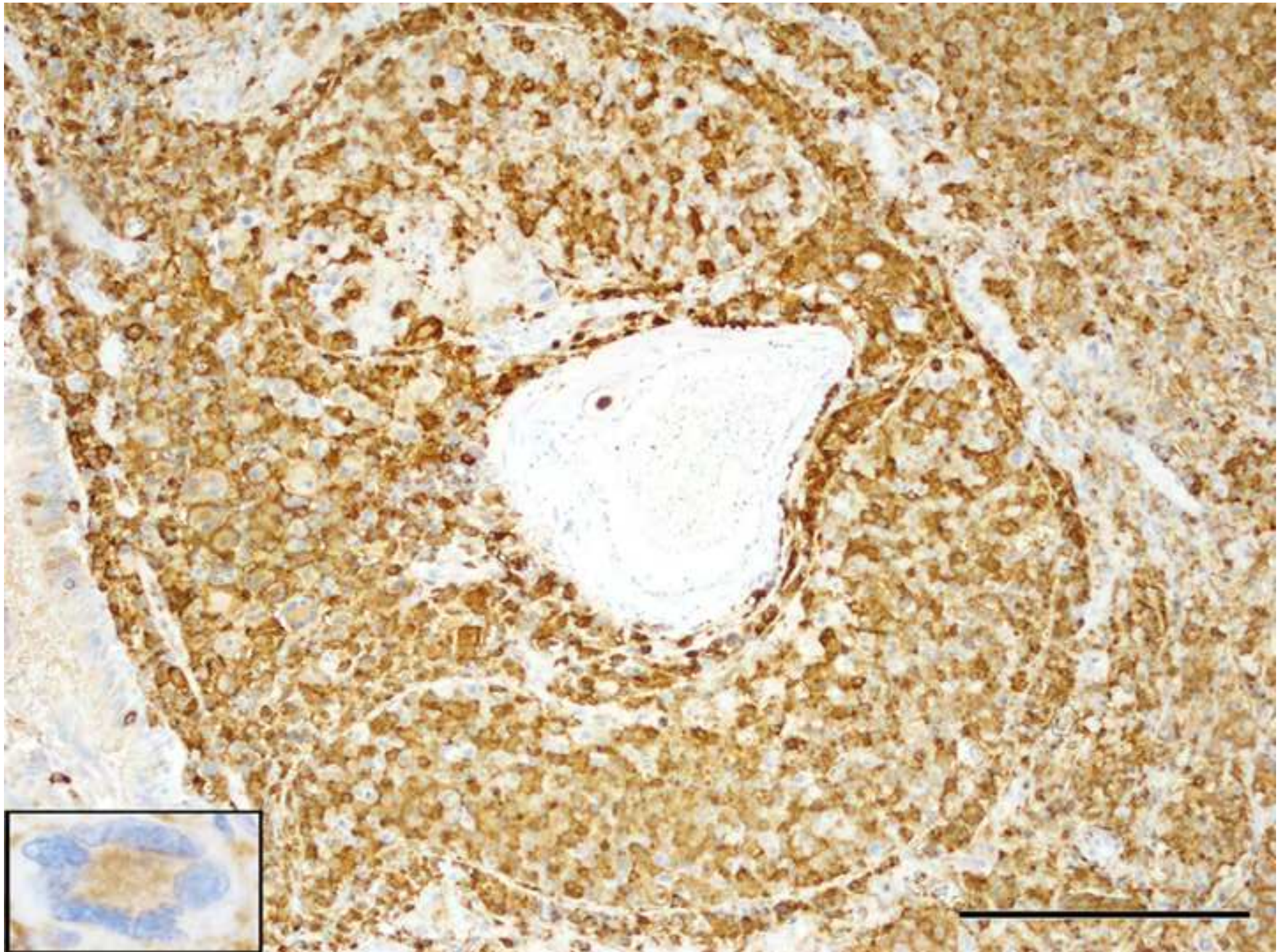


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