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Immunohistochemical analysis of smooth muscle actin and CD31 in feline post-injection site fibrosarcomas: association with tumour grade, vascular density, and multinucleated giant cells

Mateusz Mikiewicz[1*] and Iwona Otrocka-Domagała[1]

Abstract

Background Multinucleated giant cells are commonly observed in various malignancies; however their clinical and biological significance remains largely unexplored and it has been hypothesised that the cells may play a role in vascular mimicry, tumour progression and tumour survival. This study aimed to investigate the expression of smooth muscle actin and CD31 in feline post-injection site fibrosarcomas, focusing on relationships between multinucleated giant cells presence, tumour grade, and vascular density to elucidate their potential role in tumour progression.

Results A total of 61 feline post-injection site fibrosarcomas, histologically graded into grades I, II, and III, were examined immunohistochemically. Smooth muscle actin immunoreactivity was detected in 57/61 (93.4%) cases. Multinucleated giant cells expressing CD31 were identified in 39/61 (63.9%) cases, predominantly in high-grade tumours, with a correlation observed between multinucleated giant cell presence, tumour grade, and mitotic index. Vascular density differed across tumour grades. A negative correlation between vascular density, tumour grade and necrosis score was identified. Additionally, a negative correlation was observed between multinucleated giant cells presence and vascular density.

Conclusions The findings suggest a complex tumour microenvironment in which multinucleated giant cells and vascular mimicry may facilitate tumour survival under hypoxic conditions, potentially contributing to an aggressive tumour phenotype.

Keywords Feline injection site fibrosarcoma, Multinucleated giant cell, Smooth muscle actin, CD31, Immunohistochemistry

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Background

Mulitinucleated giant cells (MGCs) have been recognized in various malignancies, including canine and feline mast cell tumours [1, 2], histiocytic sarcoma [3], plasma cell tumour [4], soft-tissue sarcoma, and feline post-injection site sarcoma [5]. Despite being frequently observed in tumours, their clinical and biological significance remains largely unexplored. Macrophages have been identified as the primary precursors of epithelioid cells in vitro, induced to differentiate into these cells by treatment with interleukin 4 (IL-4) [6]. This is significant as a Th2 cytokine environment is commonly linked to the enhanced activation of macrophages and recent studies have confirmed that M2 macrophages can give rise to multinucleated giant cells [7].

In the tumour microenvironment MGCs may represent a senescent cells [8] or can exhibit non–senescent CD31 + immunophenotype [9]. Additionally, these cells might form in response to chronic inflammation which may be associated with the tumour, similar as can be observed in foreign body reaction [10]. This may be supported by the theory that MGCs may form in response to an attempt to eliminate a chronic irritant [11]. Although this seems a logical suggestion, many MGCs, particularly those that are poorly differentiated, are not effective at eradicating neoplasms and are now thought to contribute to tumour progression [12–14]. A previous study on human papillary thyroid carcinoma has revealed that MGCs occurrence is associated with advanced malignancy stage and therefore is connected to tumour progression [14].

While MGCs can appear in the context of tumours, their presence is generally not a direct immune mechanism against the malignancies. Instead, they often reflect a complex interaction between the tumour and the immune system, where the MGCs may represent a response to chronic inflammation or be part of the tumour's microenvironment rather than an active antitumour mechanism [11, 14]. Recent studies on human cancer cells have revealed that MGCs are metabolically active and may contribute to tumour heterogeneity and resistance to therapy [15].

Angiogenesis is essential for processes like wound healing; however it can be also seen in pathological conditions such as tumour growth [16]. The process is triggered by growth factors or low oxygen levels, which activate hypoxia-inducible factors [17]. During angiogenesis, previously inactive endothelial cells that line the blood vessels are prompted to proliferate, modify the extracellular matrix, migrate, and differentiate to form the structure of new vessels in response to angiogenic signals [18]. In solid tumours, the vasculature consists of endothelial-lined blood vessels and a non-endothelial microcirculatory network. This non-endothelial network is known as vascular mimicry (VM), and it is thought to arise from the differentiation of cancer stem cells into endothelial-like cells [19].

The aim of the present study was to investigate the expression of immunohistochemical markers, specifically smooth muscle actin (SMA) and CD31, in feline post-injection site fibrosarcomas. Additionally, this study sought to elucidate the relationship between tumour grade, vascular density, and the presence of multinucleated giant cells, with an emphasis on understanding these features and their potential role in tumour progression and aggressiveness.

Results

Histological examination of the tumour samples revealed a distribution across three grades, with 6 tumours classified as grade I (9.8%), 24 as grade II (39.3%), and 31 as grade III (50.8%). In all cases FISS were poorly demarcated, located in the dermis and subcutis. The cells were spindle or polyhedral with marked anisocytosis and anisokaryosis with macronucleosis, and multinucleated giant cells scattered through neoplastic mass. Mitotic count was variable. In most cases various necrotic areas in tumour mass were seen. In all tumours various degree of peripheral mostly perivascular lymphocyte and plasma cell infiltration were seen, also scattered macrophages were seen in tumour mass.

SMA immunolabelling was detected in 93.44% of cases (Fig. 1A). Specifically, SMA expression was observed in 6 grade I tumours (10.53%), 22 grade II tumours (38.6%), and 29 grade III tumours (50.88%). SMA immunolabelling was absent in 4 cases, including 2 grade II tumours (3.28%) and 2 grade III tumours (3.28%).

CD31 immunolabelling was present in multinucleated giant cells in 39 tumours (63.93%) (Fig. 1B). CD31-positive multinucleated giant cells were present in 2 grade I tumours (5.13%), 14 grade II tumours (35.9%), and 23 grade III tumours (58.97%). The mean MGCs count varied across grades, with grade I tumours showing an average of 8 ± 5 cells, grade II tumours showing 15 ± 17 cells, and grade III tumours showing 23 ± 14 cells. Statistical analysis revealed a higher count of multinucleated giant cells in grade III tumours compared to grade II ($p = 0.004$) and grade I ($p = 0.017$) (Fig. 2A). Furthermore, a positive correlation was observed between MGCs occurrence and tumour grade ($r = 0.49$; $p = 0.000$) (Fig. 2B). Additionally, the presence of MGCs varied between mitotic score (1 vs. 3 $p = 0.022$; 2 vs. 3 $p = 0.02$) (Fig. 2C), with a positive correlation between these two parameters ($r = 0.441$; $p = 0.000$) (Fig. 2D).

Vascular density showed a mean value of 21 ± 7 in grade I tumours, 29 ± 11 in grade II, and 17 ± 6 in grade III. Comparative analysis indicated that vascular density was higher in grade II tumours compared to grade III

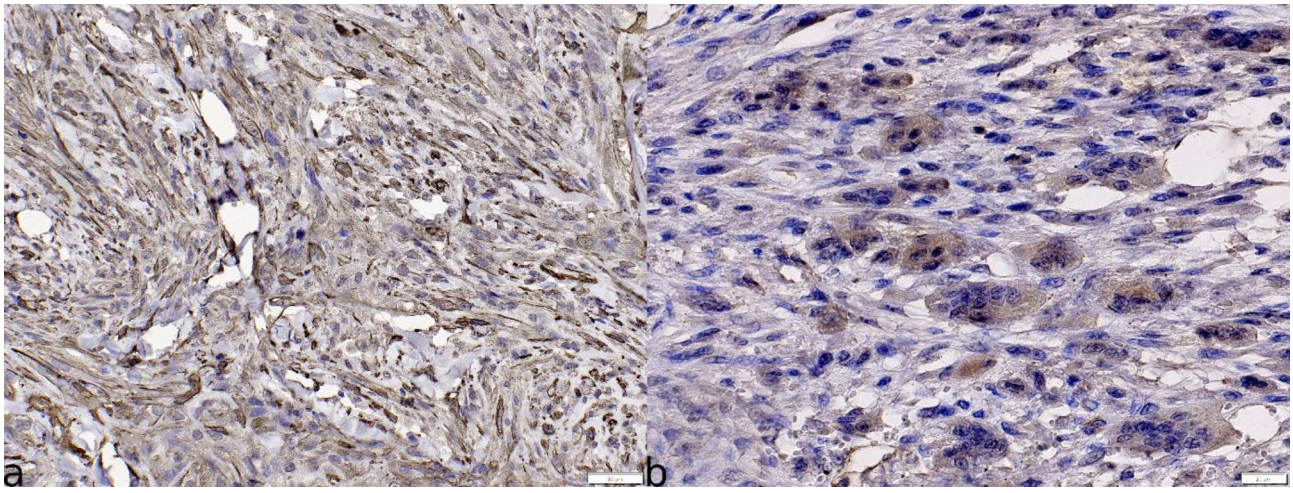

Fig. 1 Feline injection-site fibrosarcoma. (**A**) Tumour cells show positive expression to SMA. SMA immunostaining with DAB, counterstaining with Mayer's haematoxylin, 200× (**B**) Multinucleated giant cell show positive expression to CD31. CD31 immunostaining with DAB, counterstaining with Mayer's haematoxylin, 400×

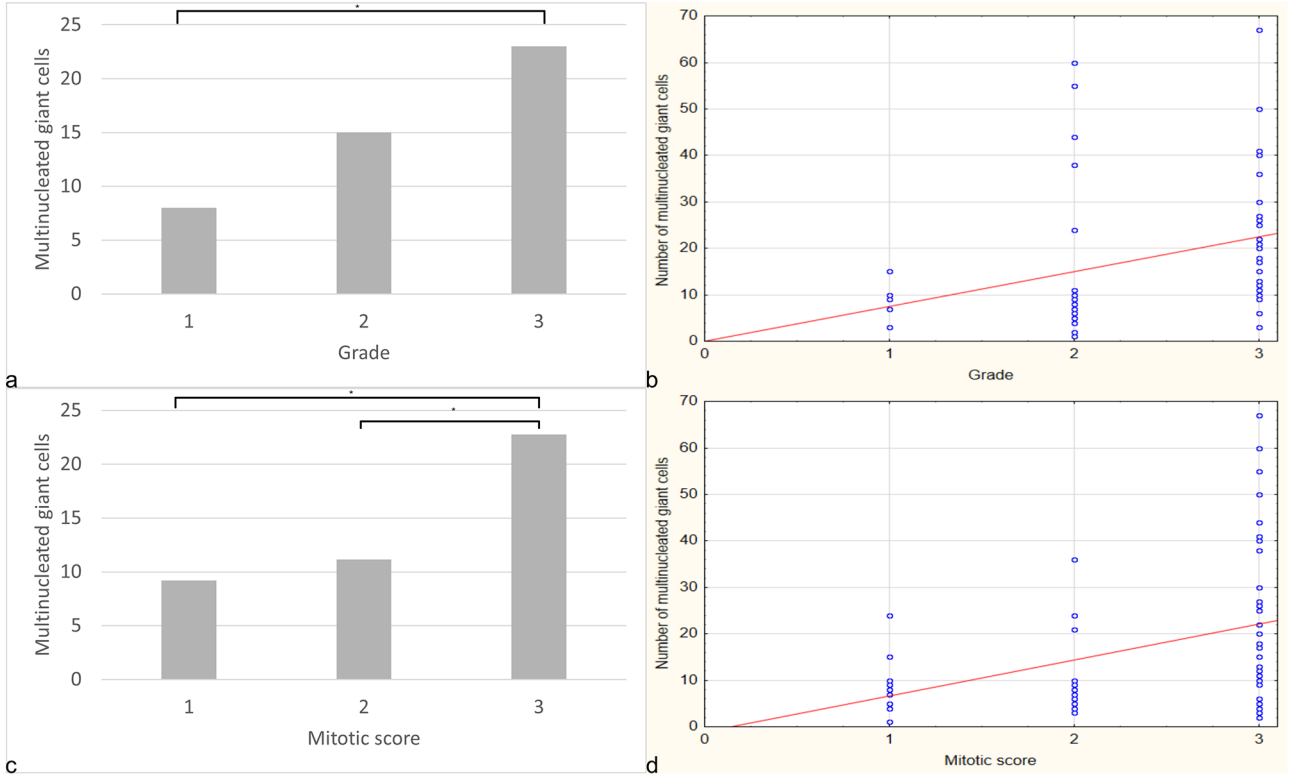

Fig. 2 Feline injection-site fibrosarcoma. (**A**) Mean multinucleated giant cell count in grade I, II, and III tumours. Significantly higher multinucleated giant cell count was observed in grade III tumours compared to grade II ($p = 0.004$) and grade I ($p = 0.017$). (**B**) A positive correlation between multinucleated giant cell occurrence and tumour grade ($r = 0.49$; $p = 0.000$). (**C**) Mean multinucleated giant cell count compared to mitotic score. Significantly higher multinucleated giant cell count was observed in mitotic score 3 compared to mitotic score 1 ($p = 0.022$) and mitotic score 2 ($p = 0.02$). (**D**) A positive correlation between multinucleated giant cell count and mitotic score ($r = 0.441$; $p = 0.000$)

($p = 0.000$) (Fig. 3A). Furthermore, a negative correlation was found between vascular density and tumour grade (r=-0.444; $p = 0.000$) (Fig. 3B). Blood vessel count was highest in with necrosis score 0 and a statistically significant difference was observed between necrosis score 0 vs. 2 ($p = 0.005$) (Fig. 3C), with a negative correlation (r=-0.411; $p = 0.001$) (Fig. 3D). Moreover, a negative correlation was found between the MGCs count and vascular density (r=-0.262; $p = 0.042$) (Fig. 4). Comprehensive

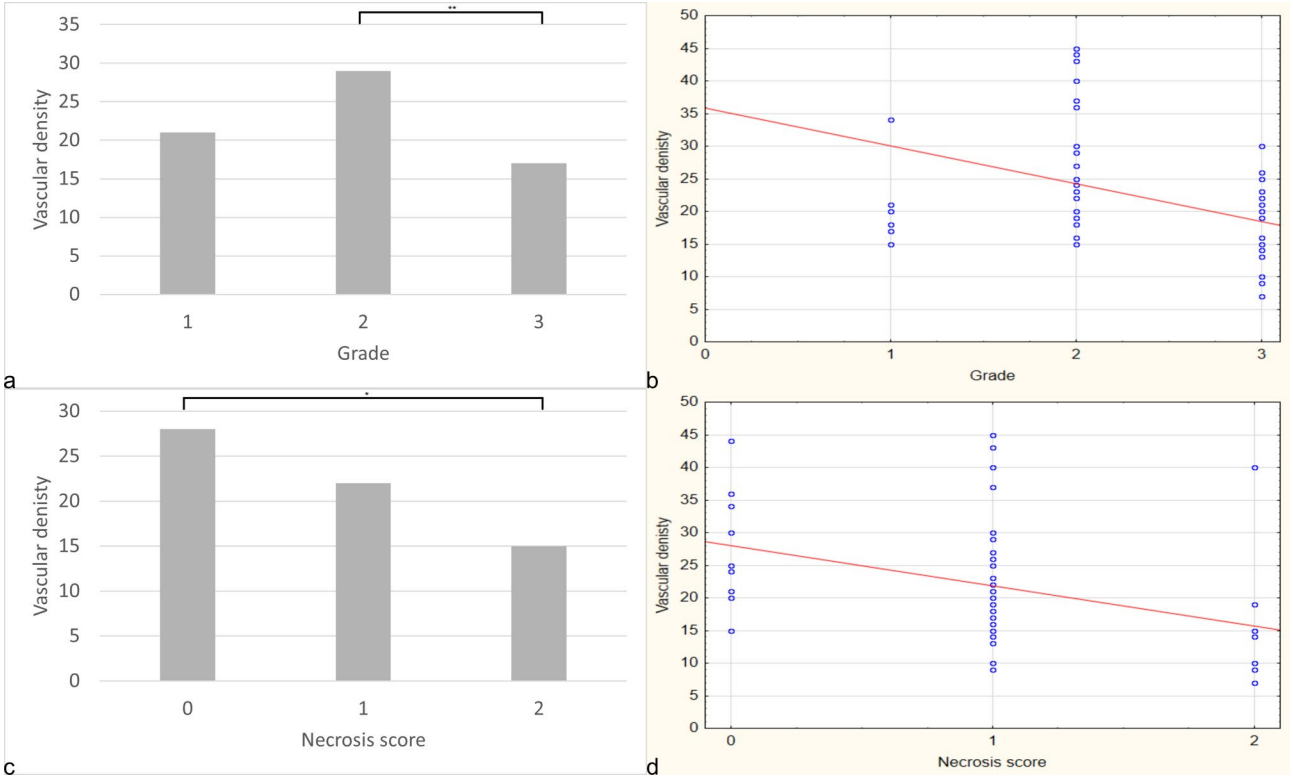

Fig. 3 Feline injection-site fibrosarcoma. (**A**) Mean vascular density in grade I, II, and III tumours. Significantly higher vascular density was in grade II tumours compared to grade III ($p=0.000$). (**B**) A negative correlation between vascular density and tumour grade ($r=-0.444$; $p=0.000$). (**C**) Vascular density compared to necrosis score. Vascular density was significantly higher in necrosis score 0 compared to necrosis score 2 ($p=0.005$). (**D**) A negative correlation between vascular density and necrosis score ($r=-0.411$; $p=0.001$)

tumour characteristics are collected in Supplemental Table 1.

Discussion

The histological and immunohistochemical findings of this study revealed an important insights into the tumour microenvironment and the progression of feline post-injection site fibrosarcomas. A significant correlation between MGCs presence, tumour grade, and mitotic score was observed. Additionally, the vast majority of evaluated cases were SMA positive, which aligns with previous studies reporting frequent SMA positivity in feline post-injection fibrosarcomas [20, 21].

The presence of MGCs in different tumour grades, with a significant prevalence in grade III tumours, may suggest a potential link between MGCs and tumour aggressiveness. The higher MGCs count in more advanced (grade III) tumours, aligns with previous studies which suggested that MGCs may contribute to tumour progression [22–25]. In human papillary thyroid carcinoma the presence of MGCs has been associated with advanced malignancy stages [14], supporting the hypothesis that MGCs, although originating from immune cells, may foster a pro-tumorigenic environment [26, 27]. This relationship between MGCs and tumour grade may reflect the immunosuppressive properties often observed in advanced tumours, where MGCs contribute to a Th2-polarized environment that favours tumour growth and immune evasion [28–30]. Moreover, a significant association was observed between MGCs occurrence and mitotic score, which further strengthens the potential link between MGCs and aggressive tumour behaviour.

The identification of CD31-positive MGCs in more than half evaluated cases, particularly within higher-grade lesions, suggests an intricate relationship between vascularization and the immune microenvironment in post-injection feline fibrosarcomas. CD31, is known as a well-established endothelial marker and it is widespread used in assessing vascular characteristics in tumours [31], and when expressed on MGCs, may indicate a non-traditional form of vasculature [16, 32]. However, we recognize, that employing CD31 alone does not definitively distinguish vascular mimicry from conventional angiogenesis. Thus, while the detection of CD31-positive MGCs suggests an endothelial-like phenotype, these findings should be interpreted with caution. Inclusion of additional markers (e.g., vascular epithelial growth factor, VE-cadherin) and functional assays would ideally provide more robust validation of vascular mimicry [33, 34]. Due to limitations in tissue availability and the scope of the

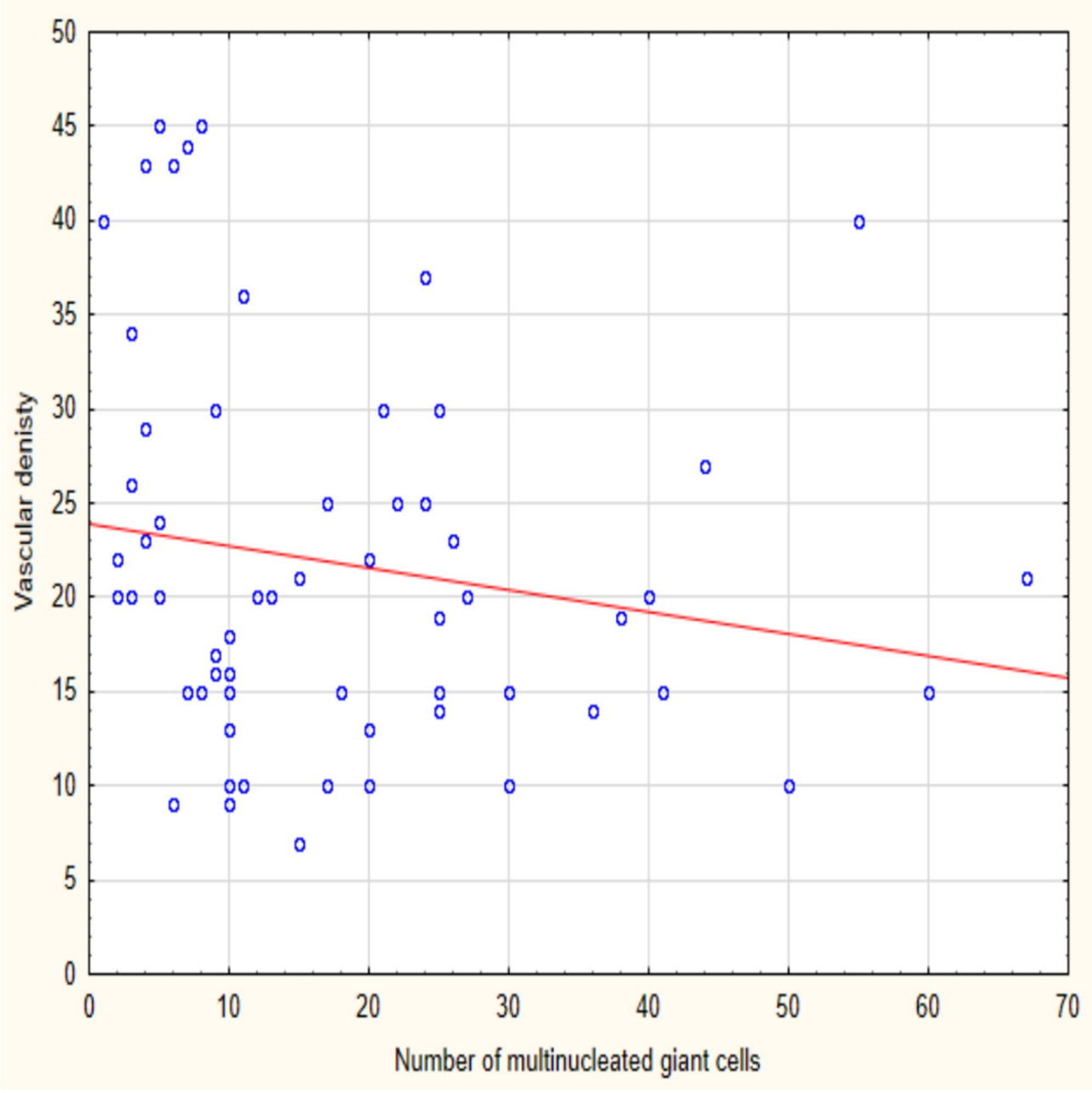

Fig. 4 A negative correlation between the multinucleated giant cell count and vascular density (r=-0.262; p=0.042)

present study, such supplementary analyses were not performed, and therefore our results represent preliminary evidence that warrants further investigation into the role of MGCs in alternative vascular network formation in feline post-injection site fibrosarcomas.

This phenomenon is in agreement with reports on VM, where non-endothelial cells acquire endothelial-like properties [19, 27]. CD31 expression in MGCs might represent an adaptive response within the tumour microenvironment to support tumour survival and progression under hypoxic conditions. The association between vascular density and tumour grade also reveals complexities in the angiogenic profile of feline post-injection site fibrosarcomas. This finding suggests that high-grade tumours may shift toward alternative mechanisms of nutrient acquisition, such as VM. The increased presence of CD31-positive MGCs in grade III tumours suggests that these cells might participate in the formation of such non-classical vascular channels, thereby facilitating tumour progression and survival within the hypoxic microenvironment characteristic of these aggressive tumours. The adaptation to hypoxia may be driven by

the activation of hypoxia-inducible factor-1 (HIF-1), a key regulator of cellular responses to low oxygen levels. Under hypoxic stress conditions, HIF-1 acts as a master regulator by promoting the transcription of numerous genes involved in angiogenesis, metabolic reprogramming, and cellular survival, which are fundamental to cancer development and progression [35, 36]. Such hypoxic conditions may not only stimulate the formation of MGCs but also induce a phenotypic shift in these cells, enabling them to acquire endothelial-like characteristics, thereby contributing to the formation of non-traditional vascular channels that help sustain the tumour despite a reduction in classical blood vessels [37]. This transition is in line with the concept of VM, where tumour cells or associated cells adopt features of endothelial cells to form alternative vascular networks independent of traditional angiogenesis [38]. In our study, high-grade feline post-injection site fibrosarcomas exhibited a paradoxically lower conventional vascular density while simultaneously displaying a significantly higher count of MGCs. Above findings are important in understanding the complex interplay between tumour vascularization and the immune microenvironment. We propose that the lower vascular density in high-grade tumours may reflect a shift towards alternative nutrient acquisition mechanisms, such as VM.

Alternative interpretations of this negative correlation are also plausible. The decreased vascular density in high-grade tumours could result from extensive necrosis and the collapse of normal vasculature, conditions that may further stimulate a compensatory response leading to the recruitment or formation of MGCs. These cells might then participate in VM, serving as a mechanism to bypass the limitations imposed by reduced angiogenesis [38].

Overall, the observed negative correlation underscores the adaptive strategies tumours may employ under hypoxic stress and highlights the potential role of MGCs in facilitating alternative vascularization. Future studies are warranted to further explore these mechanisms, which could ultimately lead to the identification of novel therapeutic targets for managing aggressive malignancies. In human malignancies VM was correlated with high tumour grade, cancer cell invasion, cancer cell metastasis, and reduced survival of cancer [39]. This inverse relationship between vascular density and tumour grade may imply a dynamic vascular remodelling process, where tumours progress toward an increasingly aggressive phenotype by reducing reliance on traditional angiogenesis and adopting alternative vascular strategies. This observation aligns with reports from other malignancies, where declining vascularity accompanies increased necrosis and hypoxia in higher-grade tumours [40]. Elevated levels of HIF-1α, frequently observed in hypoxic tumours or resulting from genetic changes, correlate with poorer patient outcomes across various cancer types [41, 42]. The findings not only highlight the plasticity of the tumour microenvironment but also underscore the potential for MGCs to facilitate tumour survival when conventional angiogenic mechanisms are insufficient. This is noteworthy since as the formation of MGCs in tumours is driven by cellular stress factors such as hypoxia, chemotherapy, radiotherapy, reactive oxygen species production, viral infections, and other factors [27, 43, 44]. Although the exact mechanism underlying MGCs induction remains unclear, other studies point to pathways analogous to those involved in VM [45, 46]. Key processes contributing to MGCs formation are connected with the HIF-1 pathway activation and DNA damage [43, 45].

Furthermore, the association of vascular density with the necrosis score strengthens the link between reduced vascularity and tumour progression. Tumours with high necrosis score exhibit pronounced hypoxic environment, what was previously reported in different malignancy types [42, 47].

The observed positive correlation between MGCs and the mitotic score supports the hypothesis that MGCs could contribute to an environment that promotes cellular proliferation. It is hypothesized that MGCs may secrete various cytokines and growth factors, such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF), which can modulate the tumour microenvironment and stimulate proliferative signalling pathways. This pro-inflammatory milieu may not only enhance the proliferative capacity of tumour cells but also facilitate further tumour progression and invasion [48]. This association could result from chronic inflammation, which may stimulate both the recruitment of immune cells and the proliferation of tumour cells, contributing to tumour growth and heterogeneity [11]. Moreover, the correlation between MGCs occurrence and tumour grade aligns with the theory, that MGCs not only indicate, but may actively contribute to the malignant progression, potentially by modulating the inflammatory microenvironment or enhancing resistance to immune clearance [27].

Above findings are important in understanding the complex interplay between vascularization and the immune microenvironment in post-injection feline fibrosarcomas. However, a limitation of our study is the absence of comprehensive follow-up data, including parameters such as tumour recurrence, metastasis, and overall survival. Due to limitations in the available follow-up information for the cases studied, we were unable to correlate our histological findings with clinical outcomes. Future studies should aim to incorporate such clinical data to better assess the prognostic significance

of these histopathological markers and to enhance the clinical relevance of these observations.

Conclusion

In summary, our study demonstrates that higher-grade feline post-injection site fibrosarcomas exhibit a lower conventional vascular density alongside an increased presence of CD31-positive MGCs. A significant positive correlation between MGC counts and mitotic scores was observed, suggesting that MGCs may be linked to increased cellular proliferation, potentially through alternative vascular mechanisms such as VM. Although the data indicate that MGCs might play a pivotal role in promoting tumour proliferation, the complexity of the tumour microenvironment precludes the identification of a single element as solely responsible for this process. We conclude that MGCs appear to play a special role in tumour proliferation; however, additional studies employing further markers and functional assays are needed to fully elucidate their precise contribution. Overall, our findings highlight the complex interplay between immune, vascular, and stromal components in feline post-injection site fibrosarcomas and underscore the need for further investigation into the roles of MGCs and vascular mimicry in tumour progression. Future studies focusing on the molecular pathways that drive MGC formation and VM could yield valuable insights into therapeutic targets, potentially improving outcomes for feline patients diagnosed with feline post-injection site fibrosarcoma.

Methods

Study design

The study included surgically excised cutaneous tumours collected from 61 cats, 32 (52%) males and 29 (48%) females, aged 4–22 (mean: 12 ± 4 years). The samples were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, cut and stained with Mayer's haematoxylin and eosin (HE). The inclusion criteria included morphological features described previously [49–51]. The tumours were located at typical injection sites: flank (34.4%; 21/61 cases), interscapular region (26.2%; 16/61 cases), lumbar area (18%; 11/61 cases), thigh (11.5%; 7/61 cases), dorsal area of the neck (8.2%; 5/61 cases), and shoulder (1.6%; 1/61 cases). Histologically, the tumours were graded into grade I, II and III, according to the grading system proposed by Dobromylskyj et al. [52] for feline soft tissue sarcomas and described previously [51].

Immunohistochemical evaluation

The sections for immunohistochemistry were mounted on silanized glass slides. Heat-induced antigen retrieval was performed in Tris-EDTA buffer pH 9.0 (EnVision™ Flex Target Retrieval Solution High pH, DAKO, Glostrup, Denmark) for 20 min at 96 °C using a PT-Link module (Dako, Glostrup, Denmark). Immunohistochemical examination of each tumour was performed using primary antibodies: α-SMA (monoclonal mouse anti-human, clone 1A4, dilution 1:50, incubation time: 30 min in a humid chamber at room temperature; Dako, Glostrup, Denmark) and CD31 (monoclonal mouse anti-human, clone JC70A, dilution 1:20, incubation time: overnight in a humid chamber at 4℃; Dako, Glostrup, Denmark) and a visualisation system based on the immunoperoxidase method, with 3,3-diaminobenzidine (DAB) as a substrate (EnVision + System-HRP, Mouse, Dako, Glostrup, Denmark). The slides were counterstained with Mayer's haematoxylin. Positive and negative control slides were processed together with the evaluated slides. For the negative control, the primary antibody was replaced by the isotype-matched mouse IgG (Dako) at the appropriate dilution. For the positive control, normal tissues were processed in the similar manner as the evaluated slides (for α-SMA and CD31– feline intestine). Brown precipitate at the antigen site was regarded as a positive reaction. The slides were evaluated under a light microscope (BX63, Olympus, Tokyo, Japan) using CellSense (Olympus, Tokyo, Japan) software.

Vascular density evaluation

Vascular "hot spots" were selected for each tumour under low magnification (×100). Subsequently, vessels were quantified within a 2.37 mm^2 area at ×400 magnification. Any brown-stained endothelial cell or endothelial cell cluster that was clearly separated form adjacent microvessels, tumour cells, and surrounding connective tissue was considered as a single, countable vessel. The presence of vessel lumens, though typically observable, was not requisite for microvessel designation, nor was the presence of red blood cells within lumens considered a defining criterion. Mean values (with standard deviation) were calculated for groups with grades I, II and III.

Multinucleated giant cells evaluation

Multinucleated giant cells "hot spots" within tumour parenchyma were selected for each tumour grade under low magnification (×100). Subsequently, the cells were quantified within a 2.37 mm^2 area at ×400 magnification. Mean values (with standard deviation) were calculated for groups with grades I, II and III.

Statistical analysis

Differences in vascular density and multinucleated giant cell in groups with grade I, II and III were analysed using Kruskal–Wallis H test (one-way ANOVA on ranks) followed by Dunn's post-hoc test. Correlations between tumour grade, MGCs count, vascular density and mitotic

index were examined using Spearman's rank correlation (r: Spearman's rank correlation coefficient); $p \leq 0.05$ was considered statistically significant, and $p \leq 0.001$ as highly significant. The statistical analysis was performed using Statistica 14 software (StatSoft Inc., Tulsa, OK, USA).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04637-8.

Supplementary Material 1: **Supplementary Table 1.** Detailed characteristics of evaluated feline injection-site fibrosarcomas.

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Author contributions
MM: writing manuscript, project administration, methodology, investigation, funding acquisition, conceptualization, validation, resources. IO-D: supervision.

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Data availability
Data used in this study are available from the corresponding author on reasonable request.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Ethical approval and consent to participate
Ethics approval is not applicable under the Act of 15 January 2015 on the protection of animals used for scientific or educational purposes– Journal of Laws of the Republic of Poland (Journal of Laws 2015 item 266) and Local Ethics Committee for Animal Experiments. The study used archival specimens collected in the Department of Pathological Anatomy. Written informed consent was obtained from the owner or legal custodian of all animals described in this work for the procedures undertaken in this retrospective study. No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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