

Microcirculation density and maturity in uterine and soft tissue leiomyosarcomas: an immunohistochemical study

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Summary. The role of angiogenesis as a hallmark of tumor progression has been poorly explored in leiomyosarcoma, a rare but aggressive mesenchymal malignancy. We aimed to characterize microvessel distribution and morphology - including pericyte coverage - in a retrospective series of leiomyosarcomas of the soft tissues and the uterus. 41 whole-block tumor slides from formalin-fixed paraffin-embedded tissues were immunostained for endothelial-specific marker CD31 and microvessel density was quantified by assigning a grade to the frequency of CD31 positive microvessels. Vessel morphology and pericyte coverage were investigated by double-labeling for CD31 and either PDGFR β , α SMA, desmin, CD90, or CD146. We found that microvessel density correlated with tumor grade in leiomyosarcoma of soft tissues, in analogy with what has been established in several types of carcinoma. This did not apply to uterine leiomyosarcoma, possibly due to the abundant myometrial vascularization. The evaluation of perivascular cell markers related to vessel stability revealed immature microvascular networks with aberrant pericyte coverage, irrespective of tumor origin or grade. Our observations substantiate the role of angiogenesis in the progression of soft tissue leiomyosarcoma. A multiple-marker approach to the assessment of pericyte coverage can identify different profiles of vessel immaturity correlated with tumor grade.

Key words: Angiogenesis, Leiomyosarcoma, Microvessel density, Pericytes

Introduction

Leiomyosarcomas (LMS) are tumors of mesenchymal origin representing 10-20% of all soft tissue sarcomas (STS) and less than 1% of newly diagnosed malignancies (Jemal et al., 2002; Toro et al., 2006). Histologically they usually appear as fascicular spindle cell tumors with smooth muscle cell-like differentiation, that mainly originate in the uterus and the retroperitoneum. LMS are clinically aggressive tumors with a high metastatic rate; traditional chemo/radiotherapies are poorly effective in treating metastatic or unresectable tumors (about 50% of the cases) due to poor specificity and development of drug resistance (Antman, 1997; Ganjoo, 2010).

The recent introduction of targeted therapies against tumor angiogenesis holds the promise of improved therapeutic strategies that have been shown to be effective in several epithelial tumor models (Ferris et al., 2010). Angiogenesis is the process by which new vessels develop from existing ones to extend a microvascular network (Adams and Alitalo, 2007); while it occurs physiologically during development and wound healing, it is also considered a hallmark of cancer, leading to leaky, immature vessels with aberrant morphology, but still able to support tumor growth and dissemination (Hanahan and Weinberg, 2011). Several studies attest the complex interplay between tumor cells and endothelial cells (ECs) lining the vessels lumen (Weis and Cheresh,

2011). More recently, attention has been given to the angiogenic and pathological roles of the perivascular/mural cells surrounding the endothelium: vascular smooth muscle cells (vSMCs) and - on the smaller vessels - pericytes (Raza et al., 2010; Armulik et al., 2011). Besides their structural 'scaffolding' function, pericytes actively regulate capillary network formation and maturity (Ozerdem and Stallcup, 2003; Yana et al., 2007; Virgintino et al., 2008). Through the interaction with inflammatory cells and the secretion of molecular mediators, they seemingly contribute to the formation of a niche facilitating tumor metastasis (Olaso et al., 1997), while defective pericyte coverage has been implicated in tumor dissemination (Yonenaga et al., 2005; Xian et al., 2006).

In carcinomas, various microvascular features and proangiogenic factors have been successfully correlated with events of prognostic significance such as tumor growth and metastasis rate (Hansen et al., 2000; Nico et al., 2008), but whether the same considerations apply for sarcomas is still largely unknown. Evaluation of microvascular density (MVD), a good predictor of metastatic disease in several carcinomas (Weidner et al., 1991; Vermeulen et al., 1996), has proven controversial as a prognostic factor in sarcoma (West et al., 2005), although none of the few works concerning STS focuses on perivascular cells.

The purpose of this study is to describe vascular morphology and pericyte coverage in a retrospective series of uterine and soft tissue LMS. We combined evaluation of microvessel density with the assessment of a series of recognized pericyte markers - namely CD90, CD146, platelet-derived growth factor beta (PDGFR β), alpha smooth muscle actin (α SMA) and desmin (Nehls et al., 1992; Nehls and Drenckhahn, 1993; Lindhal et al., 1998; Song et al., 2005; Schwab and Gargett, 2007; Crisan et al., 2008) - to describe the impact of angiogenesis in LMS from different sites.

Materials and methods

LMS cases

This retrospective study, approved by the local Ethics Committee, includes sarcoma specimens from patients diagnosed with LMS over a period of 14 years in the University-Hospital of Parma and a control group composed of 10 myometrium samples obtained from uterine prolapses. LMS samples were divided as gynecologic (uterine) or non-gynecologic (soft tissue), and grouped according to the tumor grade previously assigned at diagnosis, ranging from 1 to 3 (FNCLCC classification for non-uterine LMS, Stanford criteria (Veras et al., 2011) for uterine LMS). Tumor grade was subsequently confirmed by a second pathologist for the present study. Due to their negligible occurrence, unusual LMS histotypes were not included in the patient population.

Immunohistochemistry

Immunohistochemical analysis was performed on 4 μ m thick tissue sections cut from formalin-fixed paraffin-embedded surgical specimens. For MVD assessment, slides were stained with an antibody recognizing the EC-specific surface molecule CD31 (1:100 dilution; Abcam, Cambridge, UK). To evaluate pericyte coverage, slides were double stained for CD31 and one of the following: CD90 (1:500 dilution; Abcam, Cambridge, UK), CD146 (1:400; Abcam, Cambridge, UK), PDGFR β (1:200; Thermo Scientific, Rockford, IL, USA), α SMA (1:200; Abcam, Cambridge, UK), desmin (1:150; Abcam, Cambridge, UK). Sections were deparaffinized in xylene, rehydrated in a graded alcohol series, boiled for 40 minutes in TrisEDTA buffer pH 9 to allow heat-induced antigen retrieval, and treated with 3% H₂O₂ for 10 minutes to inhibit endogenous peroxidases. Reactions were revealed with DAKO HRP Advance system and diaminobenzidine (DAB) (DAKO, Hamburg, Germany) and counterstained with hematoxylin. Double staining was achieved sequentially by visualizing the first antibody in brown with DAB as described above, then incubating the section with the second antibody and revealing it with Vectastain ABC system and Vector RED (Vector Laboratories, Burlingame, CA, USA). For better, sharper results, CD90, CD146 and PDGFR β were stained in brown with the vascular endothelium highlighted in red, while the colours were reversed for α SMA and desmin. Large vessels with a complete wall in peritumoral regions or in necrotic areas acted as an internal control for the reaction.

Microvessel counting and pericyte coverage evaluation

Microvessels stained with CD31 were counted in vital tumor areas, excluding those bordering on fibrotic or necrotic regions in order to distinguish tumor angiogenesis proper from vascular growth as a response to necrosis (Fig. 1). Applying a variation of the method set by Weidner et al. (Weidner et al., 1991), sections were observed at 40x magnification (4x objective lens and 10x ocular lens, 15.71 mm² per field), and depending on the neovascularization density of tumor areas revealed by CD31 staining, each field was subjectively assigned a score ranging from 1 to 4 by two independent observers, blind to the tumor grade. High-density fields and fields containing smaller, higher-density areas were observed at greater magnification (400x, 1.5708 mm² per field) to perform a direct count of capillary vessels. For every lesion, the maximum number of microvessels found in any of these high-power fields (HPFs) was noted down, then the average count for each LMS grade was calculated. At least three HPFs per section were evaluated.

Pericyte coverage and vascular morphology were assessed in HPFs chosen as above, on sections double-

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labeled for CD31 and one of the relevant perivascular cell markers. Average perivascular to endothelial marker ratio was calculated for each lesion and expressed as a percentage.

Statistical analysis

The weighted average of the MVD grade was calculated for each sample. Cases in each category (gynecologic or non-gynecologic) were grouped by grade and the Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were used to compare the average MVD score between different groups. Grade 1 non-gynecologic lesions were not included in the comparison due to their inadequately low number (n=2). The effect

Table 1. Clinicopathological features of gynecologic and non-gynecologic LMS.

Case no.	Sex	Age	Tumor site	Tumor depth	Tumor size (cm)	Tumor grade (FNCLCC)
1	F	41	Uterus	Deep	n/a	1
2	F	46	Uterus	Deep	4	1
3	F	41	Uterus	Deep	n/a	1
4	F	41	Uterus	Deep	3.5	1
5	F	59	Uterus	Deep	16	2
6	F	43	Uterus	Deep	10	2
7	F	37	Uterus	Deep	n/a	2
8	F	42	Uterus	Deep	8	2
9	F	82	Uterus	Deep	7	2
10	F	53	Uterus	Deep	9	2
11	F	61	Uterus	Deep	9	2
12	F	54	Uterus	Deep	4.5	2
13	F	34	Uterus	Deep	n/a	2
14	F	66	Uterus	Deep	13	3
15	F	49	Uterus	Deep	13	3
16	F	69	Uterus	Deep	24	3
17	F	54	Uterus	Deep	10	3
18	F	51	Uterus	Deep	n/a	3
19	M	65	Extremities	Superficial	2	2
20	M	78	Extremities	Deep	8	3
21	M	81	Head/Neck	Superficial	1.2	1
22	M	56	Head/Neck	Deep	4.5	2
23	F	81	Head/Neck	Deep	9	3
24	F	74	Retroperitoneum	Deep	n/a	2
25	M	35	Retroperitoneum	Deep	10	2
26	M	67	Retroperitoneum	Deep	3	2
27	F	42	Retroperitoneum	Deep	10	3
28	F	65	Retroperitoneum	Deep	4	3
29	M	86	Retroperitoneum	Deep	2.5	3
30	F	86	Retroperitoneum	Deep	12	3
31	F	72	Trunk	Deep	n/a	2
32	F	82	Trunk	Superficial	13	3
33	F	87	Trunk	Superficial	3	3
34	F	76	Visceral	Deep	7	1
35	M	78	Visceral	Deep	10	2
36	F	45	Visceral	Deep	5.5	3
37	M	47	Visceral	Deep	18	3
38	F	46	Visceral	Deep	1	3
39	F	67	Visceral	Deep	4.5	3
40	F	81	Visceral	Deep	17	3
41	F	49	Visceral	Deep	8	3

Tumor grade: classification according to the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC).

on MVD of other variables (patient age and sex, tumor size and specific body site) was evaluated by multiple regression analysis. All computations were performed with the VassarStats online statistical package (<http://www.vassarstats.net>).

Results

Tumor specimens

We analyzed 18 cases of uterine LMS and 23 cases of soft tissue LMS; the main clinicopathological features are reported in Table 1. Non-gynecologic lesions were heavily unbalanced toward cases with high tumor grade (61% grade 3, 30% grade 2, only 9% grade 1), probably because the deep location of several soft tissue LMS makes their early diagnosis difficult. Three additional superficial lesions had to be excluded because the large presence of cartilage tissue mixed with narrow masses of invading tumor made MVD non-evaluable. Gynecologic LMS were more evenly distributed according to tumor grade (22% grade 1, 50% grade 2, 28% grade 3).

MVD assessment

MVD increased according to tumor grade in non-gynecologic LMS (Figs. 1, 2); the Wilcoxon-Mann-Whitney test confirmed a significant difference between grade 2 and grade 3 lesions (MVD score 2.31 ± 0.21 and 2.97 ± 0.58 respectively, $p < 0.05$), with very few outliers. Although the number of available grade 1 lesions (i.e., two) was inadequate for statistical analysis, these had a lower MVD score (approximately 2.00) than grade 2 or 3 tumors and displayed no vascular hotspots, confirming the perceived tendency. Conversely, uterine LMS had comparable average MVD scores among the

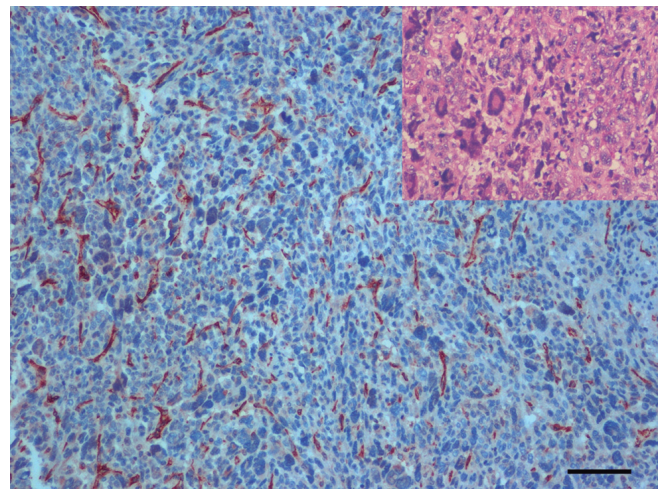


Fig. 1. Overview of the microvascular density in a high-grade LMS. Endothelial-specific immunostaining for CD31 reveals a complex and widespread microvascular network in a poorly differentiated gynecologic LMS with pleomorphic features. Bar: 100 μ m.

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three groups (no significant differences as estimated by a Kruskal-Wallis test) with an overall value of 3.12 ± 0.55 , similar to the scores of non-gynecologic grade 3 lesions (2.97 ± 0.58) and to the average MVD calculated for normal myometrium in peritumoral areas (3.17 ± 0.59) or in the control group (3.01 ± 0.42). A multiple regression test indicated that the influence of age or tumor size on MVD was negligible, and in the case of non-gynecologic LMS, specific tumor location and depth or patient age did not further affect the established correlation.

In direct HPF counts, much like in MVD scoring, the maximum number of microvessels in each lesion had a tendency to increase with tumor grade in non-gynecologic LMS only, but the considerable variance in maximum vessel number among samples allowed for no significant correlation. All data are summarized in Table 2.

Morphological assessment

Vascular morphology and maturity - as expressed by

pericyte coverage - were assessed by double-labeling for CD31 and one of several perivascular cell markers. Average prevalence of the perivascular markers for each group and grade are summarized in Table 3.

CD90 and CD146 presented a similar pattern and stained the vast majority of tumor vessels, including the smaller ones (Fig. 3C,D). There was no evident variation in their distribution across different LMS groups,

Table 2. Average MVD score and maximum number of vessels per HPF for each LMS group and grade.

Tumor grade	Average MVD score		Average max vessel count/HPF	
	Uterine	Soft-tissue	Uterine	Soft-tissue
Grade 1	3.17 ± 0.54	n/a	62 ± 19	n/a
Grade 2	2.98 ± 0.63	$2.31 \pm 0.21^*$	70 ± 3	47 ± 24
Grade 3	3.32 ± 0.43	$2.97 \pm 0.58^*$	51 ± 28	64 ± 27

*Indicates significant correlation ($p < 0.05$).

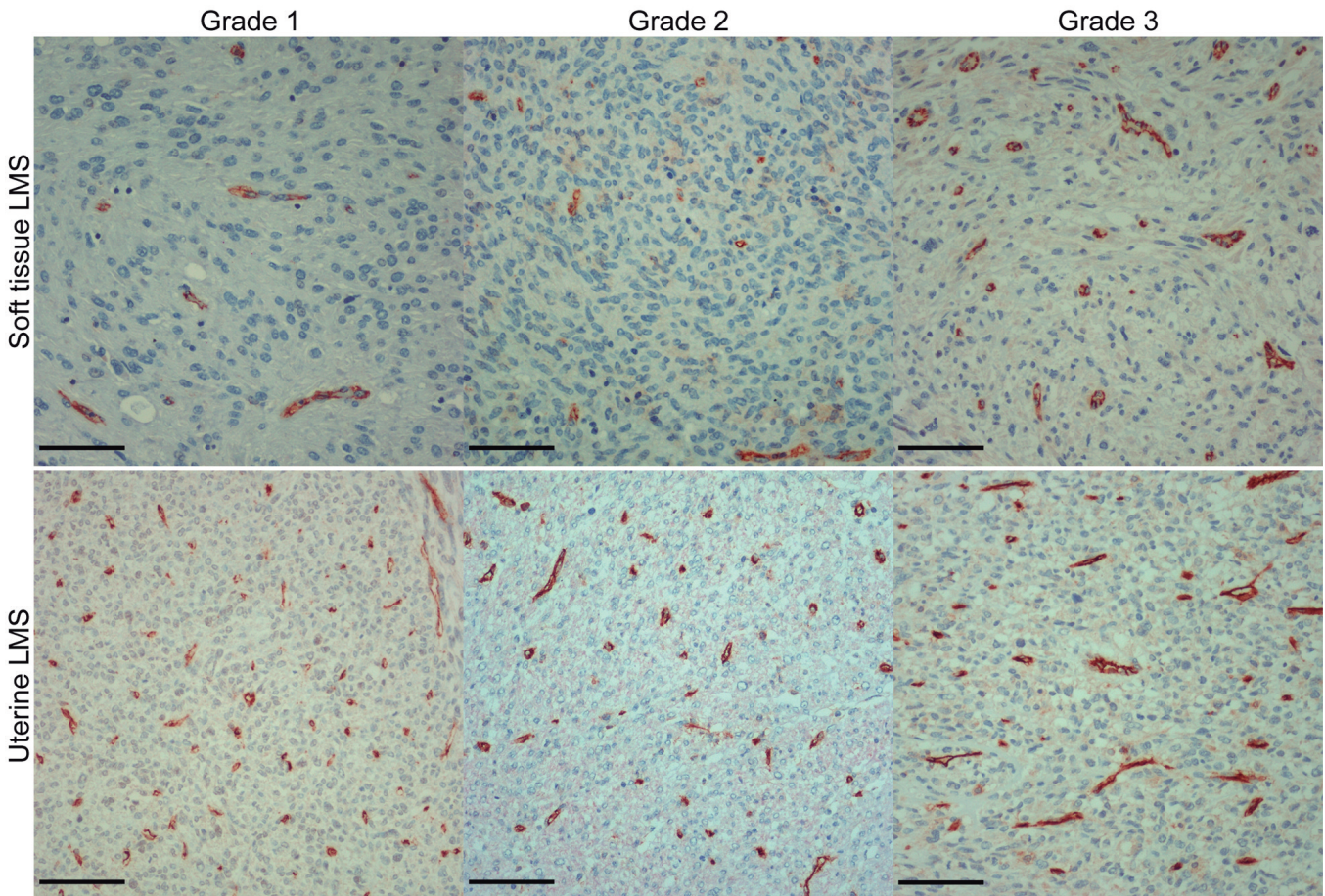


Fig. 2. MVD across different tumor grades. CD31-labeled microvascular networks in soft tissue LMS (upper row) and uterine LMS (lower row), grades 1 to 3. The fields were chosen to represent the average MVD score of any given LMS group and grade: from left to right, approximately 2, 2.3 and 3 for soft tissue LMS, and 3 or slightly above 3 for uterine LMS. Bar: 50 μ m.

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compared to the control group, or compared to a series of benign uterine leiomyomas used to optimize the immunohistochemical reactions (data not shown). PDGFR β distinctly highlighted the abluminal portion of about 70-85% microvessels regardless of lesion grade, but with a slight yet significant increase in soft tissue LMS; the coverage was generally discontinuous, with

several capillaries appearing to be naked (Fig. 3E). α SMA, a widely employed pericyte marker, was present on less than 10% of the tumor microvasculature. While larger vessels were properly enveloped by stratified α SMA⁺ vSMCs, coverage of the smaller ones was either discontinuous - similar to PDGFR β staining - or complete but sporadic, with a single layer of positive

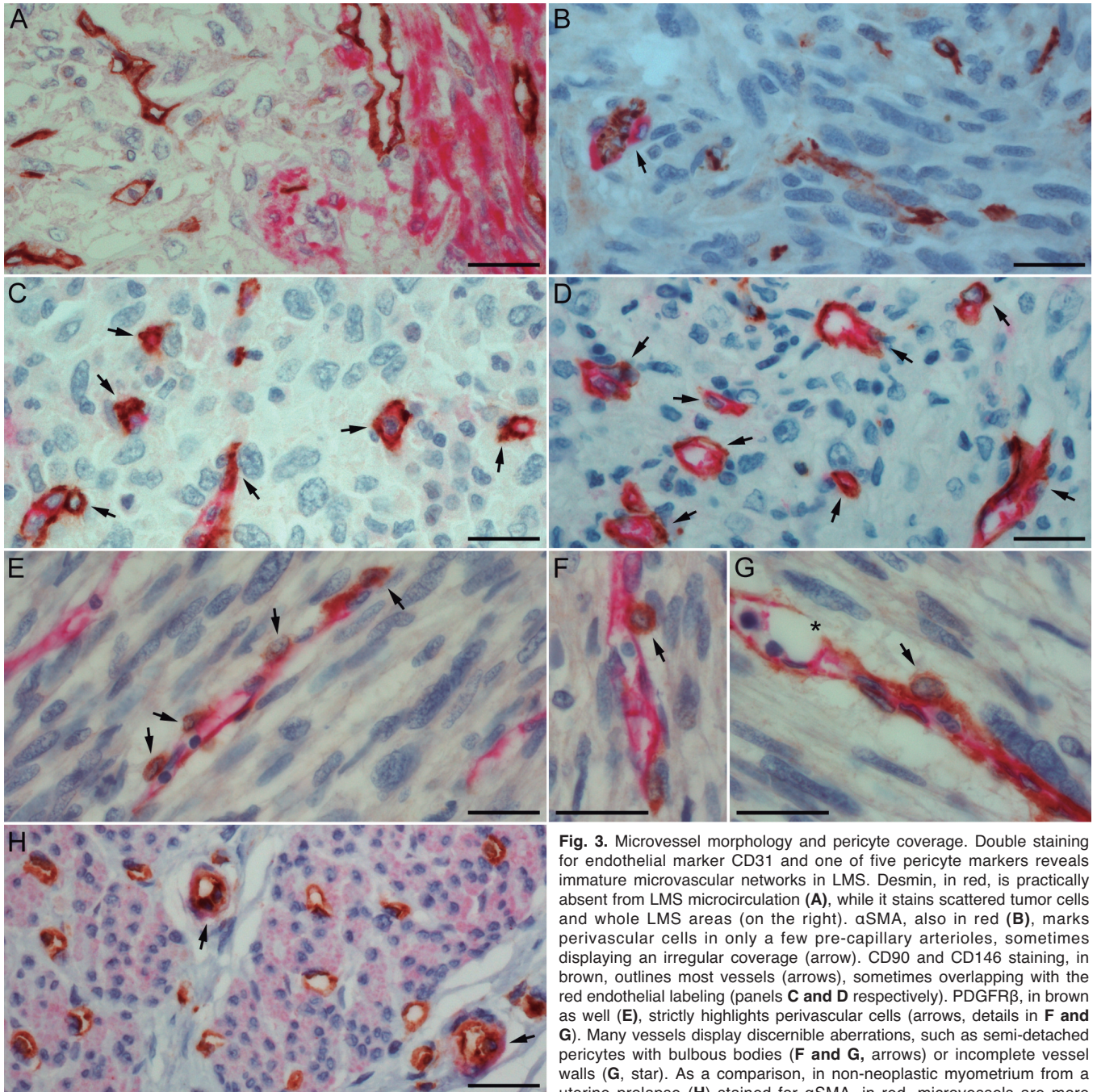


Fig. 3. Microvessel morphology and pericyte coverage. Double staining for endothelial marker CD31 and one of five pericyte markers reveals immature microvascular networks in LMS. Desmin, in red, is practically absent from LMS microcirculation (A), while it stains scattered tumor cells and whole LMS areas (on the right). α SMA, also in red (B), marks perivascular cells in only a few pre-capillary arterioles, sometimes displaying an irregular coverage (arrow). CD90 and CD146 staining, in brown, outlines most vessels (arrows), sometimes overlapping with the red endothelial labeling (panels C and D respectively). PDGFR β , in brown as well (E), strictly highlights perivascular cells (arrows, details in F and G). Many vessels display discernible aberrations, such as semi-detached pericytes with bulbous bodies (F and G, arrows) or incomplete vessel walls (G, star). As a comparison, in non-neoplastic myometrium from a uterine prolapse (H) stained for α SMA, in red, microvessels are more regular and pericytes are flattened on the CD31-stained endothelium (arrows). Bar: 25 μ m.

Table 3. Average prevalence of pericyte markers, relative to CD31 staining, for each LMS grade and group. Values are percentages, mean \pm SD.

Tumor grade	CD90 (%)		CD146 (%)		PDGFR β (%) *		aSMA (%)		Desmin (%)	
	Uterine	Soft tissue	Uterine	Soft tissue	Uterine	Soft tissue	Uterine	Soft tissue	Uterine	Soft tissue
Grade 1	91.0 \pm 3.4	n/a	90.9 \pm 3.8	n/a	73.8 \pm 9.5	n/a	7.5 \pm 6.1	n/a	1.5 \pm 1.3	n/a
Grade 2	88.0 \pm 10.1	87.7 \pm 9.2	92.9 \pm 8.7	89.5 \pm 7.7	70.8 \pm 11.2	86.4 \pm 7.8	3.1 \pm 3.6	3.7 \pm 4.9	0.9 \pm 1.0	0.6 \pm 0.7
Grade 3	90.3 \pm 4.2	89.6 \pm 7.0	92.5 \pm 4.5	92.1 \pm 5.1	73.6 \pm 9.6	87.2 \pm 9.3	4.7 \pm 3.6	2.5 \pm 3.0	3.3 \pm 4.2	1.0 \pm 1.6
Non-neoplastic	92.1 \pm 6.0		88.9 \pm 5.3		71.9 \pm 8.2		3.7 \pm 4.1		1.2 \pm 2.9	

*Indicates significant differences between uterine and soft tissue LMS ($p < 0.05$).

cells outlining the abluminal perimeter of no more than a few microvessels per low-power field, if any (Fig. 3B). These variations were usually observed in different samples rather than within the same lesion. Although a few cases showed substantial deviations in α SMA⁺ perivascular cells coverage - more specifically, one grade 1-2 and two grade 1 lesions displayed a percentage of positive vessels much higher than average - no significant pattern could be linked to tumor origin or grade. Minor intratumoral differences consisted of the preferential arrangement of the microvasculature near clusters of large vessels or in close proximity to necrotic regions, but once again this occurred indistinctly in LMS lesions from every group and grade. Desmin behaved like α SMA, but its staining was even more sparse, less than 1% of the total microvessel number (Fig. 3A).

Although no correlation was found between LMS groups (including the control group) and perivascular marker expression, from a qualitative standpoint these stainings contributed to delineate blood vessel morphology. On average, among all examined LMS sections, more than three quarters of the tumor vessels showed an immature or intermediate (partially immature) phenotype, as defined by the presence of one or more aberrant features (Gee et al., 2003): reduced or absent lumen; thin and/or discontinuous endothelium; irregular shape, usually due to activated endothelial cells with a non-flattened appearance; intussusceptive-like growths. Pericytes, as some PDGFR β or α SMA stains effectively illustrate, were often seen to form a loose and discontinuous coverage even on the larger pre- and post-capillary vessels, and in many cases were polarized on one side of the vase. Instead of being flattened against the ECs they normally envelop (Fig. 3H), some pericytes took on a rounded, bulbous shape, with shortened cytoplasmic processes, almost or completely detached from the underlying endothelium (Fig. 3F,G). We tentatively assigned a 1-4+ score to some of these features (lumen reduction; gross vessel wall aberrations including gaps; pericyte coverage), but no correlation with any tumor characteristic could be found: the allocated values seemed rather to fluctuate as an expression of intratumoral heterogeneity.

Discussion

Although angiogenesis is a prominent feature of

cancer development, MVD has been rarely correlated with tumor progression in LMS and in sarcomas in general. In the present work we compared MVD to the histological features of a retrospective series of primary LMS, divided into two groups according to the current pathological classification: uterine lesions, the most common category, and soft tissue lesions, rarer but far more aggressive (Miettinen, 2010).

We report that MVD in soft tissue LMS increases with tumor grade, in accordance with observations made in several types of carcinoma, while MVD in uterine lesions shows no significant variation and is similar to peritumoral areas. As argued by a recent clinical study (Avdalyan et al., 2012), gynecologic LMS microcirculation seems to be heavily influenced by the thick, pre-existent vascular network of the myometrium, making MVD an ill-suited parameter to describe this kind of sarcoma.

HPF capillary counts in high-density areas, though validated in other tumor types (Vermeulen et al., 1996, 2002), seem to have little significance in LMS, probably because microvessels tend to be evenly spread rather than clustered in hotspots. Our data support the angiogenic differences between carcinomas and sarcomas observed by Tomlinson (Tomlinson et al., 1999) and suggest that microvessel distribution, though often overlooked, should be taken into account when choosing a method for MVD assessment.

An unresolved issue that could impact on the design of novel antiangiogenic strategies concerns pericyte coverage of tumor vessels in sarcoma. Pericytes are notoriously lacking definite, specific markers capable of identifying them unequivocally. Moreover, as Crisan and other investigators advocate (Crisan et al., 2008), sarcoma and pericytes have a mesenchymal origin and therefore share several differentiation markers, including CD90, CD146 and PDGFR β - any of which may be expressed focally or extensively by the tumor, confounding perivascular cell staining. This is particularly true of LMS, since by definition - especially in low-grade cases - its cells may have various smooth muscle-like features, including α SMA and desmin expression. As a matter of fact, in several of the sections we examined, at least a couple of stainings per case had to be excluded from analysis because pericytes could hardly be distinguished from the surrounding, similarly-colored tumor parenchyma.

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Overall, pericyte markers showed no significant quantitative variations among our case groups. The slightly higher proportion of PDGFR β ⁺ pericytes we found in non-gynecologic lesions might be explained by tissue variability, since the prevalence of this marker in uterine lesions matches the control group. Still, staining for different pericyte-related antigens ensured that, despite tumor expression of some markers, at least one reaction for each lesion would be informative for morphological observations, crucial in revealing the aberrant vascular phenotype we described. Notably, among the few α SMA⁺ microvessels we observed in our cases, some clearly displayed perivascular cells loosely arranged along the endothelium, similar to PDGFR β staining of activated pericytes (Fig. 3F,G). This suggests non-quiescent vessels with defective coverage and questions the traditional role of α SMA as an indicator of microvessel maturity (Nehls and Drenckhahn, 1993). Consistently, Eberhard (Eberhard et al., 2000) also reported high variability in α SMA staining across different types of malignant carcinoma, while others observed that its relation to the pericyte coverage index of adjacent normal tissue still needs validation (Vermeulen et al., 2002).

In conclusion, we have documented relevant alterations in LMS vasculature that confirm the importance of angiogenesis in the pathogenesis of this rare tumor type. The aberrant pericyte coverage revealed by immunostaining with multiple pericyte markers is reminiscent of what has been observed in many carcinomas (Raza et al., 2010) and may therefore be interpreted as an important modulator of both angiogenic and tumorigenic events. The MVD analysis we present supports differences in angiogenesis between LMS of the soft tissues and the uterus. Though the size and nature of our population advise us against inferring any clinical considerations, our data suggest that combined evaluation of microvessel density and morphology on larger, selected samples could help define correlations with end-point clinical features such as patient survival, and anticipate the impact antiangiogenic treatment could have on these tumors. Furthermore, we believe that similar studies applied to a larger spectrum of tumor types might help to establish 'vascular signatures' specific for each kind of lesion.

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