# MICROVESSEL SURFACE CORRELATES WITH KI67 EXPRESSION IN EXTRAGASTROINTESTINAL STROMAL TUMOURS – A CASE SERIES

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## ABSTRACT

**Introduction.** Neoplastic microenvironment represents a frequently studied subject in numerous studies. Angiogenesis, as a part of it, is a prognostic factor and a therapeutic target in several neoplastic diseases.
**Cases presentation.** We describe the angiogenetic process in a small series of extra gastrointestinal stromal tumours (EGISTs) and corelate it with the proliferation index. We retrospectively searched our institutions databases for EGIST cases. The resulting data were reviewed by two pathologists. Immunohistochemistry (IHC) was performed to

## RÉSUMÉ

**La surface des micro-vaisseaux est en corrélation avec l'expression de ki67 dans les tumeurs stromales extra-gastro-intestinales – une série de cas**

**Introduction** Le micro-environnement néoplasique représente un sujet fréquemment étudié dans de nombreuses études. L'angiogenèse, en tant que partie de celle-ci, est un facteur pronostique et une cible thérapeutique dans plusieurs néoplasies.
**Rapport des cas.** Notre objectif est de décrire le processus angiogénique dans une petite série de tumeurs

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highlight the vascular structure of the tumours and determine the proliferative index using ki67 staining. Subsequently, the whole slide scanned digital images were used to assess micro vessel density and vascular surface. Statistical analysis of measurements data yielded a negative correlation between the tumour proliferative index and vascular surface normalized to examined surface of the tumour (r=-0.5506 and p=0.0051). A correlation between micro vessel density and proliferative index of the tumour could not be established in our series (p =0.035).

**Conclusions.** Highly proliferative EGISTs have a reduced micro vessel surface compared to low grade tumours, possibly due to a limited development rate of the angiogenesis during the tumours' growth. Due to the rarity of this sarcoma, our study is limited by the small number of cases, as well as by the preanalytical procedures performed on the tissue that may alter the vascular area in different manners. Further studies on larger series are needed to confirm our results and to investigate other morphological parameters, to assess the utility of anti-angiogenic therapies.

**Keywords:** extra gastrointestinal stromal tumour, micro vessel density, angiogenesis, vascular surface, microenvironment.

### List of abbreviations:
CD 117 – stem cell factor receptor
CD 31 – cluster of differentiation 31 protein
CD34 – cluster of differentiation 34 protein
c-Kit – receptor tyrosine kinase
CSF1R – colony-stimulating factor 1 receptor
EGIST – extra gastrointestinal stromal tumour
GIST – gastrointestinal stromal tumour
HE – hematoxylin eosin stain
IHC – immunohistochemistry
MVD – micro vessel density
PDGFR$\alpha$ – platelet-derived growth factor receptor alpha
PDGFR$\beta$ – platelet-derived growth factor receptor beta
VEGFR1 – vascular endothelial growth factor receptors 1
VEGFR2- vascular endothelial growth factor receptors 2
VEGFR3 – vascular endothelial growth factor receptors 3
VEGF – vascular endothelial growth factor

stromales extra-gastro-intestinales et de le corréler avec l'indice de prolifération. Nous avons analysé rétrospectivement les bases de données de nos institutions pour les cas de tumeurs stromales extra-gastro-intestinales. Les données obtenues ont été examinées par deux pathologistes. Une immunohistochimie a été réalisée pour mettre en évidence la structure vasculaire des tumeurs et déterminer l'index prolifératif ki67. Par la suite, des images numériques de la totalité des lames ont été utilisées pour évaluer la densité des micro vaisseaux et la surface vasculaire. L'analyse statistique des données a donné une corrélation négative entre les mesurages de l'indice de prolifération tumorale et la surface vasculaire normalisée à la surface examinée de la tumeur (r = –0,5506 et p=0,0051). Une corrélation entre la densité des micro vaisseaux et l'indice de prolifération de la tumeur n'a pas pu être établie dans notre série (p = 0,035).

**Conclusions.** Les tumeurs stromales extra-gastro-intestinales hautement prolifératives ont une surface de micro-vaisseaux réduite par rapport aux tumeurs à bas degré, cela en raison d'un taux de développement limité de l'angiogenèse au cours de la croissance et de la progression des tumeurs. En raison de la rareté de ce sarcome, notre étude est limitée par le petit nombre de cas, ainsi que par les procédures pré analytiques réalisées sur le tissu qui peuvent altérer la zone vasculaire de différentes manières. D'autres études sur des séries plus importantes sont nécessaires pour confirmer nos résultats et pour étudier d'autres paramètres morphologiques afin d'évaluer l'utilité des thérapies anti-angiogéniques.

**Mots-clés:** tumeurs stromales extra-digestives, densité microvasculaire, angiogenèse, surface vasculaire, micro-environnement

## INTRODUCTION

Extra gastrointestinal stromal tumours (EGISTs) are mesenchymal neoplasms located outside the digestive tract, which involves differentiation to Cajal-like type cells[1]. They are characterized by a spindle, epithelial, or mixed morphology and by a small amount of extracellular matrix. The tumoral environment contains vessels in varying proportions, accompanied by areas of hemorrhage and necrosis, as well as lymphocytic infiltrate. From a molecular point of view, they are characterized by mutations in either the stem cell stimulating factor gene (c-KIT) or platelet-derived growth factor receptor (PDGFR) gene. Mutations of the succinyl dehydrogenase genes encoding the constituent subunits can also be found but are uncommon. These genes and their encoded proteins are also involved in the process of tumoral and non-tumoral angiogenesis.

Angiogenesis in neoplastic proliferations represents the formation of new vessels and is part of the tumour microenvironment. The appearance of a benign tumour vascular system is the response of the host organism to neoplastic vasculogenic stimuli. The most studied stimuli are local hypoxemia, endothelial stimulation through neo agiogenesis factors, as well as local remodeling of the basement membrane following the secretion of metalloproteases. Angiogenesis has a major role in the supply of nutrients in the tumour bed, a role in distant dissemination, and in paraneoplastic syndromes. These structures are a justified therapeutic target for tumour regression, to reduce the rate of metastasis and increase tumour resection rate. Angiogenesis blockade is a therapeutic goal for several carcinomatous and sarcomatous proliferations[2].

A supplementary note should be made regarding the treatment of EGIST like the gastrointestinal stromal tumour (GIST) therapy, as both are based on a combination of tyrosine kinase inhibitors that have direct antiangiogenic effects. Thus, in practice, the therapy addressed to these neoplastic categories has anti-angiogenic results as side effects. This desirable side effect is also found in some second-line molecules but, in addition, they impact onto growth factor receptors. For example, Sunitinib was identified as an inhibitor of PDGFRα and PDGFRβ, vascular endothelial growth factor receptors (VEGFR1, VEGFR2 and VEGFR3), as well as stem cell factor receptor (c-Kit), and colony-stimulating factor 1 receptor (CSF1R)[3].

Previous English literature studies address the angiogenic issue in a more general manner, based on small case-series harbouring various histological tumour types[4–8]. Albeit the phenomenon is common to all neoplasia, it is not proven to be driven by the same amount and intensity of stimuli regardless of tumour type. Furthermore, to quantify the number of vessels per volume, the main method used is the counting of capillaries intercepting a fixed area of a grid (Chalkey method). The natural arborization of the capillaries and their serpiginous character result in quantification and classification issues when operating with this procedure. In terms of the logistics used in the previous investigations, most of the methods employ manual or semi-automatic techniques. In our opinion, in addition to the histological type, another aspect worth considering is to quantify the real vascular surface used for metabolic exchanges, or more precisely, the vascular perimeter. Most of the investigations focus on micro-vessels density (MVD) without considering their variable surface area, and the angiectatic phenomenon. From this point of view, one should not take into consideration the number of vessels alone, as they might be less significant compared to the vascular surface, since the latter participates in the exchange of metabolites and thus reflects the metabolic needs and the maturity of the tumour to assure the nutrient requirement. As a particularity of EGISTs, the micro vessels frequently suffer from angiectasia and aneurysmal dilatations, features that justify the cystic and hemorrhagic appearance of the central region in large tumours. Following these histological characteristics, we considered that either the area of capillaries or the perimeter should be more significant to characterize the micro-vessels tumoral environment.

## CASES PRESENTATION

The objective of this retrospective study was to quantify the homogeneity assessment of micro vascularization in EGISTs using the vascular surface and endothelial perimeter exposed to metabolite exchange. We intended to confirm a correlation between this parameter and the tumoral proliferative index.

We analysed the archive of the National Institute of Pathology "Victor Babes" from Bucharest, Romania, between 2005-2020, selecting cases with a histopathological diagnosis consistent with EGIST, and having a written informed consent from the patients. Besides full histopathological and immunohistochemical arguments, the inclusion criteria incorporated the existence of reserve material to perform additional stains and presence of quantifiable features (increased mitotic activity, areas of necrosis and hemorrhage), as well as a previously established proliferation index.

The study was approved by the Ethics Committee of the National Institute of Pathology "Victor Babes", Bucharest, number of approval 83/ 2020.

The paraffin sections were stained by hematoxylin-eosin (HE), and by immunohistochemistry (IHC),
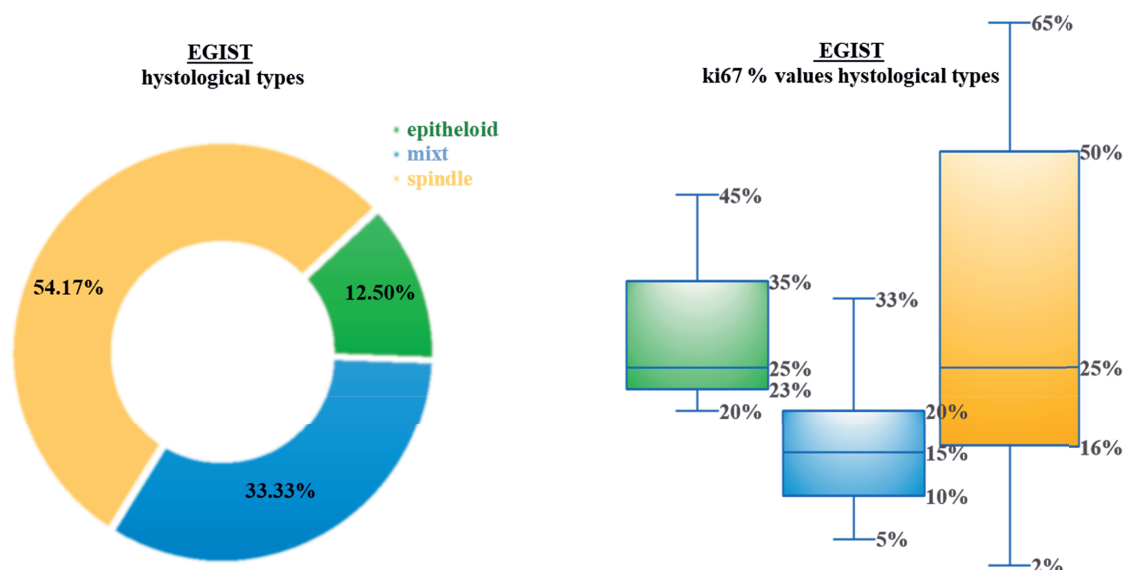
**Figure 1.** EGIST histological variants presented in the study: left panel graphic representing each histological EGIST variant included in the series: epithelioid (12%), spindle cell (54.17%), and mixed type (33%). On the right panel the proliferative index appreciated by ki67 is represented for each variant in the series. Similar colour coding. Large overlap, including high- and low-grade tumours for each category with an average of 22% for the whole series (CI: -16%+28%).

using CD31, CD34 and ki67 antibodies as necessary, to complete the pre-existent tests. The IHC techniques were performed by the indirect method with primary anti-CD34 and CD31 antibodies (monoclonal clone QBEnd10 and clone 2H8), on Leica Bond 2 type platforms with double secondary reaction for amplification. Exposure of antigenic epitopes was done by wet heat (Heat-Induced Epitope Retrieval – HIER) in 10mM citrate trimolar solution at 97 degrees Celsius, pH 9. Counter-staining was performed using Mayer Hematoxylin and detection with diaminobenzidine (DAB). The resulting histological slides were digitally scanned to obtain the corresponding digital whole slide files ".SCV" type: Scanners used: Aperio T2 and Aperio LV1. The obtained files were examined using QupathM6 image analysis software and Aperio-ImageScope (freeware version 12.03) by subtractive methods[9,10]. The regions of interest were established manually, considering the area of interest of the tumour and the apparent distribution of vascularity (at least 2 rectangle – ROI – at the periphery and central, fields without necrosis, totalizing at least 2mm squared). Inside the regions of interest, micro vessels were automatically detected using the positive pixel detection method. By subtractive methods, we established neutral zones (vascular lumens, as well as other optically empty spaces) and the tumoral areas. Subsequently, the positive pixels identified corresponding to the morphology of the endothelial nuclei

were transformed into editable polygonal (annotation objects) – regions using Public domain Java scripts[11]. These were manually reviewed. We reviewed the junctions and nodes for open or incomplete geometry, and excluded false positive markings in stroma and myofibroblasts, to correct areas of concern by reanalyzing each region of interest using a manual digital circumscribing method. An analogous method was used for assessing ki67, detecting positive cells with similar morphology. Nuclear size and cellular area were used as parameters to distinguish tumour cells from activated lymphocytes and/or endothelial cells.

The following structural parameters were collected: tumour surface, the number of marked vessels and the normalized vessels parameter per unit area (in µm squares: the total vascular area, the capillary perimeter and their equivalents, normalized to the tumour surface). The ratios between perfusion area and tumour surface were plotted and compared relative to the ki67 index. To assess the homogeneity of the distribution of the capillary network, the average density of neighboring capillaries was collected on distinct regions (peripheral and central).

Subsequently, the measurements obtained were correlated among each other using statistical package SPSS and MS Office Excel365 with statistical analysis package addons.

We selected 24 cases (n=24) compatible for the study, after eliminating the cases incompatible for
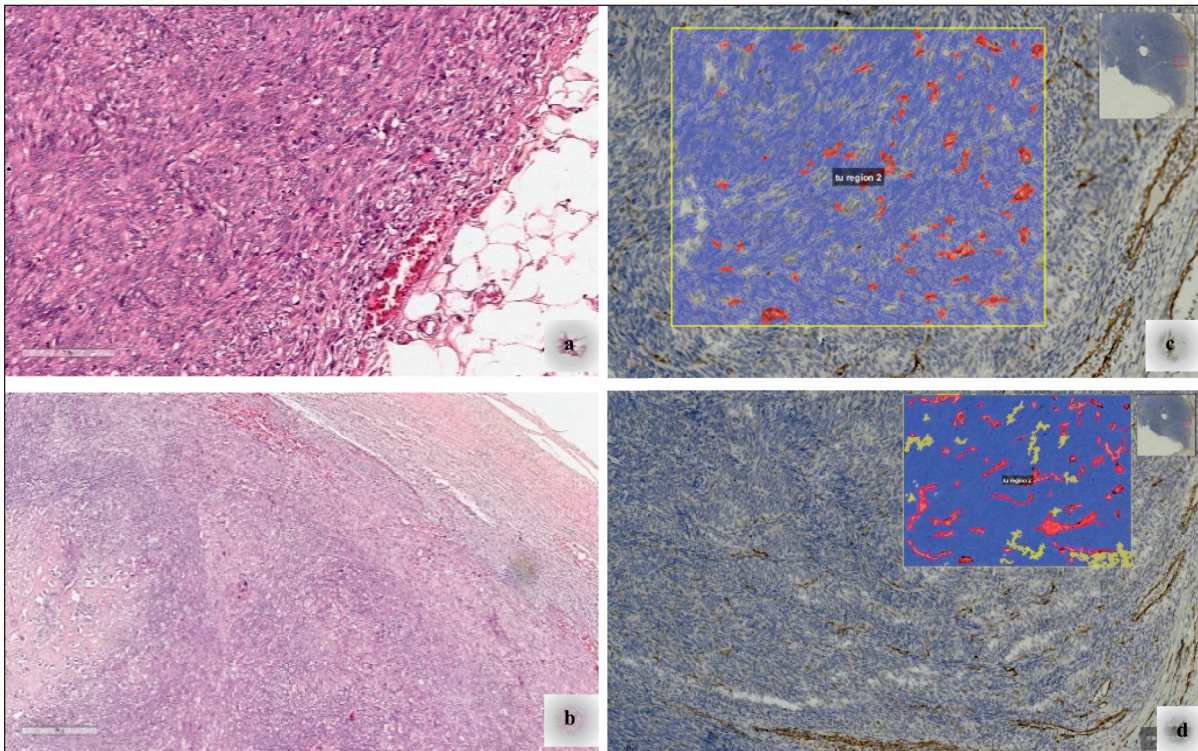
**Figure 2.** Histological and immunohistochemical (IHC) aspects of whole scanned slides. From top to bottom and from left to right: a) Spindle cell morphology variant EGIST, highly proliferative. Image at 20 X, scale bar 700 μm, HE stain. Periphery of the tumour exhibits flattened endothelial cells in the capillaries (mature type ones, gradually incorporated by the tumour lead. b) Epithelioid EGIST - highly mitotically active tumour, the periphery showing a low MVD. This case presented a large area of necrosis in the centre, as well as small islands of chondroid metaplasia (left corner of the panel). c) Screen capture of the digital analysis process of the scanned slides, positive cell detection step. Region of interest defined by the yellow rectangle, negative cell highlighted in blue, while positive on IHC with anti CD31, DAB-hematoxylin counterstain. Inserted "slide map" pointing the periphery of the tumour (second region analysed in this case). d) The conversion process of positive cell detection into measurable annotations. Red colour code for identified and measurable vessel surface, blue colour code for negative measurable area of the tumour, yellow code for neutral regions containing non-measurable stroma.

full digital scanning, because of broken slides or technical artifacts. The average age of the study group was 56.15 years. The series included three cases of EGIST of epithelioid type (12.5%), eight cases with mixed morphology (33%) and 13 with spindle cell morphology (54.5%) (Figure 1, left panel).

The series ki67 results were computed digitally, showing a range from 2 to 65% (average 22%, standard deviation 0.157). Inside the series, it was identified a subpopulation with increased mitotic activity (ki67 superior or equal to 20%) representing 33% of the series (9 cases out of 24) (Figure 1, right panel).

The parameters of interest were obtained using the specified digital analysis methods. A total of 57.31mm$^2$ tumoral surface was examined, comprising 31.666 positive vascular structures stained with CD34 and/or CD31. The average vascular density calculated this way resulted in 5.5 structures/mm$^2$. The vascular surface and vascular perimeter were also calculated, resulting an average examined vascular surface of $67.85*10^3*\mu m^2$ for each case and an average perimeter of $27*10^3\mu m$ (Figure 2).

The distribution of vascularization was heterogeneous, being more abundant in the peripheral areas compared to the central regions. Using the SPSS statistical analysis program and the MS Office Excel 365 statistical analysis package, we applied the Pearson test of statistical correlation among the tumour proliferation index (ki67), the number of capillaries, and the vascular surface normalized to tumoral area. The results showed that the correlation between the number of capillaries per unit area, and the tumour proliferation index for the analysed EGISTs is a negative type of association with moderate potency (r=-0.4319, p=0.035, null hypothesis cannot be rejected). The correlation between the tumour proliferation index and the ratio between the perfusion area and the investigated tumour area demonstrated a proportional negative type of association (r=-0.5506, p=0.0051) statistically significant (Figure 3).
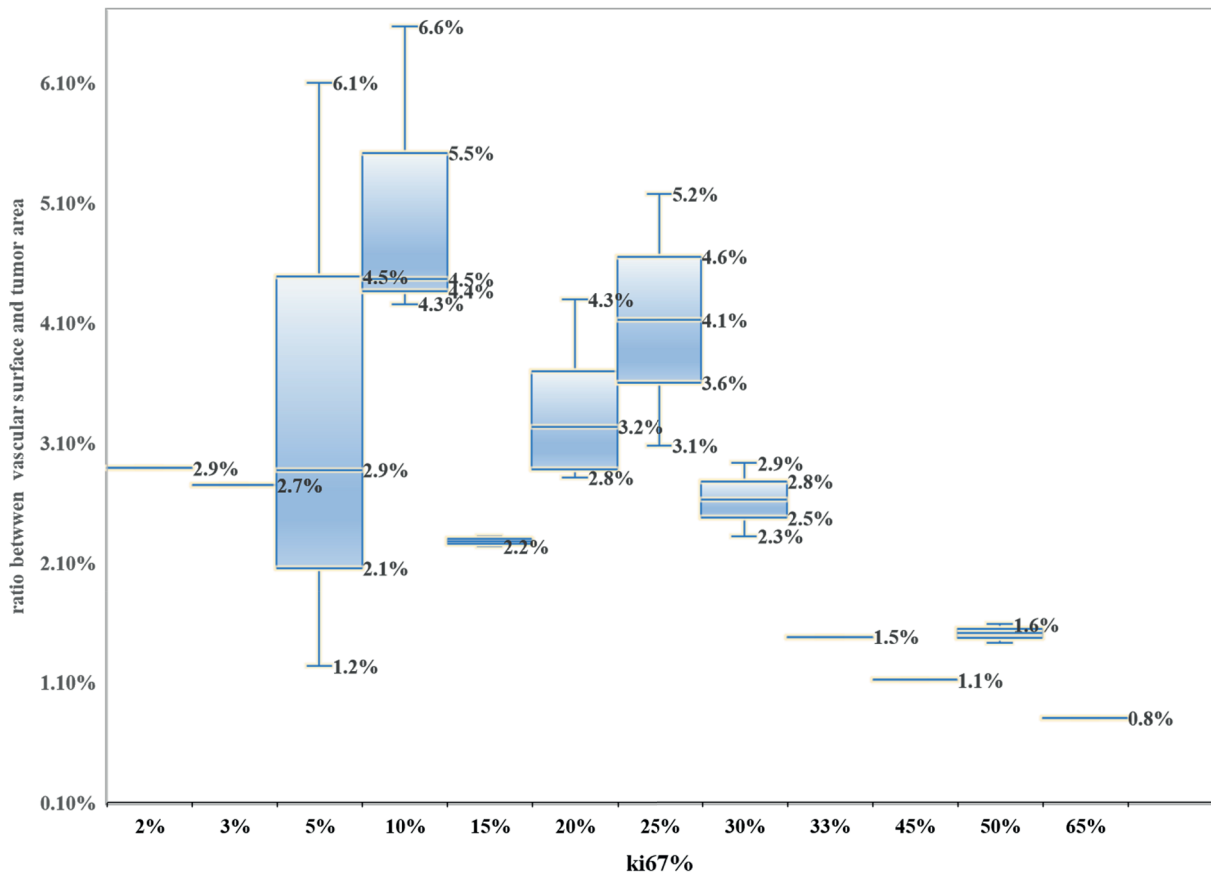
**Figure 3.** Plotting the ratio between vascular surface and tumoral surface area to proliferative index estimated by ki67 labeling. The descendent trends of ki67 percentage suggest a negative relationship between these parameters.

## DISCUSSION

The analysis of the tumoural micro-environment is a matter of concern for many researchers. The angiogenesis, as part of it, represents the base for the nutrient supply of all neoplastic processes, but also has a structural role in the progression of the tumour front. Studies published until now have several directions of research, such as: a) fundamental research of the molecular biology mechanisms involved in the process of angiogenesis to identify therapeutic targets; b) morphological studies that focus on the comparison between various tumour types of capillary density and c) correlation studies between the imaging aspect and the histological substrate of the neoplastic process. The last types of studies are used to estimate the aggressiveness of the tumour and to determine the surgical operability of the tumour, with direct applicability in tumour hemostasis in the surgery field.

Descriptive studies of tumour micro-vascularization by distinct histological categories are relatively scarce. Malignant soft tissue neoplastic proliferations are characterized by expansive centrifugal loco-regional growth. Unlike the carcinomatous neoplastic process, they are characterized by the lack of hot spots of angiogenesis and by a variety of metastatic mechanisms, blood metastatic pathway being favoured to the lymphatic one. Previous methods of investigation refer to well-known references[12] including parameters such as MVD, the distribution of pericytes, and the evaluation of endothelial proliferation using double labelling with CD31 and CD34, subsequently correlated with ki67 expression in the endothelium and/or endothelial proliferation factors.

Considering this approach, West et al.[7] demonstrated by using a CD31 staining for micro vessels, that MVD alone does not predict primary tumour metastasis and that the peripheral edge of the tumour has the lowest average MVD, followed by the centre of the tumoral mass and the necrotic areas. These findings indicate a heterogeneous distribution of the micro vessels, outcome that overlaps with our results. However, the conclusions drawn by the West's study are somehow more difficult to integrate, given the variety of histopathological tumour types included in their study, such as leiomyosarcomas, synovial sarcomas, pleiomorphic sarcomas and

malignant peripheral nerve sheath tumours, with different degrees of differentiation. Another aspect that should be taken into consideration was their sampling method, as well as the MVD counting in the regions of interest using the Chalkey's method. More recent publications[13] have focused on the evaluation of MVD looking for defined histological categories starting from the premise of a greater homogeneity of the batch and seeking more objectivity into digital analysis methods[14]. Up to date, the use of Chalkey's method of estimating tumour surface area and capillaries' number remains the favoured method for investigators. Nonetheless, it is difficult to apply this method in practice because of the subjective interpretation of the type of capillary that interests the study grid, the sampling method of the examined surface, and the heterogeneity of the sarcomas.

So far, the theory that any malignant process requires neo-angiogenesis[15,16] is only partially supported and can be explained only for some neoplastic processes. The hypothesis is that neo-angiogenesis is a mutual process in which both the host organism, as well as the tumour process, participate in different ways that complement each other. Thus, in response to the hypoxic factors released by the tumour, the host increases its own vascularization at the periphery of the tumour front. On the other side, the tumour tissue has the intrinsic ability to generate new vascular channels that produce vascular endothelial growth factor (VEGF)[17-20].

According to our investigations, the group of EGIST neoplasms had a subpopulation with increased mitotic activity and increased tumour proliferation rate (ki67 = 25-60%). This percentage is significant, representing 33% of the cohort (9 cases out of 24). The group is characterized by a decrease in the angiogenic process compared to well-differentiated tumours, with a ki67 threshold of less than 15%, (Figure 3). This reverse correlation between the surface of angiogenesis and the increasing tumoural growth rate can be interpreted as a difference between the development rate of the newly formed capillary network compared to tumour expansion. Consequently, an increased peripheral invasion will take over the pre-existing vascular structures of the host organism, thus suggesting a reduced ability of high-grade EGIST to develop its own vascular network. In this sense, the necessary tumour metabolites are supplied by the pre-existing vascular architecture of the affected region, including the lymphatic and blood capillaries. Thus, the process of tumour development is not dependent on its own vascular network and therefore the ability of neo-angiogenesis is not a mandatory condition for highly proliferative EGIST. Consequently, a diagnosis of a high-grade EGIST

established on multiple biopsies would rather benefit from a first-line treatment of a second-generation anti-angiogenic drug that will limit the ability of the host organism to respond to tumoural hypoxic stimuli. This hypothesis (perfusion surface inversely related to tumour grade) gets indirect arguments from high-grade carcinoma studies, showing flattened capillaries and reduced angiogenesis, furthermore, including larger distances from capillaries[20,21].

## CONCLUSIONS

Digitized semi-quantitative investigation of neoplastic proliferations should bring increased objectivity and reproducibility. This assumption is currently an ideal state due to the need for a pathologist to verify the automated detections, fact that brings subjectivity to the process. The pathologist must verify and decide on the accuracy of a region, and certify a certain tissue category, repeating in a digital form the subjectivity of manual interpretation. Nowadays, artificial intelligence and machine-learning techniques have entered a new era with the potential to remove the subjectivity of the investigator at least partially, but also to increase the processing speed and analysis of the investigated areas[22,23].

Our findings suggest that high-grade EGISTs have an inverse correlation between the degree of tumour activity and the surface of vascularization. A confirmation of the results of our study on larger groups is needed. Also, evaluating the processes and the evolution of angiogenesis under therapy are opportunities for future investigations. All of these should be assessed considering the limitations imposed by the series and rarity of this sarcoma, without forgetting that our digital histopathology study reflects a snapshot of a dynamic structure as all the vessel walls are, that responds to a large variety of stimuli.

**Compliance with Ethics Requirements:**

*"The authors declare no conflict of interest regarding this article"*

*"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as*

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