YEAR
2024

UNIVERSITY OF MEDICINE AND PHARMACY "CAROL DAVILA" BUCHAREST
DOCTORAL SCHOOL

FIELD OF STUDY: MEDICINE

The involvement of digital morphometric and molecular pathology in the study of mesenchymal tumors

PhD THESIS SUMMARY

PhD supervisor:
PROF. UNIV. DR. SAJIN MARIA

PhD student:
MOLDOVAN VALENTIN TIBERIU

2024

THESIS TABLE OF CONTENTS

Introduction

Oncological maladies, conditions related to the formation and development of malignant neoplasms, have been recognized and documented since antiquity, evidenced by historical writings and early medical observations. Among those of mesenchymal origin, the first mention is related to the Ebers Papyrus from 1500 B.C., which contains the earliest reference to a soft tissue tumor (1). This is described as "a fatty tumor," most likely of adipose origin, such as a lipoma. The father of medicine, Hippocrates (460–375 B.C.), distinguishes between carcinomas and sarcomas: "superficial and deep tumors at the level of the arm and thigh in elderly individuals." Charles Bell, a British surgeon (1774–1842), highlights the distinctive features that differentiate mesenchymal neoplasms from those of carcinoma type: "Soft cancer can attack any of the structures of the body..., it certainly appears in the soft substance of the limbs"(2). Currently, sarcoma is accepted as a malignant tissue proliferation constituted by cells of mesenchymal origin, excluding hematopoietic cells. The list of malignant and benign conditions with this connotation is significant, encompassing over 80 entities that have been included in the classification of soft tissue and bone tumors (3–5).

Mesenchymal neoplastic proliferations of the EGIST and GIST types have been the subject of constant interest by the teaching team led by Professor Sajin M., which has directly and indirectly contributed to the realization of numerous articles in the field of gastrointestinal stromal tumors. EGIST requires investigation using morphological, immunohistochemical, and molecular biology methods. EGISTs and GISTs are known for their molecular diversity, particularly their association with KIT or PDGFRA gene mutations. This aspect provides opportunities for studying molecular mechanisms. Understanding the anatomical and pathological particularities of GISTs can be beneficial for both scientific research and the improvement of clinical management of patients affected by these mesenchymal tumors.

Computer-assisted diagnostic methods are focused on image recognition and identifying decision trees to appreciate semi-quantitative histomorphometric parameters. The statement that they will become indispensable tools in the near future is plausible, as they are already being experimentally utilized in various fields, such as differential diagnosis and therapy in internal medicine and hematology.

The term "GIST" was first used in 1983 by Mazur and Clark to describe gastric tumors with mesenchymal differentiation (6). For those with a morphology similar to those originating from the neural crest, the generic term "gastric autonomic nerve sheath tumors (GANT)" was preferred, first described by Harrera (7,8). The discovery in 1998 by Hirota and colleagues that GIST tumor cells often have mutations in the KIT gene marked a moment in the reclassification of gastrointestinal sarcomas (9). Most tumors previously diagnosed as gastrointestinal autonomic nerve tumors (GANT) have been reclassified as GIST following the demonstration of KIT mutations. Extra-gastrointestinal stromal tumors (EGIST) represent sarcomas with differentiation towards cells resembling Cajal cells but with an extra-gastrointestinal location. The incidence of EGIST is estimated to represent a smaller percentage of less than 5% of all digestive stromal tumors (10–12). They exhibit a profile similar to that of GISTs both in terms of mutational and morphological and immunophenotypic aspects(8,13). KIT mutations are also found in sub-centimeter GISTs, suggesting that KIT mutation is the initial tumorigenic event in most GIST (14).

Aim and Objectives

The aim of the study is to analyze the morphological and immunophenotypic spectrum of extra-gastrointestinal stromal mesenchymal tumors with a digital semi-quantitative analysis of the tumor microenvironment.

To achieve this aim, we have established the following objectives:

- Evaluating the prevalence of EGIST cases in the working laboratory of the department of pathological anatomy.
- Analyzing and assessing the working techniques for the modern diagnosis of EGIST-type tumors.
- Analyzing the tumor microenvironment of EGIST concerning induced neo-vascularization, across histological categories, using processes and techniques specific to digital pathology to achieve comparable semi-quantitative parameters.
- Evaluating the host immune response to the neoplastic process by identifying and quantifying the principal actors in native cellular anti-tumor immunity, including localization and age.

s

Materials and Methods

The studies are based on the investigation of repositories, database and archives of the National Institute of Pathology "Victor Babeș" (INCDVB) as well as the University Emergency Hospital Bucharest (SUUB). The research was conducted with the approval of the ethics committees of the mentioned institutions. The study period for EGIST includes all diagnosed cases from 2005 to 2021 (16 years). Histopathological studies and immunohistochemical (IHC) tests were performed on paraffin-embedded tissue fragments. IHC examinations were conducted using an indirect protocol on the Leica Bond II platforms, with ready-to-use antibodies from Leica Novocastra, Agilent, and Abcam. Depending on the case, cocktails of pancytokeratin, Vimentin, S100, Desmin, CD34, CD117, DOG1 were used. Immunohistochemical examinations of each slide batch were performed with external positive control tissue slides simultaneously with the study batch.

The obtained slides and pre-existing ones were fully scanned using Aperio T2 and Aperio LV1 systems, manufactured by Leica Microsystems. The resulting files were examined using image analysis software such as QupathM6 and Aperio-ImageScope. Image analysis was conducted to estimate the perfusion area as well as the host's immune response. Semi-quantitative parameters for each objective were designed and checked through repeated manual sampling. The resulting data were statistically processed using both the GNU PSPP (v 1.6) statistical analysis package and the spreadsheet software from MSOffice 365 – Excel, extended with the statistical analysis package for graph generation and standardized uniform tabular data collection.

Results

The investigations conducted on the institutions' databases produced a cohort of 51 EGIST cases (n = 51). The age at diagnosis ranged from 26 to 80 years (mean age 56.15 years). The gender ratio, male/female, was 1:2. The primary location of EGIST in the study cohort was predominantly in the abdominal region (42 cases - 82%). Most cases (50%) demonstrated a conventional IHC profile: DOG1 expression, along with positivity for Vimentin, CD117, and CD34. In 10 cases (20%), cKIT expression was absent. The number of analyses performed varied significantly, primarily influenced by the unusual location of these neoplasms, ranging from 7 to 18 tests per case.

For the study of micro-vascularization, we selected twenty-four cases (n = 24). The mean age was 56 years (56.15 years) with a gender ratio of 1:1. The parameters of interest were obtained using digital analysis methods. Vascular area and vascular perimeter were also calculated. Statistical analysis results showed a negative proportional correlation between the tumor proliferation index and the ratio of perfusion area to the investigated tumor area (r = -0.5506 and a p-value of 0.0051).

Investigation of intratumoral lymphocytic infiltrates via image analysis techniques showed a predominance of the T cell line over the B cell line in EGIST cases. On average, CD5 positivity was 1.6*10^3 cells/mm2. TIL positivity for CD20 showed an average density of 263 B lymphocytes/mm2. Counting TAM using nuclear size restriction parameters and CD45 detection did not yield consistent results. The quantification of lymphoid cell aggregates per sample recorded an average of 2.41 foci/sample.

Discussions

The present study highlighted the variable immunophenotypic and morphological spectrum of EGISTs, increasing awareness of the sites of these entities. The IHC marker of choice for EGIST is DOG1, along with an additional marker to highlight differentiations from a similar Cajal cell profile (CD117 and secondary PDGFRA). CD34, CD10, and SMA have limited use in confirming the cell line. Histopathological diagnosis remains the most practical and reliable tool, with lower costs compared to molecular biology (extensive sequencing). The series of cases is substantial compared to other case series published in the specialized literature. Morphologically, the study revealed classic aspects of tumor proliferations but also less common aspects, identifying the first EGST with chondroid metaplasia—a feature reported only in GIST until the time of the study's publication.

Our findings suggest that in high-grade EGISTs, there is an inverse correlation between the degree of tumor activity and the area of vascularization. Confirmation of the study results on larger cohorts is necessary. The study suffers from the limitations imposed by the rarity of this sarcoma, but digital histopathological examination reflects a snapshot of a dynamic vascular structure that responds to a wide variety of stimuli.

In our study, we were able to highlight the variable morphological immunophenotypic spectrum of TIL EGISTs. The main cellular actor in the immune response to the presence of EGIST is the TIL of T cells. The distribution of TIL and TLS is heterogeneous in EGIST.

We highlighted the potential of TIL density as an independent histological parameter from the morphological variant, as well as the proliferative rate.

Overall, the present work adopts an approach to the particularities of the EGIST tumor microenvironment. In general, the studies conducted have an interdisciplinary character, employing image processing and analysis techniques.

First study: Analysis of the immunophenotypic spectrum of extra-digestive mesenchymal stromal tumors

Gastrointestinal stromal tumors are neoplasms that differentiate towards cells with morphology and immunophenotype similar to Cajal cells. The extra-abdominal localization of these tumors is difficult to diagnose in current anatomopathological practice. Molecular biology studies and expert consultation in this field are often required. The advantages of pathology in this regard, whether morphological-histopathological or molecular, are undeniable.

In this regard, we retrospectively investigated a series of 51 cases of EGIST presented at the INCDV Babeș Bucharest and SUUB with the aim of exploring the expressed immunophenotypes and analyzing the procedures, number, and utility of antibodies required for a positive diagnosis. Additionally, we examined data regarding the frequency of this pathology in current practice followed by a review of the specialized literature to improve current diagnostic practices.

The working method was based on querying the institutional electronic and scriptural databases of cases presenting a diagnosis of EGIST between 2005 and 2021. Slides were fully scanned, and the resulting images were reviewed by two pathologists with experience in general pathology diagnosis and a particular interest in mesenchymal-origin tumors. For cases with diagnostic difficulties or improper preservation of histological slides, immunohistochemical examinations were redone, as needed, for various markers (e.g., pancytokeratin, ki67, DOG1, and PDGFRA). Analysis of the databases from the two institutions revealed a total of 54 cases of EGIST tumors. Following case selection by removing duplicates/recurrences, cases with uncertain diagnoses, secondary/metastatic determinations, a total of 51 cases remained. In the study series, the primary tumor location was in the abdominal region in 43 cases (84%). Most tumors exhibited spindle cellularity, followed by mixed and epithelioid types - the graphical representation is shown in Figure 1.

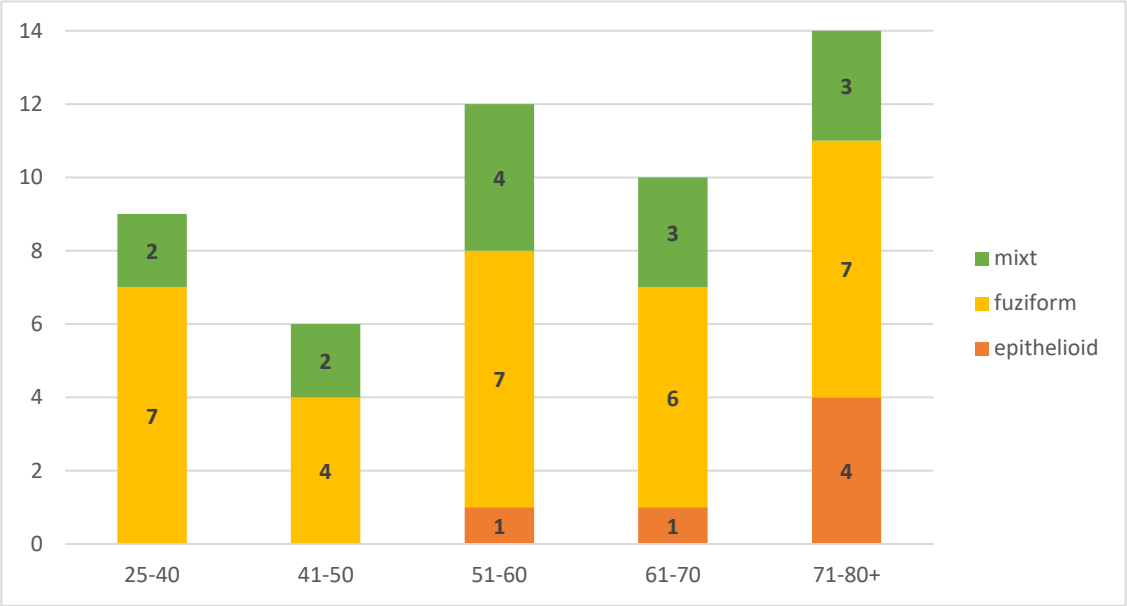

Figure 1 Chart representing the age distribution categories of the EGIST study group. Peaks of incidence are noted in the 50s decade and a second one after 70 years

Some of the microscopically analyzed cases exhibited particular histopathological aspects unusual for conventional morphology. Thus, some of these are unexpected in the case of EGIST: such as syncytial areas and myxoid degeneration (present in three cases, see Figura 2), while in others exceptional, rare aspects are encountered that may induce diagnostic traps in current practice. Thus, areas containing hyaline chondroid metaplasia were identified in one (1) case, and for three cases, the presence of scattered multinucleated tumor cells was noted. Remarkably, for cases with multinucleated cells, positivity for tumor markers was observed, and negativity for histiocytic markers.

It is noteworthy that only 26 cases (51%) demonstrated a complete conventional immunohistochemical profile: positivity for Vimentin, CD117, CD34, and DOG1. In 10 cases, c-KIT expression was absent, while the CD34 immunophenotype was preserved in all 10 cases.

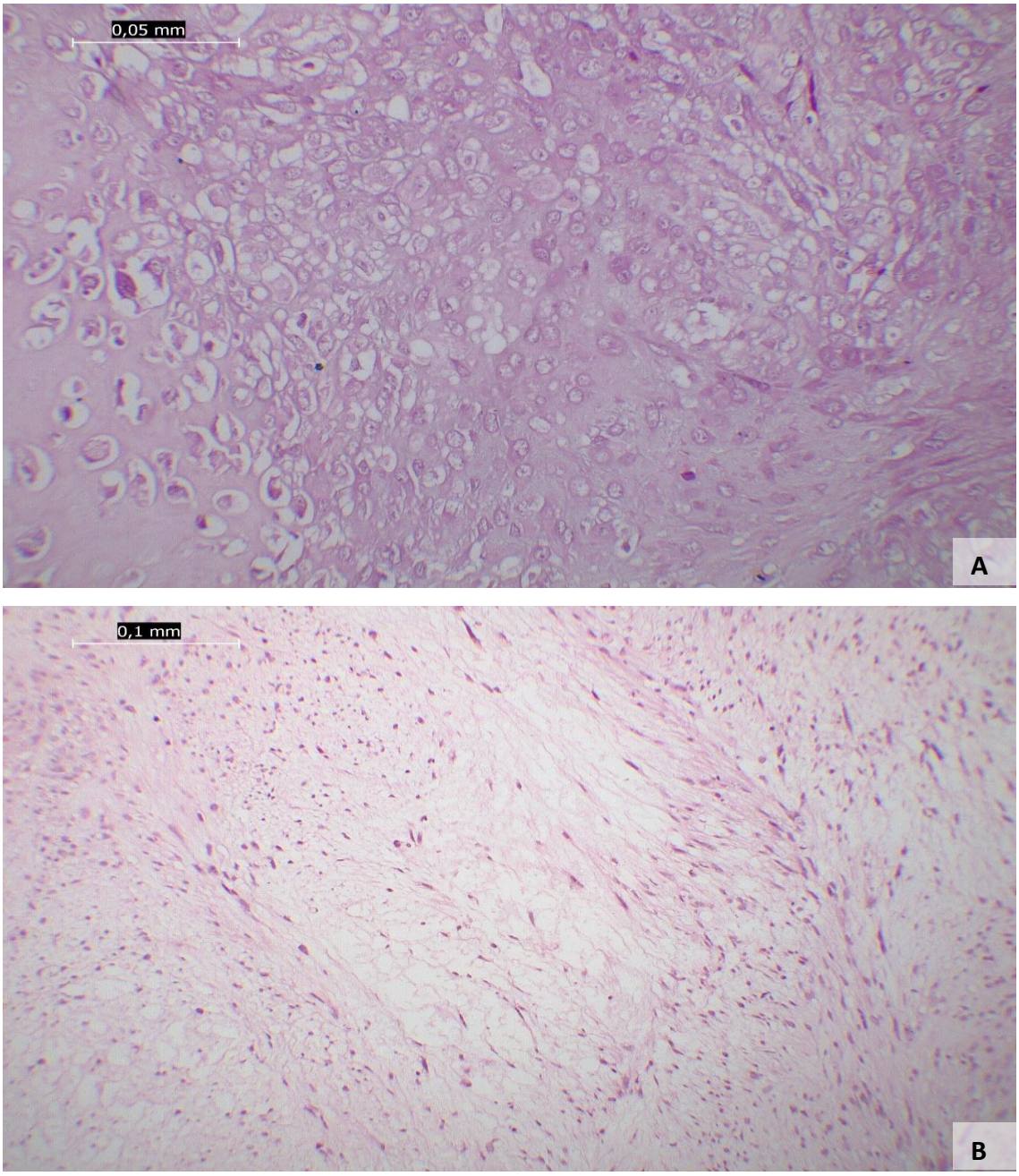

Figura 2 EGIST – particularități ale stromei. Cazuri excepționale au demonstrat modificări metaplazice de tip condroid (A). Acestea sunt vizibile ca o matrice incomplet matură în care celule tumorale se inter-mixează cu aspecte de tip loje în care se regăsesc celule tumorale retractate. În panelul de jos (B) se notează degenerescența mixoid-edematoasă a stromei cu tendința de a forma micro-cavități Colorație HE, captură foto-digitală cu reper metric în colțul din stânga sus al imaginii.

The presence of focal aberrant expressions for markers of the mesenchymal tumor line such as muscular actin, which can further shape the diagnosis - for example Figure 3. Of these cases, 8 cases tested positive for PDGFRA. In our study, we found a subgroup of 8 cases that were presented in locations not only extra-digestive but also extra-abdominal (among these, the first reported pulmonary EGIST and two cases with locations in the head

and neck area). In conclusion, from the analysis performed and the review of specialized literature, it is concluded that EGIST represents a histologically and immunohistochemically challenging subgroup, often showing aggressive behavior, more frequently cKIT negative and PDGFRA positive compared to GIST. From the analysis of the effectiveness of immunohistochemical markers, it was concluded that for a positive diagnosis, the marker of choice remains DOG1 regardless of proliferation location, while CD34 and CD117 should be considered as adjuncts in the extra-digestive localization of gastrointestinal stromal tumors.

The source of extra-digestive stromal mesenchymal tumors has long been debated. One hypothesis relates to the local maturation of precursors in the direction of the Cajal cell line, implying the appearance of an isolated mass of chondromyxoid nature plausibly during development. Subsequently, this may be exposed to a mutation in the cKIT gene and become

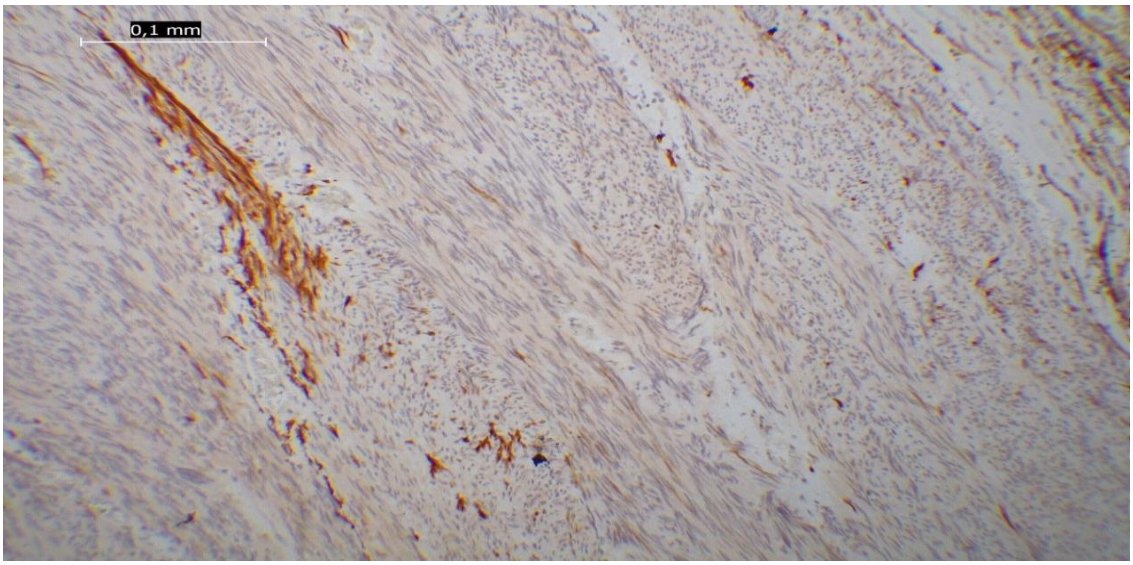

Figure 3 EGIST immunophenotype with reactivity to SMA. Intensely variable staining distributed in bundles of tumor cells. The staining can be variably or diffusely distributed but is not accompanied by other smooth muscle markers (such as caldesmone). Internal positive control in small caliber muscle vessels. Counterstaining with hematoxylin. Metric scale bar located in the top left corner.

a source of mesenchymal tumors, some with extra-digestive differentiation. In this regard, over a period of three years, the potential existence of hamartomatous or chondromyxoid sources that appeared during development was analyzed by examining histopathological analysis reports of pregnancies that ended in miscarriage and curettage products for elective terminations of pregnancy. The results were somewhat discouraging, as only one case of a mesenchymal tumor with pseudo-hamartomatous trilinear differentiation was identified (15).

Second study: Digital analysis of the correlation between the microvascular density of the tumor and Ki67 expression in extra-gastrointestinal stromal tumors

Angiogenesis in the neoplastic microenvironment is a frequently encountered area of interest in numerous studies due to its therapeutic target potential. The basic principle that there is no neoplastic proliferation without neo-angiogenesis is reasonably justified by practical experience in pathology. Neoangiogenesis, as part of it, has become a prognostic factor and a therapeutic target in several neoplastic processes. In the case of EGIST, this component is relatively underexplored, justifying the endeavor to describe and characterize the tumor vascularization process in this particular category of tumors.

The study aims to provide an overview of the vascular status as it relates to neo-angiogenesis in a subset of extra-gastrointestinal stromal tumors identified in the initial investigation. The primary goal is to identify quantifiable parameters using digital pathology techniques and correlate them with the tumor grade. These parameters will characterize the angiogenic process, including the quantification of capillary numbers and vascular surface area per unit area. Subsequently, the gathered results will undergo statistical analysis to establish correlations between common histoprognostic factors, the proliferation index, degree of differentiation, and histological variant.

Method: We conducted a retrospective analysis of our institutions' databases for cases of extra-gastrointestinal stromal tumors processed and analyzed in the initial study, with case selection to form a significant study cohort for the identified EGIST tumor population. The resulting data were reviewed by two pathologists. Immunohistochemical tests were performed to highlight the vascular structure of the tumors (CD31, D2-40, and CD34) and determine the proliferative index using immunohistochemical staining for Ki67 (see Figure 4. The histopathological slides were fully scanned with AperioLV1 Leica. Digital images from the scanning process of the entire slide were analyzed using the image analysis software Qupath to assess microvessel density and vascular surface area with the region of interest. The numerical data obtained were statistically analyzed using Microsoft Excel Statistical Package 365.

Results: Statistical analysis of the data resulting from the measurements revealed a negative correlation between the tumor proliferation index and the vascular surface area, normalized to the examined tumor area (r = -0.5506 and a p-value of 0.0051). A correlation

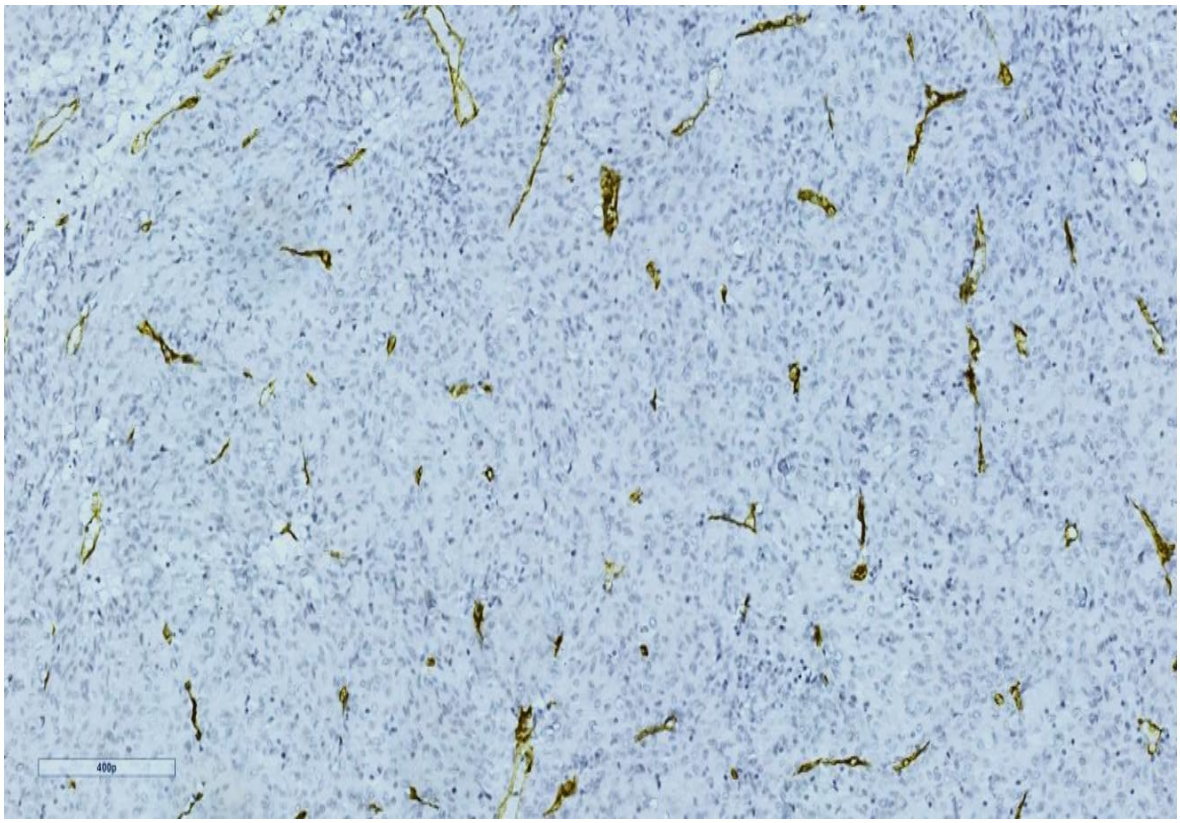

Figure 4 Epithelioid EGIST with CD31 staining shows easily identifiable small capillaries. Calculating the vascular surface area requires correlating with the tumor area. Image captured using AperioImagescope with a size bar in micrometers.

between microvessel density (number of capillaries per unit area) and the tumor proliferative index could not be established in our EGIST series (p = 0.035). The graphical representation is depicted in Figure 5.

Conclusions: Extra-gastrointestinal stromal tumors with high proliferative activity exhibit a reduced microvascular surface compared to low-grade tumors. This observation is plausible in the context of tumor expansion exceeding the angiogenesis rate. The process of angiogenesis and its correlation with tumor proliferation requires further investigation into the factors determining tumor metabolism for correlation with morphological structures, considering that both the vascular area and the permeability of the newly formed endothelium are influenced by endogenous vasoactive factors (constrictors or dilators) of the host organism, as well as intraoperative therapeutic-anesthetic agents. Following the confirmed hypothesis of a relationship between vascular parameters and tumor proliferation

rate, the study suggests that anti-angiogenic therapy would have a minor contribution in the treatment of high-grade EGISTs.

Third study: Computer-aided analysis of immune factors in the microenvironment of extra-gastrointestinal stromal tumors

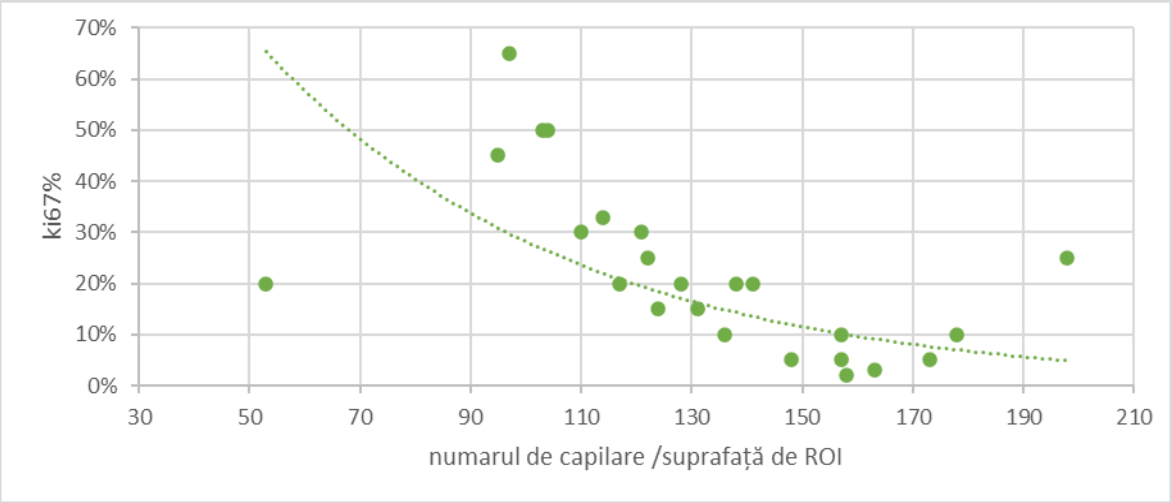

Figure 5 Graph depicting the distribution of cases based on the variables Ki67% and the total number of capillaries per ROI per case (n=24) shows a clustered pattern around a central cluster within the range of 2-33% Ki67 on the vertical axis and 95-178 capillaries/ROI, after excluding outliers (53x20y, 97x65%y, and 198x25y).

EGIST are sarcomas that are predisposed to various events triggering the host inflammatory response. Examples of events that can trigger such inflammatory phenomena include variations in perfusion flow, whether in the form of blood flow tensions or vascular caliber under the influence of various vasoconstrictive or vasoplegic factors. Neoformation vessels have low structural resistance, initially developing as simple endothelial channels without a branched arrangement. Such vascular structures will be characterized by increased fragility to pressure variations and mechanical stress from the surrounding environment. Variable vascularization, as mentioned, external mechanical compressive factors, as well as the appearance of areas with dual vascularization nearby (shunts), promote the emergence of hemorrhagic phenomena followed by inflammatory reactions. A similar inflammatory response can be found in tumors characterized by rapid growth, where areas of inflammation coexist with zones of tumor necrosis. Regardless of the mechanism of production, the

inflammatory response will recruit a leukocytic population that influences the pace of tumor growth.

Objectives: In this study, we retrospectively analyzed the type and quantity of intratumoral lymphocytes in a total of 22 cases from the EGIST series evaluated in study I. The study focused on the primary lineage composition as well as cellular density. Statistical correlation studies were conducted to highlight any specificities between host immune response and proliferation index on one hand, and between lymphocytic density and morphological changes on the other.

Methods: Using antibodies against CD5, CD20, CD45, we highlighted the intratumoral immune cells as well as the lymphoid structures through immunohistochemical techniques. The histopathological slides were fully scanned and digitally analyzed using QuPath software to select and calculate the density of infiltrating tumor lymphocytes (LTI). Tertiary lymphoid structures (TLS) were reported in each sample. Statistical analysis was performed using corrected Kruskal-Wallis test and Pearson coefficient. Statistical analyses on a subset of 19 cases included Pearson correlation, Kruskal-Wallis tests, and Bonferroni corrections to establish correlations between Ki67 expression and TIL densities, as well as associations related to age.

Results: From the series of cases identified in study I, we selected 22 patients with an average age of 51 years. Histologically, the sample consisted of EGISTs of spindle, epithelioid, and mixed histologic types (in proportions of 63%, 14%, and 23%, respectively). The numerical analysis of immunohistochemical slides showed an average proliferation index, calculated using Ki67 immunostaining, of 19%. The average density of T and B lineage TILs was 1.6*10^3 cells/mm2 (detailed calculation presented in Figure 6) and for B lymphocytes was 263 cells/mm2. TLS were identified with an average of 2.41 foci/sample examined (example is presented in Figure 7)). The correlation between Ki67 and T and B cell density as TILs shows a $p > 0.05$ in the selected series of EGISTs. The correlation between histologic variants of EGISTs and TIL density demonstrated a $p > 0.05$. Applying Bonferroni correction for small case series showed significant associations between Ki67 and CD05/CD20 positive TIL cells. Correlations related to age were noted, highlighting the complexity of the tumor microenvironment - the graphical presentation of these distributions is clearly visible in Figure 8.

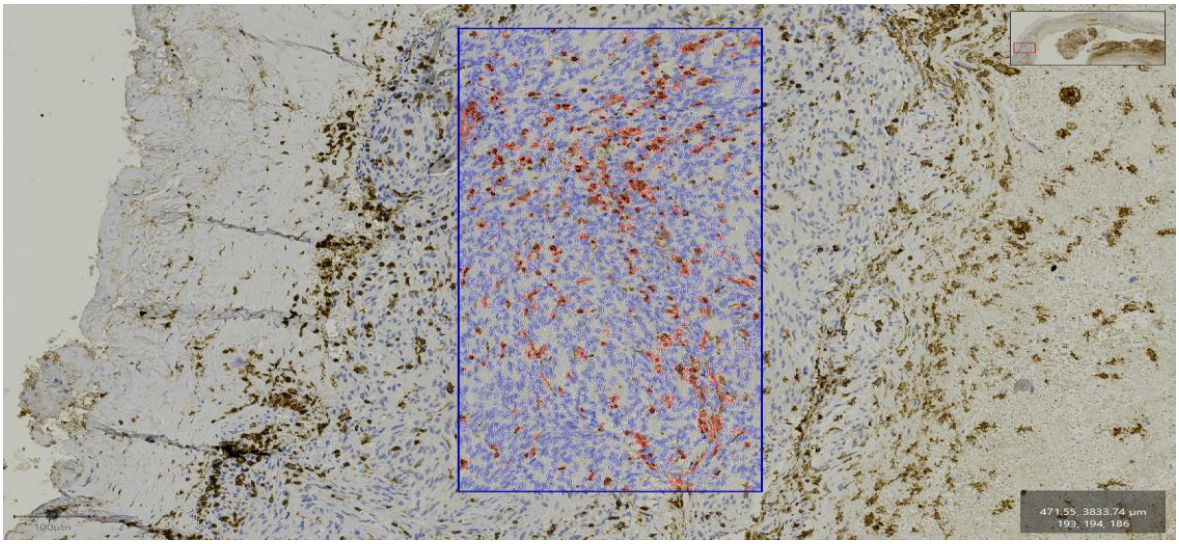

Figure 6 Spindle cell EGIST with parieto-abdominal localization. Detection of tumor cells by image analysis, positive staining for CD5 in red. On the right periphery, a cystic area with an accumulation of partly degraded lymphocytic infiltrate partially degraded by numerous macrophages with tangential bodies. Counter-staining with hematoxylin, DAB revelation. Scale bar located at the bottom left.

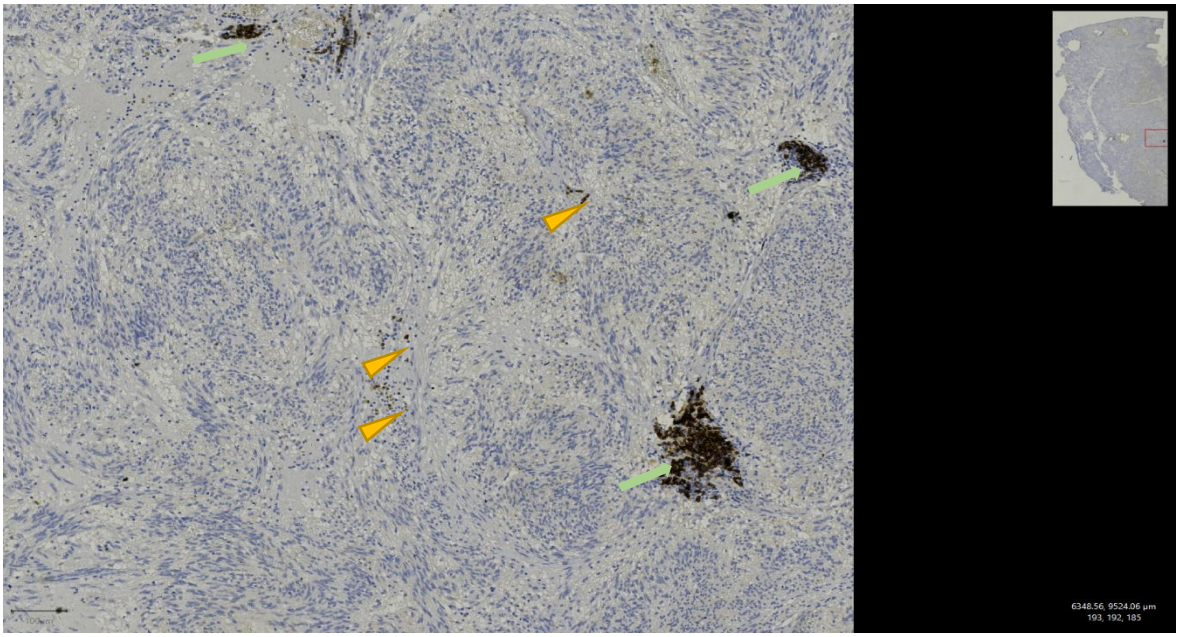

Figure 7 Positive staining for CD20 in intratumoral leukocytes in an EGIST. Tertiary lymphoid structures can be observed, marked with green arrows, and isolated B lymphocytes marked with yellow arrowheads. Screenshot by exporting fully scanned histopathological slide analyzed in Qupath v0.3.2. Scale bar located at the bottom left. Counter-staining with hematoxylin, DAB revelation.

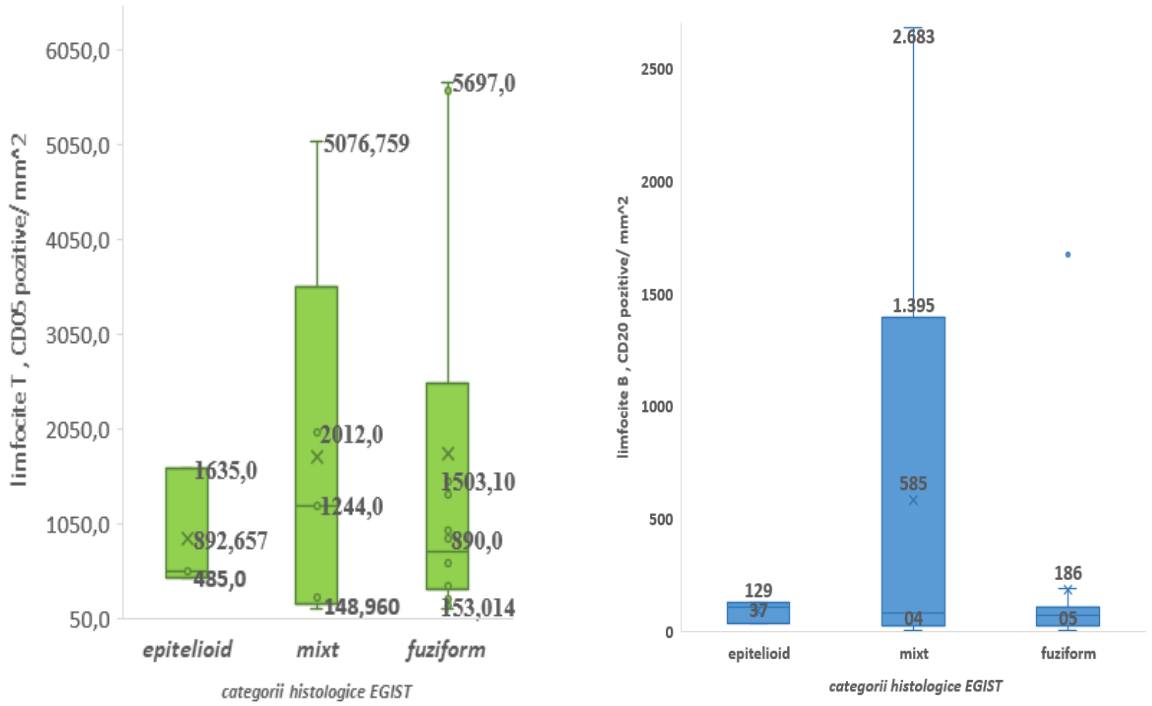

Figure 8 Graphic representation of the intratumoral density distribution of T and B lymphocytes, based on histological categories. Minimum values were observed in epithelioid and spindle-cell EGISTs, while mixed variants occupied a wide range of values for both B lymphocytes (right panel) and T lymphocytes (left panel)

Conclusions: TIL and TLS density are histological parameters independent of Ki67, proliferation rate, and histological type of EGIST. The TIL infiltrate in EGISTs is heterogeneous, predominantly composed of T lymphocytes. Tertiary mature lymphoid structures are heterogeneously distributed within EGISTs. Both TLS and TIL can be quantified using digital pathology techniques and can be used as parameters in assessing immediate immune response and recruitable immune capital in tumor immunotherapy.

§

Conclusions and personal contributions

Throughout the entire research process, we punctually addressed diagnostic methods and analyzed the tumor microenvironment using digital pathology techniques concerning image analysis as well as semiquantitative studies. The conclusions of the three studies on the examined case series can be summarized as follows:

1. EGIST is a tumor entity that can be located intra- and extra-abdominally. Morphological diagnosis of EGIST is imperative and should be supported by extensive IHC tests, complemented by testing for characteristic mutations: cKIT, PDGFR. Similar to GIST tumors, EGIST tumors have a similar immunohistochemical profile, including the mutational spectrum. The preferred IHC marker for the positive diagnosis of EGIST is DOG1, accompanied by an additional marker to confirm differentiation towards a Cajal cell profile (secondary CD117 and PDGFRA). Other markers such as Nestin, SDH, and PDGFR have limited use. They are useful in particular cases: double-negative CD117 and DOG1 cases - negative cell line markers. Conventional immunohistochemical markers in GIST diagnosis, such as CD34, CD10, and SMA, have limited use for confirming the cell line and reduced utility in direct EGIST diagnosis.

2. The combined histopathological and immunohistochemical tools remains the most practical and reliable approach in clinical diagnostics. Costs are significantly lower compared to those of molecular biology methods (with extended sequencing), making it useful in diagnosing at least 95% of EGIST cases - as confirmed by the conducted study..

3. In our study, using digital evaluation of Ki67 with the help of morphometric filters proved to be challenging to execute, requiring individual revision of each region of interest. The technique is plausible to implement but requires standardized pre-analytical working protocols.

4. From the research conducted in the first part of the study, it resulted that diagnosing EGIST located outside the abdominal cavity is approximately 50% more laborious compared to the number of tests for diagnosing EGIST. Therefore, the number of IHC tests for the morphological diagnosis of an abdominal EGIST is estimated at around 10 tests per case, while for an extra-abdominal localization, it can easily exceed 16 tests for each case, requiring reconfirmation through molecular biology analysis.

5. The second study of the thesis focused on evaluating the microvascularization of a selection of cases from the EGIST study group. Image digital analysis allowed the

characterization of the efficiency of digital detection of vascular structures in EGIST through various computer-based methods (subtractive using CD34 versus additive through CD31). Under similar cost conditions, the most consistent results, and consequently the most useful marker for digital analysis of vascular structures in EGIST, proved to be with primary antibodies against CD31.

6. In the second study mentioned in the previous section, data collected from digital image analysis demonstrated a relationship between intratumoral microvascular density and tumor proliferation rate, showing a non-linear negative correlation. Based on this finding, supported by personal research data, it can be deduced that the vascular proliferation rate and tumor proliferation rate are not synchronized (not directly proportional/positively correlated) to support the metabolic needs of EGIST. This perspective calls for further studies regarding tumor metabolic rate correlated with angiogenesis; research at the frontier between morphological and functional metabolomics and nutrigenomics domains. This conclusion also represents a personal contribution mentioned below in the corresponding section.

7. Our study focused on lymphocytes found in EGIST cases. B-lineage and T-lineage lymphocytes (TILs) are identifiable throughout the tumor front but in an unequal manner, predominantly at the periphery of the tumor front compared to the tumor center..

8. From the data obtained through digital image analysis in the third study, it resulted that the density of TILs (T-lineage and B-lineage) is a histological parameter independent of the proliferation index and morphology of EGIST. Future studies are needed to establish the potential relationship between this parameter and prognostic-evolutionary data, as well as therapeutic response.

9. The digital image analysis conducted on the tumor microenvironment of EGIST demonstrates that B-lineage and T-lineage tumor-infiltrating lymphocytes (TILs) are independent of the density of lymphoid aggregates and tertiary lymphoid structures within EGIST, as confirmed by statistical analysis.

10. In our study, we were able to highlight the variable morphological immunophenotypic spectrum of EGIST tumor-infiltrating lymphocytes (TILs). The primary cellular actor in the immune response to the presence of EGIST is the T-cell lineage TIL. The arrangement of TILs and tertiary lymphoid structures is heterogeneous in EGIST, revealing a discrepancy in immune triggers from one tumor area to another and from one case to another.

11. By correlating the results obtained in the three conducted studies, it resulted that digital image analysis is a versatile tool that can be used in diagnostic and comparative-morphological studies as well as investigational studies on semi-quantitative parameters concerning mesenchymal tumors.

Associated with the conclusions drawn from the analysis of EGIST proliferations, I contributed to the development of the theme of the EGIST tumor microenvironment and digital image analysis through the following aspects:

1. Through the morphological analysis of the entire series of EGIST cases, we highlighted unique morphological aspects, chondroid metaplasia, and multinucleated tumor cells, which have not been reported in other studies to date. (Chapter 6, 52;54).
2. Our study demonstrates the applicability of a new method for analyzing intratumoral microvascularization by using the capillary vascular area exposed to metabolic exchanges with tumor tissues. The method is innovative conceptually and technologically, utilizing digital pathology techniques and computerized image analysis. (Chapter 7, 103-109).
3. Using this method, we demonstrated in EGIST the existence of a correlation between intratumoral microvascular density and tumor proliferation rate, showing a non-linear negative relationship. (Chapter 7, p.115).
4. Through the research conducted in the third study, we highlighted in a modern way that the density of TILs and TLS are independent histological parameters of the proliferation rate and histological type for EGIST in the study group, with implications for immune therapeutic factors that can be used in EGIST therapy. (Chapter 8, p.144-145).
5. We have acquired evidence supporting the indirect reconfirmation of the immunological principle, which posits a decline in immune response intensity with advancing age. This was established by demonstrating a correlation between the density of T-cell-infiltrating lymphocytes (TIL) in EGIST and chronological age (Chapter 8, p. 148-149).
6. In a supplementary study, we have proven a relationship between the density of ki67-positive EGIST cells, indicative of mitotic activity, and patient age (Chapter 8, p. 149).

The results show that the research objectives detailed in the three studies have been fully achieved, highlighting the advantage of digital image analysis for rare or unusual cases. EGISTs are seldom encountered and challenging to diagnose in current practice. Their evolutionary potential and the management of prognostic factors are not yet well-codified,

mainly due to the lack of significant sample sizes for evolutionary studies. The advantage of digital analysis compared to other methods of lymphocyte population and tumor microvascular analysis lies primarily in the significantly reduced working time compared to manual quantification, which involves sequential analysis by one or more observers.

Future morphological research perspectives on EGIST may focus on identifying distinct precursors between PDGFR-competent and deficient EGISTs, given the different anatomical separation between interstitial cells with different immunophenotypes. Investigations may also delve into the functional analysis of these tumor formations, considering the local regulatory muscle properties of Cajal cells. Likewise, in the context of the tumor microenvironment, analyzing the vascular bed response to various vasoactive factors is a practically intriguing topic to highlight technical solutions that can be exploited as therapeutic adjuvants or in preoperative preparation. Another development direction is investigational studies that correlate angiogenesis progression under therapy with metabolic tumor data (aerobically deficient versus SDH-competent tumors proliferating in an environment with relatively low nutrient concentrations).

§

Selective bibliography

1. PAPYRUS EBERS. Bull Med Libr Assoc. 1912 Jul;2(1):12.
2. Bell Charlles. Surgical observations. 1817.
3. Choi JH, Ro JY. The 2020 WHO Classification of Tumors of Soft Tissue: Selected Changes and New Entities. Advances in Anatomic Pathology. 2021 Jan;28(1):44–58.
4. Jo VY, Fletcher CDM. WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition. Pathology. 2014 Feb;46(2):95–104.
5. Soft Tissue and Bone Tumours [Internet]. 5th ed. [cited 2022 Jun 30]. (WHO Classification of Tumours; vol. 3). Available from: https://tumourclassification.iarc.who.int/chapters/33
6. Mazur MT, Clark HB. Gastric stromal tumors. Reappraisal of histogenesis. Am J Surg Pathol. 1983 Sep;7(6):507–19.
7. Herrera GA, Cerezo L, Jones JE, Sack J, Grizzle WE, Pollack WJ, et al. Gastrointestinal autonomic nerve tumors. “Plexosarcomas.” Arch Pathol Lab Med. 1989 Aug;113(8):846–53.
8. Herrera GA, De Moraes HP, Grizzle WE, Han SG. Malignant small bowel neoplasm of enteric plexus derivation (plexosarcoma): Light and electron microscopic study confirming the origin of the neoplasm. Digest Dis Sci. 1984 Mar;29(3):275–84.
9. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science. 1998 Jan 23;279(5350):577–80.
10. Monabati A, Safavi M, Solhjoo F. Extragastrointestinal Stromal Tumor Presenting as Omental Cyst. J Gastrointest Surg. 2016 Jun;20(6):1275–7.
11. Angelo Paolo Dei Tos, Jason L. Hornick, Markku Miettinen, Ian R. Wanless, Eva Wardelmann. Gastrointestinal stromal tumour. In: Organisation mondiale de la santé, editor. Soft tissue and bone tumours. 5th ed. Geneva: OMS; 2020. (World health organization classification of tumours; vol. 3).
12. Qian XH, Yan YC, Gao BQ, Wang WL. Prevalence, diagnosis, and treatment of primary hepatic gastrointestinal stromal tumors. World J Gastroenterol. 2020 Oct 28;26(40):6195–206.
13. Goldblum JR, Folpe AL, Weiss SW. Enzinger & weiss’s soft tissue tumors. 7th ed. Philadelphia: Elsevier; 2019. chapter 17.
14. Rossi S, Gasparotto D, Toffolatti L, Pastrello C, Gallina G, Marzotto A, et al. Molecular and clinicopathologic characterization of gastrointestinal stromal tumors (GISTs) of small size. Am J Surg Pathol. 2010 Oct;34(10):1480–91.
15. **Moldovan VT**, Sajin M, Habago SD, Ali L. Type II pleuropulmonary blastoma in a fetus: case report of a rare mesenchymal mediastinal tumor and literature review. Arch Clin Cases. 2024;11(2):41–6.

§ §
§

List of published scientific papers

1. Morphological and immunohistochemical diagnostic of extragastrointestinal stromal tumors - a 51 case series analysis. **V. T. Moldovan**, Oana Maria Patrascu, Leila Ali, Mariana Costache, Maria Sajin. Romanian Journal of Morphology and Embryology, 2021 Vol. 62, No. 4, 1011-1016. ISI; DOI 10.47162/RJME.62.4.13, PMID: 35673820, Factor de Impact: 0.833. https://rjme.ro/archive/62/4/13/ (Capitol 6, 44-64);

2. Type II pleuropulmonary blastoma in a fetus: case report of a rare mesenchymal mediastinal tumor and literature review. **V. T. Moldovan**, M. Sajin, S. D. Habago, și L. Ali Arch Clin Cases, 2024, vol. 11, no. 2, pp. 41–46, BDI doi: 10.22551/2024.43.1102.10286 PMID: 38919847
https://www.clinicalcases.eu/index.php/acc/article/view/1082 (Capitol 6, 65-70)

3. Microvessel surface correlates with ki67 expression in extragastrointestinal stromal tumours – a case series. **V. T. Moldovan**, Patrascu OM, Sajin M, Ginghina O, Eftimie L, Birceanu A, Ali L. Arch Balk Med Union. 2021;57(1):91-98. BDI.. DOI 10.31688/ABMU.2022.57.1.11. https://doi.org/10.31688/ABMU.2022.57.1.11; (Capitol 7, 99-108).

4. Tumor Microenvironment Biomarkers Correlated with Proliferative Activity and Immune Response in Extragastrointestinal Stromal Tumors: Exploring Variations in Different Age Groups. **V. T. Moldovan**, Leila Ali, Maria Sajin. MAEDICA – a Journal of Clinical Medicine, 2024, 19(2), 233-238. BDI,
https://www.maedica.ro/articles/2024/2/2024_19(22)_No2_pg233-238.pdf;
DOI https://doi.org/10.26574/maedica.2024.19.2.233, (Capitol 8,127-129; 145-148).

§ § §
§ §
§