"OVIDIUS" UNIVERSITY OF CONSTANȚA

DOCTORAL SCHOOL

DOCTORAL FIELD: MEDICINE

HABILITATION THESIS

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL CONSIDERATIONS IN TUMORAL AND NON-TUMORAL PATHOLOGY

ABSTRACT

Associate Professor Mariana DEACU, MD,PhD

2024

This habilitation thesis represents a synthesis of my professional achievements from my beginning in higher medical education until now, which equally reflects my professional experience as a pathologist, and university teaching staff, as well as my experience in scientific research.

The habilitation thesis was written in compliance with the provisions of the Order of the Ministry of Education and Scientific Research no. 3121/21.01.2015, as well as in compliance with the Regulation of the "Ovidius" University of Constanța no. 141/16.05.2016, regarding the organization and development of the process of obtaining the qualification certificate and it is composed of three parts according to the recommendations of CNATDCU and the legislation in force.

The title of the habilitation thesis, formulated holistically, proposes a perspective on the results of some studies and research projects in the Pathological Anatomy specialty, presented in a systematized manner, which targeted non-tumor and tumor pathology.

The first part of the thesis includes the main personal achievements in the professional medical, academic, and scientific fields.

On a medical professional level, I started in 1996 as a general medicine intern, so later, through the national residency exam, I became a resident doctor in the Pathological Anatomy specialty. My professional training was completed once I obtained the qualification of a specialist doctor in 2001 and then, in 2005, as a senior pathologist. During all this time, I understood the importance of pathological diagnosis and my mission to work for the benefit of the clinician and the patient, aspects that still motivate me to improve myself continuously. From the perspective of the position of a chief physician, I was constantly concerned with the development and modernization of the service through the purchase of equipment, the arrangement of the workspace, the development of the immunohistochemistry technique, the increase in the quality of the services provided, the organization of the activity and the provision of an optimal working framework for team members from which I belong and interdisciplinary collaboration.

Academically, my career began in February 1998 as an Assistant Lecturer in the Pathological Anatomy discipline, Faculty of Medicine, "Ovidius" University Constanţa. During my academic career, I acquired the title of University Assistant in 2001, in 2006 Lecturer, and since 2013 I have been an Associate Professor. As a teacher, I supported activities of teaching courses and guidance of practical works, I developed for each course and practical work materials on electronic support in Microsoft Office - PowerPoint with notions specific to Pathological Anatomy, I participated as author and co-author in the development of course books and practical workbooks for medical students of the Romanian Division, respectively the English Division. I permanently contributed to the completion and updating of the didactic material with specifics, I insisted on the fixation by the students of the acquired notions and their application by developing the capacity to recognize and diagnose lesions from a pathological point of view, as well as making correlations with the acquired notions to the other preclinical and clinical disciplines. I participated as a supervisor in the elaboration of 30 bachelor's theses, and as a coordinator in 27, all graded with the highest grade. I am a year tutor for the General Medicine specialization, third-year, series A, from which perspective I have a good collaboration with the students and their representatives.

In terms of scientific research, my debut took place with the doctoral study, completed in 2007 by the public support of the thesis with the title *"Morphological study of mammary glandular structures and their involvement in dysplastic and tumoral lesions"*, developed under the scientific coordination of the late Prof. Univ. Dan Ulmeanu. The rich and diverse case history of the Clinical Service of Pathological Anatomy, where I work, allowed me to delve into other pathologies besides that of the mammary gland, with results materialized in: 17 articles published in extenso as first author in ISI-rated medical journals; 21 articles published in extenso as co-author in ISI rated medical journals; 18 articles published in abstract in ISI rated journals; 20 articles published in extenso in journals with ISSN/ISBN; 24 articles published in extenso in B+/BDI journals; 30 communicated/abstract papers, published in the journals of national and international scientific events. Thus, my scientific activity is currently described by scientometric indices calculated by the international databases ISI Web of Science (Core Collection) Hirsh index 7, respectively Google Academic index Hirsh 7. I have worked alongside well-known names in Pathological Anatomy from Romania to national projects realized in the Romanian translation of some established pathology treatises: ***RUBIN PATHOLOGY – MECHANISMS OF HUMAN DISEASES*** – Editor Wolters Kluwer, Hipocrate Publishing House, Eighth Edition, year 2022; ***CLINICAL PATHOLOGY PRACTICAL GUIDE*** – Editor James Carton, HIPOCRATE Publishing House, 2nd Edition, year 2017. I contributed, together with the members of the SCAP-SCJU Constanța Research Team, to the elaboration of the treatise ***GENERAL PATHOLOGY***, publishing house of the Academy of Medical Sciences, Publishing House ArtPRESS Timișoara, year 2020 under the coordination of Prof. Aşchie Mariana. I have participated since 2003, together with my colleagues from SCAP and also with the Scientific Research Group from SCAP-SCJU Constanţa coordinated by Prof. Aşchie Mariana, in carrying out studies within 17 scientific research projects, as a member- -executor, scientific manager, project director, expert.

The research themes that I pursued and presented in this work, had in mind the role of histopathology and the interdisciplinary approach to restrictive alveolar and septal lung lesions, the morphometry and scoring systems used in histopathology, as well as the evaluation of the constitutive elements of the tumor microclimate.

The role of histopathology and interdisciplinarity in restrictive lung lesions with alveolar or septal location. Restrictive lung diseases represent a large group of conditions, with extrinsic (extrapulmonary) or intrinsic origin (lung parenchyma lesions), with complex pathogenesis determined by both direct lung parenchyma lesions and systemic involvement. With the onset of pulmonary injury, inflammatory cells are recruited in the damaged organ, which has the role of repairing the tissue. They release cytokines, reactive oxygen species, growth factors, and chemokines, with a role in healing and at the same time, in supporting and promoting the inflammatory infiltrate. However, the persistence of inflammatory cells damages the extracellular matrix and stimulates fibrosis. Morphological changes constitute the substrate of functional changes. Pulmonary remodeling produced by inflammatory and fibrotic lesions is responsible for decreased compliance and increased static recoil pressure. All restrictive lung diseases have in common lesions of variable intensity of alveolar structures, interstitium, and/or small airways. Therefore, their correct diagnosis necessarily involves clinical, radiological, and histopathological criteria. Interdisciplinarity must be ensured by at least one pulmonologist, one radiologist, and one pathologist. This collaboration benefits the patient by increasing diagnostic

accuracy as a result of interobserver agreement. The obligation of multidisciplinary collaboration derives from the fact that all three basic branches (clinical, imaging, and histopathology) present certain limits that can only be overcome together. Cases such as *Pulmonary alveolar lipoproteinosis associated with emphysematous areas* represent a diagnostic challenge for all the specialties involved. It is a rare pathological condition, characterized by the alveolar accumulation of lipoproteinaceous material, as a result of the inability of macrophages to free the alveolar spaces from the consumed surfactant. The most common cause of this clinical-pathological syndrome is the development of autoantibodies that neutralize GM-CSF (granulocyte-macrophage colony-stimulating factor), which induces the primary form of the disease. The secondary form is determined by exposure to toxins or induced by other pathological conditions such as: enzyme defects, autoimmune diseases, immunosuppressive diseases, or respiratory infections. Clinical manifestations are generally nonspecific. The imaging investigation of the lungs brings suggestive information for diagnosis and at the same time draws attention to the less common association of some lesions. Confirmation is achieved through the anatomical-pathological examination of the product obtained by bronchial lavage, supplemented by the histopathology of the lung tissue taken by biopsy, which through the use of usual and special stainings provides information on the clinical presumption and certifies the presence of emphysema. The association of the two lesions, each with a different pathogenesis, is a rare one, and the correct diagnosis was possible through a close interdisciplinary collaboration.

The constellation of restrictive lung diseases was rapidly and provocatively completed by the SARS-CoV-2 virus infection which is of the RNA type and which causes an immune response with significant release of cytokines and chemokines, causing acute respiratory distress syndrome and multiple organ failure. Medical knowledge, limited at the time, about the evolution of the infection with the new Corona, as well as the rules imposed during the COVID-19 pandemic, made case management difficult. Thus the questions were born: Is the SARS-CoV-2 infection the cause of death? Is it just a circumstantial or enabling factor? Is it a special condition? In this idea, the autopsy remains a basic method, considered the "gold standard", both in terms of the definite establishment of the diagnosis, and for the purpose of scientific research regarding the mechanisms of the disease. Collaboration with the team of the County Forensic Clinical Service in Constanţa made possible the study of *Morphopathological features induced by SARS-CoV-2 infection - a series of 57 autopsies*, the second number of cases after that of Edler et al. which is larger and included 80 cases, complements the knowledge about the pulmonary morphological changes induced by the new strain. The pulmonary injury observed in autopsied cases of SARS-CoV-2 infection is dominated by diffuse alveolar damage (DAD) with different patterns of evolution (exudative, proliferative, fibrotic) and a hypercoagulable status reflected by frequent and extensive thrombosis, mainly in the level of the small and medium pulmonary arteries, which is an important cause of morbidity and mortality. Other morphological features associated with SARS-CoV-2 infection are cytopathic changes in pneumocytes, hyperplasia and/or detachment of type II pneumocytes, multinucleated giant cells, squamous metaplasia, and organizing pneumonia. In our opinion, pneumonia caused by the infection of COVID-19 combines the classic aspects of alveolar and interstitial pneumonia, with some particularities represented by the predominant presence of alveolar hyaline membranes, extensive thrombosis, and non-systematized fibrosis.

Three years after the onset of the pandemic, prospective studies on long-term changes in SARS-CoV-2 infection have begun to appear, as well as a comparative analysis of SARS-CoV-2-induced pulmonary fibrosis (PPFC) with idiopathic pulmonary fibrosis (IPF). The experience conferred by the morphological evaluation of lung tissue with lesions induced by SARS-CoV-2, allowed us to contribute together with the team of pulmonologists of SCJU Constanţa to the elaboration of the review *Clinicopathological Outlines of Post-COVID-19 Pulmonary Fibrosis Compared with Idiopathic Pulmonary Fibrosis*. There are many similar features between FPPC and IPF in terms of clinical aspects, risk factors, respiratory tests, imaging, and histopathological changes, as well as some notable differences. In terms of histopathology, FPI and FPPC both cause pulmonary DAD and fibrosis. Findings of pulmonary fibrosis in COVID-19 are limited and require further morphological research. FPPC causes significant, irreversible consequences that affect the quality of life of patients after SARS-CoV-2 infection.

Morphometry and histopathological scoring systems. The histopathological examination is the basis for studying inflammatory, degenerative, and tumoral lesions to establish a diagnosis. Histopathological quantification methods are supported by techniques such as: immunohistochemistry, immunofluorescence, or in situ hybridization. The three types of evaluations that can be carried out are qualitative, quantitative, and semi-quantitative. The qualitative assessment includes the examination of the examined morphological structures, a descriptive part (definition of the examined elements), and a visual part. Quantitative assessment lends vigor to working data by allowing much more precise correlations with clinical and biological data and is based on digital pathology. The semi-quantitative evaluation is the most used method, it serves as a complementary approach to the quantitative one, and the main role is played by the observer who assigns a score/grade to the examined tissue in order to perform statistical analysis through various methods. Morphometry, as a quantitative method, primarily targets the changes that interest the nucleus, because it is at this level that the first changes in the carcinogenic process occur. Nuclear morphometry is an ancillary tool that helps distinguish benign from malignant lesions by measuring nuclear sizes, shapes, textures, and density. In the *Nuclear comparative morphometric study between DCIS and normal resting mammary gland tissue*, the use of this method in the evaluation of resting mammary tissue showed that the average values obtained for the morphometric parameters describing the size and shape of the epithelial nuclei do not show significant differences between the follicular phases and luteal phase of the menstrual cycle, in accordance with international studies. We used these results as benchmarks to compare the data obtained by applying morphometry on the mammary glandular tissue with CDIS. Thus, in the ductal epithelial cells of the CDIS, the nuclei show variable polymorphism and increased values of the nuclear area compared to those of the normal tissue of the mammary gland in functional rest, and the increase of the nuclear area induces increased values of the other morphometric parameters. These results provide information on tumor aggressiveness, invasion, and prognosis.

Whether it is semi-quantitative measurements (scoring systems) or quantitative measurements (morphometry), their last but not least role is related to clinical applicability for patient therapy. Thus, based on scoring systems, many cancers benefit not only from diagnostic tools but also from tumor response scores. They evaluate the rate of therapeutic response that

various medications or techniques (adjuvant or neoadjuvant) exert on malignant cellularity, the purpose being twofold: evaluating the effectiveness of the treatment and the patient's prognosis.

The paper *The Predictive Role of the Histopathological Scoring System in Adipose Tumors-Lipoma, Atypical Lipomatous Tumor, and Liposarcoma*, is the result of a 10-year retrospective study, carried out together with the team of researchers from SCAP-SCJU Constanţa on neoplasms originating in adipose tissue, which represents, moreover, the most common type of soft tissue tumors. The histopathological re-evaluation, combined with the immunohistochemical examination using MDM2, CDK4, CD34 and CD31/PECAM-1 monoclonal antibodies, as well as with chromogenic in situ hybridization (CISH) for the detection of MDM2 gene amplification at the 12q15 region, using the ZytoDot SPEC MDM2 probe, allowed us to categorize the cases with greater accuracy into benign, atypical lipomatous tumors and liposarcomas. Through this study, we propose a score based on histopathological features: severity of nuclear atypia, cellular pleomorphism, proliferation of fibrous tissue and lipoblasts, as well as quantification of mitotic activity. Thus, we observed that this score guides the histological diagnosis, helping to achieve the differential diagnosis ($p<0.001$). An advantage of this score is represented by the possibility of differential diagnosis between all three major entities, not just between lipoma and ALT. Moreover, we found that this score has a statistically significant value in predicting the results of immunohistochemical and genetic tests of MDM2 and CDK4 ($p<0.001$) supporting the histopathological diagnosis. Another important element that we took into account was the density of the tumor microvascularization, which, after the radiotherapeutic treatment in the cases that lend themselves to it, does not undergo changes. We observed that a cut-off value of ≥ 38.75 vessels/mm^2 is highly suggestive of the diagnosis of liposarcoma, with a sensitivity of 94.1% and a specificity of 100%, this cut-off has a higher fidelity in the diagnosis of liposarcomas than mitotic activity. Another observation is that, when the threshold of 16.25 vessels/mm^2 is exceeded, nuclear atypia are much more frequent. This threshold can be considered the turning point to a malignant lesion.

Evaluation of the constituent elements of the tumor microclimate. The tumor microclimate represents a complex structure made up of a varied range of cellular elements arranged in a modified extracellular matrix. The non-cellular components of the matrix are represented by collagen, fibronectin, laminin, and hyaluronan, and the cellular components at this level are represented by tumor cells, stromal cells, and immune cells. Immune cells present in the tumor microclimate are a determining component and have the ability to either suppress or promote tumor growth and are divided into two broad categories, innate immunity and adaptive immunity. The first category includes neutrophils, macrophages, and dendritic cells. The second category needs activation, which is exposure to specific antigens to form an immune memory that enhances the response. The active components are represented by T-lymphocytes, B-lymphocytes, and natural-killer (NK) cells. One way in which the malignant cell can escape the immune defense is through the programmed death receptor 1 (PD1) axis and its ligand [programmed death ligand-1 (PD-L1)]. The paper *Assessment of programmed death-ligand 1 receptor immunohistochemical expression and its association with tumor-infiltrating lymphocytes and p53 status in triple-negative breast cancer* is the result of a research project that considered the analysis of associations between PD-L1 immunoexpression and the distribution of TILs stromal cells (stTILs) in relation to the clinical-morphological characteristics of patients with triple-negative breast cancer (TNBC), as well as the evaluation

of the value of PD-L1 immunoexpression as a prognostic factor and its correlation with p53 immunoexpression. In conjunction with the histopathological examination that established malignancy, immunohistochemistry allowed us to categorize the cases as triple-negative by evaluating ER, PR receptors, and HER2/neu status, as well as evaluating PDL-1, CD8, and p53. Our study showed a statistically significant association between PDL-1 expression and age over 50 years, pT tumor size, lymphovascular invasion, stTILs level, the presence of CD8+ TILs, as well as p53 immunoexpression. Most tumors with an immunopositive reaction for PD-L1 were associated with a high Ki67 index. TNBC is a relatively rare molecular subtype of BC that is associated with a poor prognosis, and its morphological and molecular characteristics make it difficult to treat, being responsible for up to 25% of deaths in breast cancer patients. Because it does not respond to hormone therapy or Trastuzumab, along with chemotherapy and radiotherapy, immunotherapy is a new approach for treating extremely "immunogenic" tumors such as triple-negative breast cancer. The tumor protein p53 (TP53) gene plays an important role in carcinogenesis and can have two types of mutations leading to aberrant immunoexpression of the p53 protein: "null" mutations and "nonsense mutations" with an "overexpression" for the p53 protein. Mutation of the TP53 gene can lead to a poor CD8+ T cell response, which can affect the response to immunotherapy. In our group, six cases with p53 overexpression were identified, 85.7% of which were also positive for CD8 TILs, consistent with international studies showing that "nonsense" mutations of the TP53 gene can trigger an immunological response very different from "null" mutations of TP53 in TNBC. Identification of cases with aberrant immunoexpression of p53 is important because it may predict a favorable response to immunotherapy and develop new therapy targeting this pathway of carcinogenesis. The research project on the aggressiveness of astrocytic neoplasms, from which I presented the paper *Implications of Cellular Immaturity in Necrosis and Microvascularization in Glioblastomas IDH-Wild-Type*, also falls within the same sphere of morphological approach to the tumor microclimate. Currently, IDH-wildtype glioblastoma is a tumor entity distinct from central nervous system gliomas, and two of the criteria that define its diagnosis are tumor necrosis and microvascular proliferation. Our study is a retrospective one, carried out over 10 years, on 114 glial tumors of which 39 were classified as IDH-wildtype glioblastoma. The aim was to observe the prognostic role in patient survival of the degree of cellular maturity responsible for hypoxic processes, which lead to the appearance of necrosis and microvascularization, noting their associations with imaging and morphogenetic parameters. Along with the information obtained by histopathological, immunohistochemical, and fluorescent in situ hybridization (FISH) evaluation, we considered the imaging parameters provided by the computed tomography (CT) examination (location, tumor size, tumor volume, peritumoral edema, and midline displacement), as well as and the type of resection assessed postoperatively by magnetic resonance imaging (MRI). The most important aspects regarding the negative prognosis in patient survival were represented by advanced age, the presence of residual volume, and the absence of at least one therapeutic method from the standard trimodal treatment. The presence of a predominantly immature cell population was an independent risk factor for death. In addition to this aspect, immaturity combined with acute onset (less than 1 month) of symptoms or age over 50 years or with the presence of residual tumor represented major negative risk factors in terms of patient survival. Percent necrosis quantification was not

a risk factor, whereas associations of microvascular density with age over 50 years and cellular immaturity were risk factors for mortality.

The second part of the habilitation thesis presents the main development directions of my activity in terms of professional medical, academic, and scientific research.

- **From a professional medical point of view**, I am considering the implementation of the project to expand our service by building a new building next to it, in addition to the existing spaces; diversifying the activity by introducing new investigations into the activity of our service, such as the DNA-HPV detection method; broadening the range of monoclonal antibodies used in immunohistochemical evaluation and introducing the in situ hybridization method; the engagement of SCAP-SCJU Constanţa in scientific research projects. In my view, the fulfillment of these objectives will materialize in increasing the performance and addressability of SCAP-SCJU Constanta and in the provision of high-quality medical services. Achieving the proposed objectives is possible through teamwork, together with the members of the organization I belong to, in an organized and transparent way, in a collegial atmosphere.
- **Academically**, I propose to optimize the teaching methods for the course by creating new educational materials in electronic format, revised and supplemented with new data from the specialized literature; the development of student's practical skills and reflexive competencies through more active participation in conducting courses and practical works, as well as their better presence at diagnostic activities in SCAP; stimulating the responsibility of students, with an emphasis on the development of moral and ethical values from the perspective of the profession they are going to practice. An important objective on which I direct my attention is the professional training of resident doctors. Their involvement in the diagnostic activity by approaching all the methods used in Pathological Anatomy, the detailed discussion of each case with the presentation of the arguments for and against, with a special emphasis on differential diagnosis, represent, from my point of view, effective methods for fixing notions and augmenting their practical experience. Another essential objective, on which I will focus, is the publishing activity in the didactic sphere. In the long term, I have in mind the publication of the "Practical Course of Systemic Pathology" which is about to go to print, as well as two other course volumes currently in the works. Through their content, these works will be useful both to medical students and resident doctors in Pathological Anatomy, as well as to doctors of other specialties interested in the universe of this specialty.
- **In terms of scientific research**, the future studies that I want to develop will address the tumor pathology of soft tissues. In this sense, I will focus on continuing the study of the tumor microclimate, namely the immune cellularity, with the objective of highlighting the role of the inflammatory infiltrate and the importance of each constituent element. It is important to objectify the association and ratio of these inflammatory cells. Thus, from this perspective, the future studies in soft tissue pathology that I have in mind will focus on: the role of the immune tumor microclimate in oncological therapy; the ratio of infiltrating tumoral lymphocytes; the mechanism of action of inflammatory cells; the role of neutrophils, eosinophils, histiocytes or macrophages and dendritic cells in the regulation of lymphocyte activity. In the same sphere of the tumor microclimate, I will continue the study of microvacularization. Methods of quantifying microvascular density are varied and remain at the discretion of the observer. Even under these conditions, many studies have highlighted its prognostic role in neoplastic disease. Based on these studies, antiangiogenic therapies were put into practice. The most studied

marker is vascular endothelial growth factor – VEGF. It has been given special importance, especially for the implications it has during pathogenesis, through the development of endothelial cells associated with tumors. In the last decade, more than five anti-VEGF drugs have been approved, the most recognized being Bevacizumab. Unfortunately, the adverse effects that these drugs have, make their practicability difficult, and their indications are reduced. From this perspective, I believe that studies on microvascular density using other immunomarkers are needed. In this sense, I propose the development of a project related to the identification of scoring and grading systems on microvascularization in the pathology of soft tissue tumors, which would represent the basis of future antiangiogenic therapies.

Regarding the non-tumor pathology, I will continue the study of pulmonary changes induced by non-specific and specific inflammations. The participation of fibroblasts in lung remodeling is a process that affects the function of the respiratory system and not only that. Pulmonary fibrosis itself represents a resource in terms of the further development of tumor pathology. In this sense, I propose to improve the diagnostic conditions, with an emphasis on multidisciplinarity. Thus, the corroboration of all the pathological data (histopathological examination, histochemical examination, and immunohistochemical examination) with the clinical-imaging data is a primary process in establishing a diagnosis, as close as possible to the truth. Along with correctness, this collaboration increases the speed of providing the diagnosis. In conclusion, supporting and improving multidisciplinarity brings immeasurable results in patient management.

Delivering in scientific research projects of the ideas I have presented, I will achieve together with the team of pathologists of which I am a part, of the scientific researchers who make up the Research Collective from SCAP-SCJU Constanţa, as well as in collaboration with colleagues from CEDMOG – Center for Research and Development for Morphological and Genetic Study in Malignant Pathology.

I will capitalize on the results obtained from the research activity in the form of scientific works, contributing in this way to increasing the academic prestige and visibility of the Faculty of Medicine of the "Ovidius" University of Constanța, as well as the Clinical Service of Pathological Anatomy of the Emergency County Clinical Hospital where I carry out my work as a university teacher and a pathologist.

The achievement of the proposed objectives in the three directions: professional-medical, didactic, and scientific research is motivated by the permanent desire for improvement, professional growth, and overcoming personal limits and at the same time, it is possible thanks to perseverance, the team spirit that I have developed over time, the interdisciplinary collaboration that I always call for and the openness that I have towards young researchers.

The last part of the habilitation thesis is represented by the bibliography.