UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

HABILITATION THESIS

From Gynecological and Obstetrical Pathology to Dermatopathology and beyond

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CONTENTS

ABSTRACT

This habilitation thesis includes my professional, academic and scientific achievements that materialized in the postdoctoral period (2005-2022), as well as some of the future projects, which will take shape in the coming years.

According to the National Council for Attestation of University Degrees, Diplomas and Certificates (CNATDCU) recommendations and to the methodology of the Doctoral School of "Grigore T. Popa" University of Iași, the thesis is structured in 4 sections as follows:

Section I presents my professional, academic and scientific achievements, in a chronological order, being organized separately but interrelated with each other.

Section II represents the widest and most important part of the thesis, comprising the scientific achievements obtained following the integration of knowledge from the three fields of activity and which is organized in three chapters, every one of them starting with a special subchapter, named *State of the Art*, with a recent overview of the scientific literature for each area of interest.

Chapter 1 focuses on gynecological pathology, whose areas of interest have been structured, besides *State of the Art*, in 5 subchapters. The first subchapter, entitled *HPV-associated carcinoma and its precursor lesions*, presents the studies, which assess p16, COX-2, EGFR, cyclin D1, and p53 immunoexpression, in correlation with HPV L1 capsid protein immunoexpression in HPV-associated precursor lesions as well as related invasive squamous carcinomas and adenocarcinomas. The next three subchapters, entitled *Endometriosis and the relationship with cancer*, *Aggressive behavior in endometrial carcinoma – particular aspects*, and *Endometrial stem cells*, include my original research as well as two reviews, as a continuum of the topic of my doctoral dissertation on endometrial carcinomas and endometrial pathology in general, governed by its particular microenvironment and molecular mechanisms, which have a pivotal role in the endometrial lesions progression dynamics. The last subchapter of gynecological pathology, entitled *Granulosa cell ovarian tumours*, presents the research carried out on adult ovarian granulosa cell tumors (AGCTS), tumours with heterogeneous morphology and unpredictable behavior, in which we evaluated the immunohistochemical expression of ER alpha, inhibin A, calretinin, and Ki67.

The results of my research in the field of gynecological pathology have been disseminated as articles in the following publications: *Curr Pharm Des* (IF: 3,31), *Int J Mol Sci* (IF: 5,924), *Rom J Morphol Embryol* (IF: 0,523, 0,912, 1,033, and 1,5).

Chapter 2 is dedicated to obstetric pathology and is distinctly structured, besides the *State of the Art*, in two more subchapters. The first, entitled *Placenta and its relationship with IUGR*, presents a study that evaluates the morphological and developmental particularities of the placenta encountered in intrauterine growth restriction (IUGR), which reflects the relationship between maternal and fetal circulation. The second subchapter includes particular aspects of the multifaceted placenta, presenting 4 case reports which describe particular and rare aspects related to the placenta accreta spectrum (PAS), as well as fetal and placental changes found in various genetic defects, like autosomal recessive disorder and chromosomal abnormality, such as Roberts' syndrome and triploidy respectively.

The results of this research materialized in papers published in the following journals: *Rom J Morphol Embryol* (IF: 0,811 and 1,033), *Rev Rom Med Lab* (IF: 1,027), *In Vivo* (IF: 2,155), and *Diagnostics* (IF: 3,706).

Chapter 3 includes, in addition to the *State of the Art*, two reviews in the field of dermatopathology, related to *Particular aspects in autoimmune bullous diseases* and *Tumor microenvironment components in malignant melanoma*. Although apparently unrelated, recent studies describe an interplay between malignant melanoma (MM) and bullous pemphigoid (BP). Considering a common pathophysiology for the two forms of BP, drug-induced and conventional, researchers describe two possibilities of the BP-MM relationship: (i) drug-induced BP, as a side effect in patients with MM treated with checkpoint inhibitors; (ii) a direct association between BP and melanoma, allocating a role of BP autoantigens in melanoma carcinogenesis.

The reviews research materialized in papers published in the following journals: *Medicina (Kaunas)* (IF: 2,430) and *Exp Ther Med* (IF: 2.447).

Section III includes my future academic, professional and scientific projects.

Future research will focus on the expansion of the presented studies in the field of gynecological pathology, characterized by HPV-associated and HPV-independent cervical neoplasms and precursor lesions, endometrial, and ovarian pathology, aimed to identify new molecular markers with impact on the prognosis and pathogenic mechanisms decoding, as well as the assessment of the pregnancy-carcinogenesis relationship, especially for endometrial tumors.

Obstetric pathology also remains a priority, willing to continue the studies related to implantation mechanisms, but also to the placental infections pathology and to the gestational trophoblastic disease.

Dermatopathology will continue to exist in the landscape of my scientific projects, both through the pathology of malignant melanoma and non-tumoral pathology, as well as through new research opportunities arising from my medical practice of histopathology.

Beyond the above domains, my interest is directed towards tumoral and non-tumoral mammary pathology, evaluated both histopathologically, on specimens of mastectomy, lumpectomy or punch biopsy, but also cytologically, by evaluating fine needle aspiration specimens, and also to the pathology of the uterine cervix evaluated by cervico-vaginal cytology, wishing to continue the assessment of the pathogenic and morphological aspects through correlations on the cytological-morphological-molecular axis, with impact on the clinical and therapeutic management of patients.

Section IV contains the bibliographic references used as a scientific reference for my presented research topics.

REZUMAT

Această teză de abilitare cuprinde realizările mele profesionale, academice și științifice care s-au concretizat în perioada postdoctorală (2005-2022), precum și câteva dintre proiectele viitoare, care vor lua contur în următorii ani.

Conform recomandărilor Consiliului Național de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) și a metodologiei Școlii Doctorale a Universității „Grigore T. Popa" din Iași, teza este structurată pe 4 secțiuni, după cum urmează:

Secțiunea I prezintă realizările mele profesionale, academice și științifice, în ordine cronologică, fiind organizate separat, dar interrelaționate între ele.

Secțiunea II reprezintă cea mai amplă și mai importantă parte a tezei, cuprinzând realizările științifice obținute în urma integrării cunoștințelor din cele trei domenii de activitate și care este organizată în trei capitole, fiecare dintre ele începând cu un subcapitol special, introductiv, cu o prezentare recentă a literaturii științifice pentru fiecare domeniu de interes în parte.

Capitolul 1 se concentrează pe patologia ginecologică, ale cărei arii de interes au fost structurate, pe lângă partea introductivă, în 5 subcapitole. Primul subcapitol, intitulat *Carcinomul HPV-asociat și leziunile sale precursoare*, prezintă studiile care evaluează imunoexpresia p16, COX-2, EGFR, ciclinei D1 și p53, în corelație cu imunoexpresia proteinei capside HPV L1 în leziunile precursoare HPV-asociate precum și în carcinoamele scuamoase invazive și adenocarcinoamele corespunzătoare. Următoarele trei subcapitole, intitulate *Endometrioza și relația cu cancerul*, *Comportamentul agresiv al carcinomului endometrial – aspecte particulare* și *Celulele stem endometriale*, includ cercetările mele originale, precum și două recenzii, reprezentând un continuum al subiectului tezei mele de doctorat legat de carcinoamele endometriale cât și patologia endometrială în general, guvernată de micromediul endometrial particular și de mecanismele moleculare, care au un rol esențial în dinamica progresiei leziunilor endometriale. Ultimul subcapitol de patologie ginecologică, intitulat *Tumori ovariene cu celule granuloase*, prezintă cercetările efectuate asupra tumorilor ovariene cu celule granuloase forma adultă (AGCTS), tumori cu morfologie heterogenă și comportament imprevizibil, la care am evaluat expresia imunohistochimică a ER alfa, inhibinei A, calretininei și Ki67.

Rezultatele cercetărilor mele în domeniul patologiei ginecologice au fost diseminate sub formă de articole în următoarele publicații: *Curr Pharm Des* (IF: 3,31), *Int J Mol Sci* (IF: 5,924), *Rom J Morphol Embryol* (IF: 0,523, 0,912, 1,033 și 1,5).

Capitolul 2 este dedicat patologiei obstetricale și este structurat inedit, pe lângă partea introductivă, conținând încă două subcapitole. Primul, intitulat *Placenta și relația sa cu retardul de creștere intrauterină (RCIU)*, prezintă un studiu care evaluează particularitățile morfologice și de dezvoltare ale placentei întâlnite în restricția de creștere intrauterină (RCIU), care reflectă relațiile circulatorii materno-fetale. Al doilea subcapitol include aspecte particulare ale placentei, prezentând 4 rapoarte de caz care descriu aspecte particulare și rare legate de spectrul placentei accreta (PAS), precum și modificări fetale și placentare găsite în diferite defecte genetice, cum ar fi tulburarea autozomal recesivă și anomalii cromozomiale, întâlnite în sindromul Roberts și respectiv triploidie.

Rezultatele acestei cercetări s-au concretizat în lucrări publicate în următoarele reviste: *Rom J Morphol Embryol* (IF: 0,811 și 1,033), *Rev Rom Med Lab* (IF: 1,027), *In Vivo* (IF: 2,155) și *Diagnostics* (IF: 3,706).).

Capitolul 3 include, pe lângă stadiul cunoașterii, două recenzii în domeniul dermatopatologiei, legate de *Aspecte particulare în afecțiunile buloase autoimune* și *Componentele micromediului tumoral în melanomul malign*. Deși aparent fără legătură, studii recente subliniază o interacțiune între melanomul malign (MM) și pemfigoidul bulos (BP). Având în vedere o fiziopatologie comună pentru cele două forme de BP, forma indusă de medicament și cea convențională, cercetătorii descriu două posibilități ale relației BP-MM: (i) BP indus de medicament, ca un efect secundar la pacienții cu MM tratați cu inhibitori checkpoint; (ii) o asociere directă între BP și melanom, prin alocarea unui rol al autoantigenelor BP în carcinogeneza melanomului.

Cercetarea din cadrul celor doua review-uri s-a materializat în lucrări publicate în următoarele reviste: *Medicina (Kaunas)* (IF: 2.430) și *Exp Ther Med* (IF: 2.447).

Secțiunea III include viitoarele mele proiecte academice, profesionale și științifice.

Cercetările viitoare se vor concentra pe extinderea studiilor prezentate în domeniul patologiei ginecologice, ce cuprind neoplasmele cervicale HPV-asociate și HPV-independente și leziunile precursoare, patologia endometrială și ovariană, cu scopul de a identifica noi markeri moleculari cu impact asupra prognosticului și a decodificării mecanismelor patogenice, precum și evaluarea relației sarcină-carcinogeneză, în special pentru tumorile endometriale.

Patologia obstetricală rămâne si ea o prioritate, continuând studiile legate de mecanismele de implantare, dar și legate de patologia infectiilor placentare precum și de boala trofoblastică gestațională.

Dermatopatologia va continua să existe în peisajul proiectelor mele științifice, atât prin patologia melanomului malign și a patologiei non-tumorale, cât și prin noi oportunități de cercetare care decurg din practica mea medicală de histopatologie.

Dincolo de domeniile de mai sus, interesul meu se îndreaptă către patologia mamară tumorală și non-tumorală, evaluată atât histopatologic, pe specimene de mastectomie, lumpectomie sau biopsie cu punch, dar și citologic, prin evaluarea specimenelor de aspirație cu ac fin, cât și către patologia col uterin evaluat prin citologie cervico-vaginală, dorind să continui evaluarea aspectelor patogenice și morfologice prin corelații pe axa citologico-morfologic-moleculară, cu impact asupra managementului clinic și terapeutic al pacientelor.

Secțiunea IV conține referințele bibliografice utilizate ca referință științifică pentru subiectele de cercetare prezentate de mine.

Motto
Science without morality is worthless.
Ana Aslan

SECTION I.
PROFESSIONAL, ACADEMIC, AND SCIENTIFIC ACHIEVEMENTS

I.1. INTRODUCTION

The privilege of a physician to embrace an academic career gives him various possibilities and perspectives to refine his profession and specialty, as teaching involves a constant updating of shared information, representing also an anti-aging method, due to the permanent interaction with young students, which contributes to professional maturity in a background of mental and spiritual youth.

Career is a part of our existence that needs to be organized and managed in order to achieve optimal results. The key to professional development is planning. In this sense, self-assessment is the essential starting point for career planning, which can be achieved through various tools and techniques that contribute to a better understanding of personal skills.

The road to a successful career is rarely straightforward, for individual professional development requires patience, long-term vision and careful navigation through the complex and ever-changing environment in which we operate, each creating its own niche.

Another defining element in career development is building good relationships and optimizing opportunities. Strong peer relationships, built on trust and mutual understanding of priority issues, are essential in building a future career.

The habilitation thesis entitled **"From Gynecological and Obstetrical Pathology to Dermatopathology and beyond"** presents the clinical research I have conducted since I became a specialist in Pathology, most of the studies being published after completing my doctoral thesis "Morphological and molecular aspects of endometrial carcinomas in correlation with clinical aspects", coordinated by Professor Dr. Coriolan Cotuțiu (Diploma D0002905, No. 1138/13.12.2005).

Currently, I am an Associate Professor at the Histology Discipline, Department of Morpho-Functional Sciences I, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, and a consultant pathologist at Clinical Hospital of Obstetrics and Gynecology „Elena Doamna" Iasi, where I coordinate the Pathology Department.

Histology completes and supports Pathology, the academic side combining very well with the professional and scientific fields in which I carry out my activity as a doctor.

I.2. PROFESSIONAL ACHIVEMENTS

In 1997, following the Residency Competition, I started Pathology as a resident, following a 4-year training program at the „St. Spiridon" Clinical Emergency County Hospital and „Elena Doamna" Clinical Hospital of Obstetrics and Gynecology, Iasi, while I attended courses and seminars in Pathology and Cytology and other related branches, like Genetics, Immunopathology, and Hematology.

In October 2001, I graduated as a specialist in Pathology (Certificate S1/006756/The Order of M.S. F. No. 866/03.12.2001), and in 2008, I became consultant pathologist (Certificate P1/002666/The Order of M.S.P. No. 1971/2008).

During the residency, I won a 6 months government fellowship granted by the National Office of Study Abroad Scholarships and financed by the Romanian Government (Document No. 34221/543/08.06.2000), at the Department of Anatomic Pathology, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York, between 26.10.2000-30.04.2001. This fellowship allowed me to complete my doctoral studies by participating in the research project entitled „Gene Expression Profile of Endometrial Carcinomas using cDNA microarray" and to benefit from other research opportunities, by learning cDNA microarrays and immunohistochemistry techniques. Besides research activities, this fellowship offered me the opportunity to get involved in academic teaching, as well as to carry out the activity of resident in Pathology, which involved attendance of macroscopic and microscopic diagnosis sessions, attending tumour boards of Pathology, Gynecology and Gynecologic Oncology, and courses within the Pathology residency curricula.

Continuing learning and development in my field of medical practice, I attended various postgraduate training courses in Romania and other countries, like France and Italy.

In 2002, I started working as a pathologist in the Pathology Department of „Elena Doamna" Clinical Hospital of Obstetrics and Gynecology Iasi, part-time, as academic clinical integration.

In order to develop my professional connections and benefit from new research and learning opportunities, I have joined various professional medical societies, as an active member: the European Society of Pathology, la Division Française de l'Académie Internationale de Pathologie, the Romanian Society of Normal and Pathological Morphology, the Romanian Society of Clinical Cytology, the Romanian Society of Physicians and Naturalists.

I.3. ACADEMIC ACTIVITY

Following the competition promoted in 1996, I began my teaching career as a trainer/preparator at the Discipline of Histology, Faculty of General Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi. These almost three years were an important starting point for my academic training, which focused especially on theoretical and practical training in the field of histology and histopathology, as well as teaching methodology, by attending my coordinating professors's courses and delivering practical works for the 2nd year Medicine students, from both Romanian and English programs.

From March 1999 to June 2006, I fulfilled the position of Assistant Professor at the Discipline of Histology. During these seven years, I continued to improve my academic performance, participating in postgraduate courses and tutorials in clinical cytology and pathology, developing education materials and new facilities for the teaching process, for 2nd year Medicine students, Romanian and English program, as well as for students enrolled in College of Health Care, from Iași, Galați, Bacau, and Botoșani. Also during this period, I extended my teaching activity toward 1st year Dental Medicine students by delivering practical lessons and seminars and by contributing to their examination process during exams sessions.

In 2006, I became, through competition, lecturer at the Discipline of Histology. The main coordinates of this period of 8 years (June 2006 - February 2014) were represented by the extension of professional training, integrating the elements of theoretical and practical knowledge in the field of histology and histopathology at a higher level of approach, in parallel with the development of skills in quality management. In this regard, I started teaching courses, practical works, and conducting final examinations for 2nd year Medicine students and 1st year Dental Medicine students from Romanian, English, and French programs, as for residents in Pathology, Oncology, Radiology and Imaging.

In order to improve my language skills for English and French, I took part in intensive training courses at the EuroEd Center, British Council, and the French Cultural Center, which culminated with the authorized language exams for English and French, obtaining Cambridge C1 (Cambridge Certificate Nr. 0050411930/04.09.2015 Level C1) and DELF B2 (Diplôme DELF 040098-004521/02.06.2015 Niveau B2) diplomas.

This period was also marked by the completion of my PhD thesis, as well as the involvement in a large international project, which focused primarily on implementing quality management in Pathology laboratories from Romania.

Thus, during 2010-2013, I participated, as a member of the management team, at the Project ANATOMOPAT "Pathology Laboratory – Professional and organizational training through the implementation of quality management" (POSDRU/81/3.2/S/58942). The project involved medical doctors and technicians from pathology laboratories in Romania, providing a complex training program, represented by 9 sessions of professional training courses and workshops, as well as internships in European laboratories, for knowledge and implementation of quality management according to SR EN ISO 15189:007.

Within this project, all the participants, including doctors and technicians as well as the members of the management team, benefited from an internship in Pathology, held in the Pathology Department of the University of Turin, Molinette Hospital from Turin, Italy. This opportunity offered us the possibility to exchange experience with peers in the same field of activity and to learn new technologies applied in different subspecialties of Pathology.

My constant interest in implementing quality standards in the Pathology laboratory materialized also through the participation in the seminar "Accreditation of medical laboratories according to ISO 15189: 2003", accredited by DAP (Deutsches Akkreditierungssystem Prüfwesen GMBH), held in 2006, as well as the participation in the training course "Internal auditors for management systems of medical laboratories according to EN ISO 15189: 2007 and EN ISO 19011: 2002", TÜV Rheinland Romania, held in May 2010.

Involvement in academic projects continued with my enrolling in Training People Soft, MEDICALIS project, during which I participated in June 2012 at the course "Educational Management and Quality Education in the Information Society, HRD 2007-2013", "John Moore'', held in Liverpool, England.

The development of the didactic career also involved the coordination of bachelor theses and scientific papers within the student congresses, as well as the preparation of various didactic materials, in order to improve the quality of knowledge dissemination to the students.

In 2014, I became Associate Professor at the Histology Discipline, Department of Morphofunctional Sciences I, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, where I am currently working. Besides the update and improvement of the teaching act and of the specific materials for the students from Medicine and Dental Medicine, my academic activity also involved the collaboration as invited lecturer or the coordination of 21 postgraduate courses.

Moreover, I have introduced, as an optional Discipline, the Integrative Medicine course for 2nd year Medicine students, from Romanian program, which aims to introduce students to this medical concept that combines conventional (Western) medicine with complementary and alterantive medicine. The introduction of this optional Discipline also brought me the opportunity to be a lecturer, within the Doctoral School Module entitled Bio-Logical Principles in Integrative Medicine.

Another achievement worth mentioning was my involvement in the international project Erasmus + (contract 2018-1-RO01-KA202-049189), "Promoters of advanced oncogenesis open online training and multimedia raise awareness on multidisciplinary assessment of patients and their families at risk of hereditary or familial cancer (HOPE) ", held between September 2018 - August 2021, as a member of the Romanian team for the preparation of teaching materials, which

ended with the writing of a monograph in 2021. Moreover, in this Erasmus project, I participated in the online course "Training guide for advanced high-specialized intervention in oncogenetics", organized in 2 modules, between November 2020 – June 2021.

In all these years, I have responded to the invitations of being a member in the doctoral activity coordination committees, a member of the Admission or Residency commissions or other competitions, as well as tutoring activity, which allowed me to improve my communication with students and to better understand their needs.

I.4. SCIENTIFIC PROFILE

My research activity, which started with the beginning of the teaching activity at the Discipline of Histology, includes the realization of the doctoral thesis, as well as countless projects and collaborations, with research teams consisting of members of the Discipline of Histology and clinicians from Iasi and abroad, of specialties related to my medical activity. The research activity cannot be seen outside the context of the academic and medical activity, all three representing the inspiration and the starting point for any type of original research project or study.

I had the great advantage of benefiting from an excellent training in morphology, conferred by the high professional attire of the School of Histology from Iaşi, which was of real use to me in understanding, deepening, and perfecting the pathology. A continuous preoccupation in the personal research activity was directed, by the nature of my medical activity, on the pathology of the female genital tract. I consider that Gynecological Oncopathology, complemented by Obstetrical Pathology, Breast Pathology and Dermatopathology represent the main research direction that defines my scientific profile. The results of my scientific activity have been published in various papers indexed by the Web of Science Core Collection or other international databases. The research activity also included communications at local, national, or international congresses and conferences.

My PhD thesis, defended in 2005, entitled "Morphological and molecular aspects of endometrial carcinomas in correlation with clinical aspects", coordinated by Professor Dr. Coriolan Cotuțiu, marked my research activity, benefiting from the Romanian government fellowship, at the Department of Anatomic Pathology, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York.

The doctoral study, conducted mostly in USA, allowed me to assess endometrial carcinomas, studied in terms of immunohistochemistry technique, but mainly of cDNA microarray technique, a newly developed tool in the 2000s, which could analyze the gene profile and the differences in gene expression in the two major types of endometrial carcinomas. In addition, this study resulted in the publication of a monograph on the cDNA microarray technique and its applicability in tumor pathology.

The correlation of medical, academic and scientific activity has led to the publication, besides of research and review articles, of several books, monographs, manuals, treatises, 3 as main author and 12 as contribution with book chapters.

My international visibility is reflected in medical journals as follows:

- Web of Science Clarivate Analytics H-index: 9;
- total number of citations without self-citations: 358;
- in extenso ISI papers: 32, of which 14 as principal author;
- in extenso IDB papers: 32.

The dissemination of the results of scientific studies was also achieved by participating in numerous national and international scientific conferences, congresses, symposia, courses, as follows: National Symposium on Normal and Pathological Morphology, Regional Conference on Dermatology "Gheorghe Năstase", National Conference on Clinical Cytology

and Clinical Cytology Instruction Symposium, National Conference Medical Days "V. Dobrovici", European Breast Cancer Conference (EBCC), where I obtained a mobility grant from the European Cancer Organization for the participation in The 5th European Breast Cancer Conference (EBCC-5), Nice, 2005, European Congress of Pathology, with also a mobility grant for the participation at ECP in 2005, International European Society of Gynaecological Oncology Meeting (ESGO), and the postgraduate course, "Pathologie tumorale du col utérin. Carcinomes du col utérin et ses précurseurs", organized by Academie Internationale de Pathologie Division Française (2016).

Interdisciplinary collaborations continued by participating in other grants and research projects, one as a project director and the other 4 as a member of the research team:

- director and principal investigator, IDEAS research grant 2036: "Expression of the L1 capsid protein of human papilloma virus (HPV) in correlation with other molecular factors: prognostic value and impact in the management of HPV vaccination", 2008-2011;
- team member, Young Researcher grant: "The impact of COX2, HIF-1α, VEGF-C, EGFR, and XRCC1 in the management of cervical cancer with neoadjuvant therapy (NAT): bioclinical significance and prognostic value", 2018-2019;
- team member, CNCSIS research grant code 1218: "Study of the interrelation between metalloproteinases, soluble factors (hormones and cytokines) and apoptosis in the neoplastic endometrium", 2007-2008;
- team member, CNCSIS research grant code 826: "Clinical and epidemiological study on reproductive health and preventing the abandonment in Romani collectivities in Iasi County", 2005-2007;
- team member, CNCSIS research grant code 1240/4: "Computerized morphometry in the efficiency of cytopathological and anatomo-pathological diagnoses", 2004-2005.

I.5. FINAL REMARKS

Career planning and professional development is an ongoing process, which involves periodically assessing the current state, reflecting on short- and long-term goals and setting new directions and strategies when the situation requires it. The planning process is much more valuable than the plan itself. The professional development plan must be flexible and responsive to changes, opportunities and challenges that may arise throughout the career. We live in a dynamic higher education ecosystem, where adaptation has become a goal. Once continuous planning becomes a habit, the career has every chance of becoming prosperous.

As a teacher at the Discipline of Histology and a pathologist, I believe that updating information in my professional field and correlating it with specific fundamental and clinical sciences is a priority that we must consider in the process of morphological training of students. Although Histology is considered a relatively static science, at present it becomes the link of new discoveries in the top fields of scientific research, represented by Molecular Biology, Immunology and Genetics, thus acquiring a dynamic character, in continuous modernization. From a methodological point of view, this update implies an adaptation to the level of knowledge of each trained category (undergraduate studies, postgraduate studies: residents, specialists), appropriately adjusting the volume and complexity of information, connections with other medical fields and practical applicability.

SECTION II.
SCIENTIFIC ACCOMPLISHMENTS

CHAPTER 1.
GYNECOLOGIC PATHOLOGY

1.1. STATE OF THE ART

Pathology remains a key specialty in medicine, especially for oncology, due to the essential information that can be obtained by morphological evaluation of tissues. New technological advances in pathology contribute to the facilitation of the histopathological diagnosis and the refinement of the therapeutic management [Farnell et al., 2020].

In this regard, gynecologic oncopathology benefits from new trends and concepts due to changes in the frequency of tumor localizations in the female genital tract and to new molecular classifications, which lead to new therapeutic approaches specific for each tumour subtype [Rekhi, 2020].

New WHO classification of female genitale tumours emphasizes the key molecular events that led to the emergence of new types of tumors or the refining of certain tumor categories, like endometrial carcinoma, highlighting the importance of a morphological-molecular approach, conventional pathology remaining the basis of interpretation [Herrington et al., 2020].

The main changes to the WHO classification of cervical tumors relate to several morphological entities [Herrington et al., 2020] which are described below.

For squamous lesions, invasive forms are currently classified into 2 broad categories, namely: HPV-associated and HPV-independent. Although rare, HPV-independent squamous cell carcinomas have been considered separately because they have a much more aggressive behavior compared to lesions associated with HPV infection.

Glandular lesions, both preinvasive and invasive, are classified into the same 2 main categories as squamous lesions: HPV-associated and HPV-independent. Adenocarcinomas due to HPV infection can be further classified with the terminology used in the previous classification. The most common HPV-independent, in situ or invasive adenocarcinomas are of gastric type. The other types of HPV-independent adenocarcinoma are clear cell carcinomas and mesonephric carcinomas.

The WHO 2020 classification also includes a category of squamous cell carcinoma NOS, which could be used when molecular or p16 IHC tests are not available [Herrington et al., 2020]. However, there is a substantial decline in cervical cancer incidence and mortality due to the association of the three main diagnostic techniques represented by cytological screening, HPV genotyping, and colposcopy [Holcakova et al., 2021].

Human papillomavirus (HPV) is a double-stranded DNA virus, member of papillomaviridae family, with two coding regions (E and L) and one long control region (LCR), which has regulatory functions upon viral gene expression [Calaf et al., 2018]. The expression of HPV early (E1, E2, E4, E5, E6 and E7) and late (L1 and L2) oncoproteins is the result of polyadenylated and polycistronic mRNAs transcripts [Calaf et al., 2018]. HPVs include five genders: alpha (α), with mucosal and cutaneous tropism, being responsible for genital warts

[Calaf et al., 2018], beta (β), gamma (γ), mu (μ), and nu (ν), which share differences in L1 gene [Flores-Miramontes, 2020].

High-risk (HR) human papilloma virus (HPV) infection is the trigger for high-grade squamous intraepithelial lesions (HSILs) or adenocarcinoma in situ (AIS), these precursor lesions representing the starting point in cervical carcinogenesis [Reich et al., 2020]. Of all four major HPV classes defined by the WHO according to their carcinogenic potential, which include group 1 (carcinogenic HPV subtypes), group 2A (probably carcinogenic HPV subtypes), group 2B (possibly carcinogenic HPV subtypes), and group 3 (low risk HPV subtypes), group 1 (mainly HPV16, 18, and 45) is the main factor responsible for cervical squamous and glandular carcinogenesis [Reich et al., 2020].

Among all available HPV genotyping tests used for primary cervical cancer screening, only the most sensitive HPV-DNA and/or HPV E6/E7 mRNA tests will detect HSIL (cervical intraepithelial neoplasia grade 3, CIN3) and AIS [Reich et al., 2020; Petry et al., 2016].

In order to infect the squamous epithelium, HPV must reach the dividing basal keratinocytes through epithelial micro-wounds found in the thickness of the epithelium [Regauer, Reich, 2021; Doorbar, 2016]. Human papillomavirus is infecting the basal cells, which express specific HPV receptors, through an endocytosis-mediated mechanism, using later host replication in the nucleus [Calaf et al., 2018].

The viral infection has several phases: (i) an initial phase with genome amplification in basal mitotically active cells; (ii) a maintenance phase of the viral genome replication, without a clinical lesion (non-productive/subclinical infection) [Regauer, Reich, 2021]. From this point, the infection can be latent, sometimes with clinically normal mucosa, koilocytosis (HPV-cytopathic effect), most of the cases resolve histologically and HPV tests become negative, or productive, represented by a minority of HPV-infections, which may regress (LSIL/CIN1) or persist for a long time, culminating in a clonal expansion (transforming infection) that produce a HSIL (CIN3) [Regauer, Reich, 2021].

Initiation of cervical carcinogenesis stands out through the ability of HPV to modulate several signaling pathways associated to DNA damage response, apoptosis, and cell cycle control, being able to induce p53 and pRb degradation. HR-HPV integration into the host genome leads to E6 and E7 overexpression, which represents a crucial event in HR-HPV-mediated oncogenicity. Accordingly, numerous studies report that E6 increases telomerase expression and activity, while E7 binds to pRb proteins, inducing overexpression of p16INK4a [Calaf et al., 2018].

In this context, the uniform or block p16INK4a-immunostaining in all dysplastic cells is considered a surrogate marker for a persistent HPV infection, although there are cases of HPV-independent precancerous lesions or invasive squamous carcinomas with immunoexpression for p16INK4a [Regauer et al., 2022; Regauer, Reich, 2021; Sacco et al., 2020]. Moreover, the Standardization Project LAST (Lower Anogenital Squamous Terminology) recommends the testing of this surrogate marker for differential diagnosis between HSIL (CIN 2/3) and its mimickers, as reactive/reparative changes, squamous metaplasia, atrophy, or tangential cut sections [Rekhi, 2020; Darragh et al., 2012].

HR-HPV persistence into the host cells is associated with other cofactors, like genetic, host immune status, or environmental carcinogens, responsible for both promotion and progression, overexpression of HR-α-HPV E6 and E7 oncoproteins being not enough for cancer progression [Calaf et al., 2018]. Moreover, another cofactor considered as having a role in cervical carcinogenesis is oxidative stress (OS), which acts independently or in association with HR-α-HPV infection. In this regard, previous studies showed a higher level of oxidative DNA damage in high-grade squamous intraepithelial lesions compared to control samples [Calaf et al., 2018].

The classification of endometrial carcinomas has undergone significant changes, from the dualistic model that divided them into type-1 (endometrioid) and type-2 (including serous,

clear cell, mucinous, and mixed) to recent WHO molecular classification according to the Cancer Genome Atlas (TCGA), which redistributes them in four molecular subtypes, namely, POLEmut, MMRd, p53abn, and no specific molecular profile (NSMP), identified based on genomic architecture (ultramutated, hypermutated, microsatellite instability hypermutated, high copy-number, low copy-number). These molecular groups are heterogeneous compared to histotypes, with the largest variation being observed in p53abn endometrial carcinomas. [Rekhi, 2020; Kim et al., 2020].

Although a benign lesion, endometriosis can undergo malignant transformation toward endometriosis-related ovarian neoplasms [Wei et al., 2011; Farolfi et al., 2022].

The first histopathological description of endometriosis was given by Von Rokitansky, as early as 1860 [Von Rokitansky, 1860]. By 1896, the name of "endometriomas" or "adenomyomas" had been proposed, due to the lesion resemblance to the mucous membrane of the uterus [Cullen, 1896a; Cullen, 1896b].

A combined anatomical and histopathological classification recognizes three main types of endometriosis, as follows: endometrioma, peritoneal, and deep infiltrative lesions [van der Linden, 1996]. Endometrial cysts or endometriomas are usually involving the ovaries, exhibiting bilateral location in around one third of cases and developing until almost completely replace the ovarian parenchyma [Vang, Wheeler, 2011]. They are most commonly associated with fibrous walls, adherences to neighboring structures, and usually chocolate-colored inspissated or semifluid content [Vang, Wheeler, 2011]. If larger than 15 cm in diameter or associated with polypoid projections or solid areas, a developing neoplasm should be considered [Vang, Wheeler, 2011]. Peritoneal endometriosis may involve, in decreasing order of frequency: ovaries (30%), uterosacral and large ligaments (18-24%), fallopian tubes (20%), pelvic peritoneum, Douglas pouch, and gastro-intestinal tract [De Ceglie et al., 2008; Cacciato Insilla et al., 2014]. Deep infiltrative endometriosis has been identified in 30-40% of patients diagnosed with endometriosis [De Ceglie et al., 2008], involving pelvis and gastrointestinal tract [Cacciato Insilla et al., 2014].

Numerous studies showed that even ovarian and extraovarian endometriosis carries different somatic mutations in cancer associated genes, the vast majority of endometriotic lesions with cancer driver genes do not undergo malignant transformation [Anglesio et al., 2017; Suda et al., 2018; Guo, 2020], as there are different protective environmental factors against malignant progression [Suda et al., 2018; Farolfi et al., 2022]. Recent data suggest that there are epithelial ovarian cancer (EOC) subtypes directly related to endometriosis, mainly endometrioid ovarian (EnOC) carcinoma and ovarian clear cell (OCCC) carcinoma, this relation being confirmed at the molecular level by the presence of cancer driver genes mutations, as atypical endometriosis is not always present in endometriosis associated ovarian cancer (EAOC), which may have the origin in endometrial cysts (ECs) [Wiegand et al, 2010; Jones et al., 2010; Murakami et al., 2020; Samartzis et al., 2020]. It is considered that alterations in MMR genes could lead the endometriotic lesions toward malignancy, these endometrioid tumours presenting a heterogeneous genotype, with new gained oncogenic mutations occurred in normal endometrium, rather than direct malignant progression [Fuseya et al., 2012; Farolfi et al., 2022; Murakami et al., 2020].

Endometrial carcinoma (EC) is a diversified malignancy according to its morphology, molecular features, clinical aspects, treatment response and prognosis [Karabag, 2021].

The depth of myometrial invasion (MI) as well as tumor spread in neighbouring organs are criteria used in the 2009 FIGO staging system for endometrial carcinoma (EC). There are several morphological patterns of MI, the most aggressive types being diffusely infiltrative pattern, single-cell invasion (SCI), and microcystic, elongated, and fragmented glands (MELF) [Mateva et al., 2021]. The correct assessment of the depth and pattern of myometrial invasion is very important, as the aggressive MI patterns are compatible especially with high-grade

endometrial carcinomas, which are approached with more aggressive therapeutic management [Park et al., 2019; Mateva et al, 2021].

A systemic study demonstrated that a significant loss of INI1 expression, a subtype of SWI/SNF complex, involved in transcriptional regulation and chromatin remodeling, characterizes the molecular profile of high-grade endometrial carcinoma, confirming the role of poor prognostic factor of INI1 loss, thus reinforcing the different pathogenesis of high-grade EC [Karabag, 2021].

Moreover, it was prooved that loss of E-cadherin expression defines type 2 endometrial carcinomas, being associated with aggressive phenotype represented by increased metastatic potential, dedifferentiation, and deep myometrial invasion [Setiawan et al., 2013; Rubesa-Mihaljevic et al., 2019; Youseff, Mohamed, 2019; Karabag, 2021]. Other studies reported the decrease in E-cadherin expression and related molecules on in vitro endometrial carcinoma cell line (HEC-1-A cells/HE cells), by ETV5 transcription factor overexpression [Monge et al., 2007], EMT (epithelial-to-mesenchymal transition)- related molecular changes [Colas et al., 2012], higher expression of type I transmembrane glycoprotein, FXYD5/dysadherin (FXYD5/Dys) proved to be associated in other solid tumours with lower E-cadherin expression and decreased cell-cell adhesion [Ino et al., 2002; Nakanishi et al., 2004; Batistatou et al., 2005; Kyzas et al., 2006; Nam et al., 2007; Besso et al., 2019]. This last aspect was shown to be related with poor prognosis and distant metastasis in other tumors [Nam et al., 2007; Besso et al., 2019].

The basal layer of the endometrium has regenerative capacity due to the resident stem/progenitor cells [Cousins et al., 2021]. These cells have been the subject of numerous researches that described, using different functional methods (multi-lineage differentiation assays, long-term culture, and clonogenicity) and several stem cell markers (SUSD2/W5C5, LGR5, NTPDase2, N-cadherin, CD146, SSEA-1), three main cathegories: (i) endometrial epithelial stem/progenitor cells; (ii) endometrial mesenchymal stem cells (eMSCs); (iii) side population cells (SPs) or endothelial stem cells [Miguel-Gomez et al., 2021; Kong et al., 2021]. Menstrual stem cells (MenSCs) represents endometrial progenitor cells from the menstruation blood, which, due to their autotransplantation capabilities and excellent proliferation, are considered good candidates for cell-based therapy in immune-related diseases, inflammation, or regenerative medicine [Kong et al., 2021]. Endometrial stem/progenitor cells are also involved in the development of endometriosis by retrograde menstruation mechanism, becoming overreactive and undergoing clonal expansion. Moreover, exogenous population of stem cells representd by circulating bone marrow mesenchymal stem cells (BMDSCs) can participate in the process of deep invasive endometriosis, endometriosis-derived cells being able to form circulating stem cell-like endometrial cells (CECs) with properties of migration and implantation into distant sites [Kong et al., 2021; Miguel-Gomez et al., 2021].

Ovarian cancer, as well as the endometrial counterpart, are usually diagnosed in advanced stages, after the onset of symptoms, as there are no available screening methods for this pathology [Jacobs et al, 2016; Morice et al., 2016; Costas et al., 2019; Holcakova et al., 2021]. Therefore, the discovery of a panel of new biomarkers is necessary, given that CA-125 detection and ultrasound examination cannot detect early stages of the disease [Holcakova et al., 2021]. Given that many of the ovarian malignancies, especially the most common, epithelial ovarian cancers (EOCs) such as high-grade adenocarcinomas, are recurrent despite their chemosensitivity, it has become necessary to investigate new markers of prognostic value. Thus, epidermal growth factor (EGF) receptor, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 receptor (IGF1R), and hCtr 1 (drug influx pump) were studied [Huang et al., 2010; Deo et al., 2019; Rekhi et al., 2020].

Adult granulosa cell tumor (AGCT) represent a rare type of ovarian neoplasm, accounting for 2%–5% of ovarian malignancies, but the most common endocrine-active ovarian

stromal tumour (70%) [Bjorkholm, Evans et al., 1980; Silfversward, 1981; Babarovic et al., 2018; Dridi et al., 2018; Yang et al., 2018]. There are two types of granulosa cell tumors (GCTs), AGCTs, more frequent, usually in postmenopausal women, and juvenile granulosa cell tumours (JGCTs), characteristic for younger women than 30 years or premenarchal girls [Stenwig et al., 1979; Babarovic et al., 2018; Dridi et al., 2018]. Clinically, patients with AGCT often present with abnormal uterine bleeding, the characteristic hyperestrogenism status being related to excessive hormone secretion represented by estrogens, inhibins, and anti-Müllerian hormone [Li et al., 2018; 4; Babarovic et al., 2018; Dridi et al., 2018; Yang et al., 2018].

Although AGCTs are usually low grade at presentation, they have a tendency for late recurrence [Fox et al., 1975; Malmstrom et al., 1994; Schumer, Cannistra, 2003; Babarovic et al., 2018]. Surgery remains the treatment of choice for AGCT, chemotherapy and radiotherapy being reserved for patients with advanced stage tumours or who present with inoperable recurrent disease [Thomakos et al., 2016; Dridi et al., 2018].

Several prognostic factors for AGCT are described, among which the patient's age, tumor size, mitotic activity, differentiation grade, and lymphovascular invasion, the most important being tumour stage. However, other histopathological or biological prognostic criteria are required, on which gynecological oncologists can rely in the therapeutic management of these unpredictable heterogenous tumours [Fox et al., 1975; Stenwig et al., 1979; Evans et al., 1980; Malmstrom et al., 1994; Schumer, Cannistra, 2003; Babarovic et al., 2018].

Although the pathogenesis of these tumors is not fully elucidated, many authors consider early ovarian mesenchyme as the starting point for tumor development, because of the AGCT heterogenous cellularity [Thomakos et al., 2016; Dridi et al., 2018].

Several signaling pathways characterize the development of GCT, as follows: TGF-β signaling pathway, Notch signaling pathway, PI3K/AKT, and the fork head box protein L2 (FOXL2) signaling pathway, influencing both cell proliferation and apoptosis, by forming a complex pathogenic network [Anttonen et al., 2014; Färkkilä et al., 2014; Chang et al., 2014; Leung et al., 2016; Hua et al., 2016; Li et al., 2018].

Numerous studies confirmed that the somatic point mutation of transcription factor FOXL2 is characteristic for AGCTs, representing an important differential diagnosis tool for these tumours. However, it is still unclear how dysregulation of FOXL2, with aromatase upregulation represent a driver of the tumour pathogenesis [Shah et al., 2009; Jamieson et al., 2010; Jamieson, Fuller, 2012; Kommoss et al., 2013; McConechy et al., 2016; Babarovic et al., 2018; Yang et al., 2018].

My interest for this research direction was materialized in the following achievements:

Articles

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Liliac L, Amălinei C, **Balan R**, Grigoraş A, Căruntu ID. Ovarian cancer: insights into genetics and pathogeny. *Histol Histopathol* 2012; 27(6): 707-719.

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Book chapters

Amalinei C, **Balan RA**, Lozneanu L. Induction of oxidative stress: a promising approach in female gynecological cancer therapeutic arsenal. In S. Chakraborti (ed.). *Handbook of Oxidative Stress in Cancer: Therapeutic Aspects*. Springer Nature Singapore Pte Ltd. 2022, 1-20.

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1.2. HPV-ASSOCIATED CARCINOMA AND ITS PRECURSORS LESIONS

1.2.1. INTRODUCTION

Given the known overwhelming role of HPV infection in the neoplastic epithelial pathology of the uterine cervix, the current WHO classification divides precursor lesions and cervical carcinomas into two broad categories, HPV-associated and HPV-independent [Herrington et al., 2020]. Although numerous studies demonstrated that almost all squamous cervical carcinomas and a vast majority of adenocarcinomas are associated with HPV infection, there is a remarkable proportion of carcinomas, especially adenocarcinomas who are not related to HPV infection, these HPV-independent types being more aggressive [Herrington et al., 2020]. In addition to morphological criteria, HPV testing and/or p16 immunoexpression are required to diagnose HPV-associated cervical tumors [Herrington et al., 2020].

The incidence of adenocarcinoma of uterine cervix (ACC), represented by a heterogeneous group of neoplasms with a variety of histological patterns, has changed, with several papers reporting increased rates of ACC among young women [Zheng et al, 1996; Smith et al, 2000; Wang, Lu, 2004; Witkiewitz et al, 2011; Ganesan, 2018]. Epidemiologic studies have shown a strong association between high-risk human papillomavirus (HR-HPV) and adenocarcinoma of the cervix [Pirog et al, 2000; Andersson et al, 2001; Castellsague et al, 2006; Kusanagi et al, 2010; Ganesan, 2018; Bogani et al., 2020], HPV-16 being the most important type and HPV-18 playing a greater role in endocervical adenocarcinoma than in squamous cell carcinoma [Jenkins, 2008; Ganesan, 2018]. Despite these data, some investigators revealed that uncommon types of endocervical adenocarcinoma are HR-HPV-negative [Pirog et al, 2000; Park et al, 2008; Kusanagi et al, 2010]. However, the immunoexpression of HPV L1 capsid

protein was demonstrated only in the early productive phase of cervical carcinogenesis (low-grade squamous dysplastic lesions), but not in high-grade squamous lesions, squamous cell carcinoma, and cervical adenocarcinoma [Gu et al., 2007; Wu et al, 2011]. The assessment of tumour supression protein p16 completes HPV L1 quantification, as p16 represents a valuable biomarker of squamous and glandular types of cervical carcinoma providing supplementary information about HPV related precursor lesions as well as malignancy progression [McCluggage, Jenkins, 2003; Jenkins, 2008; Wu et al, 2011].

These results are reinforced by another study that revealed that 36.4 % of the low-grade squamous intraepithelial lesions expressed L1 capsid protein, 9.1 % of high-grade squamous intraepithelial lesions were L1 positive, and the invasive squamous carcinoma and adenocarcinoma were without L1 immunoexpression, which indicates that HPV L1 expression decreases to absence with advancing of the lesion degree [Izaadi-Mood et al., 2014].

As previously stated, human papilloma virus (HPV) plays an essential role in cervical carcinogenesis, the presence of high-risk HPV types being documented in nearly all invasive cancers and in up to 90% of precancerous lesions [Munoz et al, 2003; Stanley, 2010; Carter et al, 2011; Ganesan, 2018; Herrington et al., 2020; Cangara et al., 2021]. L1 viral capsid protein is considered a major target of the cellular immune response [Melsheimer et al, 2003; Stanley, 2006; Norman et al., 2013]. LSILs without immunochemically detected L1 are correlated, in more than 80% of cases, with neoplasia progression [Griesser et al, 2004; Norman et al., 2013]. Most probably, the lack of HPV antigen is determined by a weak protein synthesis, under a minimum level of the immunoexpression. The immunochemical detection of L1 capsid protein, on Papanicolau smears, may demonstrate the locally induced defense status against HPV infection and may offer prognostic information in different squamous intraepithelial lesions.

Different authors demonstrated the presence of L1 expression in most of HR-HPV-associated LSIL, but not in HR-HPV [Melsheimer et al., 2003; Norman et al., 2013]. Moreover, one of the mentioned study showed a mixture of various biological stages of HPV infection, either clearance or progression, in different cytological lesions, as ASCUS and LSIL. This conclusion may affect the prediction of clinical outcome of early intraepithelial lesions, creating a differentiation between transient and progressive HPV infections, with significant impact upon the management algorithm of squamous intraepithelial lesions [Norman et al., 2013].

The tumor suppressor protein, p16 is a cyclin dependent kinase inhibitor that regulates G1-S transition in the cell cycle [Ortega et al, 2002; Walts, Bose, 2009]. Several studies showed the characteristic immunohistochemical p16 overexpression (block staining) in dysplastic and neoplastic epithelium of the uterine cervix [Klaes et al, 2001; Bibbo et al, 2002; Volgareva et al, 2004; Regauer, Reich, 2021]. The viral oncoprotein E7 accounts for the major activities of transformation and immortalization in high-risk types of HPV. E7 contains also a binding site for retinoblastoma gene (Rb) [Fiedler et al, 2006]. Rb is involved in regulating cell proliferation, undergoing several stages of phosphorylation throughout the cell cycle. Retinoblastoma protein (pRb) inhibits also the transcription of the inhibitory gene of cyclin-dependent kinase p16 (INK4A), with impact on cell proliferation. P16 (INK 4A) is overexpressed in the cells, by blocking pRb function. Recent studies have proposed that p16 is a useful marker for HR HPV-type related cervical neoplasia and for predicting SIL progression [Murphy et al, 2005; Guimaraes et al, 2005; Reich et al., 2020; Regauer, Reich, 2021].

Cyclin D1 is responsible for the cellular proliferation, initiation and progression through the G1-S cell cycle phase transition by forming a complex with the cyclin-dependent kinases. Cyclin D1 exhibits a distinct expression pattern in endocervical adenocarcinoma, suggesting an alteration in cell cycle regulation confined at the tumour-stromal interface [Little, Stewart, 2010; Stewart et al, 2011].

Moreover, cyclin D1 represents also a major target gene of Notch pathway, the abnormal Notch signaling pathway modulating cyclin D1 controled cell cycle of HPV-16-

associated adenocarcinoma of the uterine cervix [Tripathi et al., 2019]. ACC features an important combined impact of Notch proteins-cyclinD1, JAG1, and Notch-3. These findings suggest that inhibition of cyclin D1 protein could have a tremendous therapeutic impact in HPV-associated ACC patients [Tripathi et al., 2019].

The overexpression of tumour suppresor gene p53, which plays a major role in cell cycle control and growth arrest following DNA damage, is correlated with a poor prognosis in cervical adenocarcinoma [Baalbergen et al, 2007]. Although HPV 16 and 18 are major etiopathogenic factors in endocervical glandular malignancies, the involvement of HPV infection might be a separate event in the development of endocervical adenocarcinoma [Yoon et al, 2001], with p53 mutation representing a late stage in endocervical carcinogenesis [McCluggage et al, 1997].

The Epidermal Growth Factor Receptor (EGFR) is a member of the ErbB family, the tyrosine kinase receptors with growth promoting effects [Rogers et al, 2005], including an angiogenic potential. Human EGFR gene is located on chromosome 7 and codifies a surface transmembranar glycoprotein, which binds Epidermal Growth Factor (EGF), Transforming Growth Factor alpha, amphiregulin, and Heparin-binding Growth Factor. It is considered that HPV-E5 oncogene is involved in EGFR activation, without an increase in the number of receptors [Pim et al., 1992; Gonzales Martin, 2007; Basto et al., 2020]. Subsequently, HPV-E6 oncogene increases EGFR mRNA level, with protein stabilization, promoting the cellular transduction signal [Chen et al., 2018].

EGFR overexpression is considered to be negatively associated with cervical cancer survival, being observed a significant correlation between high EGFR levels and poor disease-free survival in cervical squamous cell carcinoma patients [Nicholson et al., 2001; Tian et al., 2016]. Thus, the altered EGFR expression could be a reliable predictive biomarker of poor survival in cervical carcinoma, resulting in more efficient targeted therapies for these patients. [Tian et al., 2016].

Epidermal growth factor receptor (EGFR) is overexpressed in ACC and adenosquamous carcinoma due to its involvement in cell cycle, apoptosis, angiogenesis, and regulation of invasive and metastatic potential [Soonthornthum et al, 2011; Iida et al, 2011], the immunoexpression being weaker than in squamous cell carcinoma [Baltazar, 2007; Soonthornthum et al, 2011]. Despite the EGFR overexpression, the EGFR gene activating mutations are absent [Iida et al, 2011]. Moreover, EGFR is associated with HPV infection, but not correlated with HPV type [Soonthornthum et al, 2011].

The cyclooxygenase 2 (COX-2) regulates the prostaglandins synthesis and carcinogenesis through several mechanisms, such as the inhibition of apoptosis and immune surveillance, and the increase of the neoangiogenesis [Ferrandina et al, 2002]. Consequently, COX-2 is involved in the onset and progression of malignancies, including the cervical carcinoma, and is considered a marker of tumor aggressiveness [Ferrandina et al, 2002; Kim et al, 2009]. Unfortunately, only a small number of reported studies focus on the relationship between the COX-2 expression and the HPV detection, in preinvasive cervical lesions [Ferrandina et al, 2002; Sarian et al, 2006; Subbaramaiah, Dannenberg, 2007].

Cyclooxigenase-2 (COX-2) exhibits an enhanced expression in cervical adenocarcinoma [Kulkarni et al, 2001; Chen et al, 2003], possibly due to deregulation of the EGFR signaling pathway [Kulkarni et al, 2001].

Other studies confirmed the overexpression of COX-2 in different types of cervical neoplasia, like cervical intraepithelial neoplasia (CIN), squamous cell carcinoma, and adenocarcinoma [Kim et al., 2013; Ye et al., 2020]. The correlation between COX-2 expression and uterine cervix carcinoma development and progression was immunohistochemically proved more significant for adenocarcinoma than squamous cell carcinoma [Kim et al., 2004; Ye et al., 2020].

Aim

Considering our published data and the current research developments, the aim of our study was to comparatively assess the immunohistochemical expression pattern of several markers (p16, p53, cyclin D1, EGFR, COX-2) in benign and malignant cervical glandular lesions, as well as in low-grade and high-grade cervical squamous intraepithelial lesions, the results being related to L1 HPV capsid protein immunoexpression, in order to determine the relationship of these tumoral markers with the infection status of HPV, and their practical applicability in patients diagnosis and follow-up.

1.2.2. MATERIALS AND METHODS

The study included two groups of patients, selected from the files of the Pathology Laboratory of the "Elena Doamna" Clinical Hospital of Obstetrics and Gynecology, Iasi, Romania. The study was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy and "Elena Doamna" Clinical Hospital of Obstetrics and Gynecology, based on the patients' informed consent on the usage of their biologic material leftover after diagnostic testing, in accordance with the ethical standards of Helsinki declaration.

Patients

The first group comprises 35 cases selected from the files of the Pathology Laboratory of the "Elena Doamna" Obstetrics and Gynecology University Hospital of Iasi, Romania.

The specimens were obtained from endocervical curettage, polipectomy or total hysterectomy. Within the study group, there were 7 cases of endocervical adenocarcinoma in situ (AIS), 8 cases of adenosquamous carcinoma, and 15 cases of invasive adenocarcinoma of endocervical type. We also included in this study group 5 cases without malignant lesions (normal and/or benign endocervical epithelium), corresponding to hysterectomy specimens performed for uterine leiomyomata. One case with AIS was diagnosed within an endocervical polyp. The adenocarcinomas of usual endocervical type were classified as well-differentiated (11 cases) and moderately differentiated (4 cases). The depth of invasion was assessed through the percentage of carcinomatous involvement of the uterine cervix wall.

All tissues were fixed in neutral-buffered formalin, routinely processed and paraffin-embedded. Serial sections of 4 μm were dewaxed and stained with Hematoxylin–Eosin, or furthermore prepared for immunohistochemistry.

The second group included 50 women with cytological and histopathological confirmed LSIL (low grade SIL) (CIN1, cervical intraepithelial neoplasia) (n=32) and HSIL (high-grade SIL) (8 cases of CIN2 and 10 cases of CIN3) (n=18). The immunoexpression of L1 HPV protein was assessed on conventional cervicovaginal smears and EGFR, COX-2 and p16 were immunohistochemically evaluated on the corresponding cervical biopsies. The cervico-vaginal smears were previously fixed and stained with Papanicolaou method.

Immunohistochemical exam

For both groups, all tissues were fixed in neutral-buffered formalin, routinely processed and paraffin-embedded. Serial sections of 4 μm were dewaxed and stained with Hematoxylin–Eosin, or furthermore prepared for immunohistochemistry. Table 1.1 summarizes the characteristics of the primary antibodies, and the antigen retrieval technique. After blocking the endogenous peroxidase and non-specific binding, the samples were incubated with the primary antibodies for 30 minutes, at room temperature, followed by the amplification with the appropriate secondary antibody and the Streptavidin-Biotin-Peroxidase HRP complex (code K5001, DAKO, Denmark).

Finally, the sections were developed with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB, code K5001, DAKO, Denmark), counterstained with Lillie's modified Hematoxylin, dehydrated with ethanol and permanently coverslipped. Positive and negative controls were simultaneously run.

HPV L1 immunoassessment was performed on all histological specimens from the first study group. Supplementary, the patients with malignant endocervical glandular lesions (AIS, endocervical and adenosquamous carcinoma) underwent genotyping for HPV 16/18, by in situ PCR, because of the abnormal glandular lesions identified in liquid-based Pap test.

After the routine cytodiagnosis, the cervicovaginal smears from the second group of patients were used to detect HPV L1 capsid protein by immunocytochemistry, using the monoclonal antibodies (Cytoactiv HPV L1 High Risk Set REF SCA0850, Cytoimmun Diagnostics GmbH). Epithelial cells with positive nuclear staining were scored as positive, considering one stained nucleus enough for scoring.

Table 1.1. Antibodies and antigen retrieval technique

ANTIDOBY	SOURCE	CLONE	DILUTION RANGE	ANTIGEN RETRIEVAL
HPV L1 protein	Viroscreen Virofem Diagnostics GmbH (first group) Cytoimmun Diagnostics GmbH (second group), Germany	VAHK1006 (first group) SCA0850 (second group)	RTU	HIER, 20 minutes
p16	Santa Cruz Biotechnology, USA	2D9A12	1:100	HIER, 30 minutes, pH 6
p53	DAKO, Denmark	DO-7	1:50	HIER, 30 minutes, pH 9
Cyclin D1	DAKO, Denmark	DCS6	1:40	HIER, 30 minutes, pH 9
EGFR	DAKO, Denmark	E30	1:50	PIER, 5 minutes, RT
COX-2	DAKO, Denmark	CX-294	1:100	HIER, 30 minutes, pH 9

HIER, Heat-induced epitope retrieval; PIER, Proteinase-induced epitope retrieval; RT, room temperature; RTU, ready-to-use.

Semi-quantitative immunohistochemical assessment

The semi-quantitative assessment was performed using different scoring systems, as follows.

For HPV L1 capsid protein, the presence of at least one epithelial cell with strong nuclear staining represented the criterion for the immunopositive detection, both for cytological or histopathological specimens [Griesser et al, 2004].

The semi-quantitative evaluation of p16 and COX-2 used two criteria: the percentage (P) of positive cells (0 – no staining; 1 for less than 1% positive; 2 for 1-10% positive; 3 for 11-33% positive; 4 for 34-66% positive; and 5 for more than 66% positive) and the intensity (I) of staining (0 – no staining, 1 – weak, 2 – moderate, 3 – strong), resulting a total P+I score (0-8 : 0-1; 2-3; 4-6;7-8) (Table 1.2) [Allred et al, 1998; Leong, 2004].

The evaluation of p53 was made considering only the percentage of positive cells: 0 for up to 5%, 1 for 5-25%, 2 for 26-50%, 3 for 51-75%, and 4 for more than 76%. The intensity of the reaction was not considered (Table 1.3) [Baalbergen et al, 2007].

The EGFR immunohistochemical expression was quantified according to a proposed score system, as follows: 0 (negative): no staining/membrane staining in <10% neoplastic cells; 1+(positive): weak complete and/or incomplete membrane staining in >10% neoplastic cells; 2+ (positive): moderate complete and/or incomplete membrane staining in >10% neoplastic cells; 3+ (positive): strong complete and/or incomplete membrane staining in >10% neoplastic cells, positive (Table 1.3) [Gamboa-Dominguez et al, 2004].

For cyclin D1, the assessment was based on the proportion of positive tumour cells, and the reaction was considered 0 for negative stain, 1 for less than 50% (focally positive stained) and 2 for more than 50% (diffusely positive stained) (Table 1.3) [Stewart et al, 2011].

Table 1.2. The Allred score (IHC assessment for p16 and COX-2)

PROPORTION SCORE (PS)		**INTENSITY SCORE (IS)**	
Value	**Significance**	**Value**	**Significance**
0	None	0	None
1	<1%	1	Weak
2	1-10%	2	Intermediate
3	10-33%	3	Strong
4	33-66%		
5	>66%		
Allred score (total score) = Proportion score + Intensity score			
0-1		Negative	
2-3		Positive	
4-6		Positive	
7-8		Positive	

Table 1.3. IHC assessment scores for EGFR, p53, and cyclin D1

ANTIBODY	**GRADE**	**CRITERIA**
EGFR	0 1 2 3	no staining/membrane staining in <10% neoplastic cells weak membrane staining in >10% neoplastic cells moderate membrane staining in >10% neoplastic cells membrane staining in >10% neoplastic cells
p53	0 1 2 3 4	< 5% 5-25% 26-50% 51-75% > 76%
Cyclin D1	0 1 2	negative stain < 50% (focally positive stained) > 50% (diffusely positive stained)

Statistical analysis

Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) v. 20 program (SPSS Inc., IBM Corporation, Chicago, IL, USA).

1.2.3. RESULTS

● ***The immunohistochemical assessment of p16, COX-2, p53, EGFR, and cyclin D1 in HPV related adenocarcinoma – first group***

General clinicopathological characteristics

The cases with normal and benign endocervical epithelium presented focal immature squamous metaplasia, tunnel clusters or Nabothian cysts.

Two of the seven cases diagnosed as AIS also had a low grade squamous intraepithelial lesion (LSIL), with evidence of cytopathic HPV effect (koilocytes). The microscopical examination revealed widely spaced or densely arranged glandular pattern in endocervical adenocarcinomas and associated focal solid growth pattern in moderately differentiated adenocarcinomas (four cases). The architectural pattern showed cribriform and papillary features. The cell morphology consisted in simple or stratified columnar epithelial cells exhibiting pleomorphism, marked atypia, with elongated, hyperchromatic nuclei, and evident mitotic figures.

The adenosquamous carcinomas presented a well-differentiated squamous component, with keratin "pearls" or individual cell keratinization. Cellular nests, aggregates or tumor cords represented the invasion pattern, which infiltrated the cervical wall.

All patients with AIS were positive for HPV 16/18. 12 patients out of 15 diagnosed with endocervical adenocarcinoma and 6 out of 8 patients diagnosed with adenosquamous carcinoma had HPV 16/18 positive test (Table 1.4).

Table 1.4. HPV 16/18 genotyping in first group HPV-associated adenocarcinomas

DIAGNOSTIC CATEGORY	HPV 16/18 GENOTYPING	
	n	%
AIS	7	100
Endocervical adenocarcinoma	12	80
Adenosquamous carcinoma	6	75

n – number; % - percent

Correlation between HPV L1 and p16 immunoexpression

The HPV L1 capsid protein immunoexpression was positive in benign glandular epithelium in a single case.

The HPV L1 capsid protein was absent in all cases of AIS, endocervical adenocarcinomas, and adenosquamous carcinomas. Although L1 expression was absent in AIS cases, it was detected in the nuclei of the LSIL component (two cases). The positive reaction was confirmed by the strong staining of the nucleus surrounded by cytoplasm, with no background. The immunoexpression was focally detected in the nuclei of the glandular epithelial cells and from the squamous component.

The HPV infection status revealed by the expression or absence of L1 respectively was correlated to p16, a marker that reflects HPV oncogenic potential. The association L1(+)/p16(+) was found only in one case of benign lesions. L1(-)/p16(+) was present in five cases of AIS, and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma. For L1(-)/p16(-), there were four cases of benign lesions and two cases of AIS. None of the patients presented the relationship L1(+)/p16(-) (Table 1.5).

Table 1.5. Correlation between HPV L1 and p16 immunoexpression in the first study group

DIAGNOSTIC CATEGORY (n)	HPV L1(+)/p16(-) n	%	HPV L1(+)/p16(-) n	%	HPV L1(-)/p16(+) n	%	HPV L1(-)/p16(-) n	%
Benign endocervical epithelium (n = 5)	1	20	0		0		4	80
AIS (n = 7)	0		0		5	71.4	2	28.6
Endocervical adenocarcinoma (n =15)	0		0		15	100	0	
Adenosquamous carcinoma (n = 8)	0		0		8	100	0	

The immunohistochemical assessment of p53, cyclin D1, EGFR, p16, and COX-2

The score values achieved in the semi-quantitative assessment of p53, cyclin D1, EGFR, p16, and COX-2 were summarized in Tables 1.6 - 1.9, each table corresponding to a subgroup of lesions, as follows: benign endocervical epithelium (Table 1.6), AIS (Table 1.7), endocervical adenocarcinoma (Table 1.8), and adenosquamous carcinoma (Table 1.9).

The p16 expression was observed in the epithelial cells nuclei of benign lesions (one case), from AIS (five cases) and from all cases of invasive malignant lesions (Fig. 1.1 - 1.2), with varying degrees of staining intensity and area extent (Tables 1.6 - 1.9).

The staining pattern of p53 was predominantly nuclear. In the subgroup diagnosed with benign lesions (Table 1.6), four cases were p53 negative, and one case presented a positive reaction in less than 25% of cells (score 1). A weak p53 positivity (score 1) was noted in three cases of AIS (Table 1.7). All cases of endocervical adenocarcinoma and adenosquamous carcinoma, except for one from each category, exhibited p53 immunoexpression (Tables 1.8 -1.9). The tumor cells showed immunopositivity in more than 76% of cells (score 4) in one case of endocervical adenocarcinoma (Fig. 1.3) and in two cases of adenosquamous carcinoma (Fig. 1.4).

The EGFR immunoreactivity was predominantly membranar, with focal cytoplasmic positivity.

A large staining heterogeneity was observed, with positive cells admixed with negative cells. However, the proportion of cases with a more intense immunoexpression of EGFR registered a progressive intensity correlated to the severity of lesion. Accordingly, a moderate immunostaining was observed in two cases diagnosed with adenosquamous carcinoma and in one case diagnosed with endocervical adenocarcinoma (Fig. 1.2.5, Tables 1.2.8-1.2.9). A weak immunostaining was also more frequent in carcinomas than in AIS and benign lesions (six and three cases for endocervical and adenosquamous carcinomas respectively, in comparison with one and two cases for benign epithelium and AIS respectively) (Table 1.2.6-1.2.7).

Cyclin D1 was diffusely positive in the cytoplasm and nuclei of only two cases showing immature metaplasia (Table 1.2.6). The immunoreactivity was patchy (Fig. 1.2.6) also in two cases of AIS (Table 1.2.7), eight cases of endocervical adenocarcinoma (Fig. 1.2.7, Table 1.2.8) and three cases of adenosquamous carcinoma (Fig. 1.2.8, Table 1.2.9).

Eighteen of the total thirty-five cases were completely unstained. COX-2 was detected in all in situ and invasive carcinomas (Tables 1.2.7-1.2.9), with a higher total score and a more diffuse immunopattern in endocervical adenocarcinomas (Fig. 1.2.9) and adenosquamous carcinomas than in AIS (Fig. 1.2.10). The epithelial cells cytoplasm in immature squamous metaplasia and normal glandular epithelium of two cases of benign lesions were stained in 10-33%, exhibiting a weak or moderate intensity (Table 1.2.6). COX-2 was expressed in both epithelial tumor cells and inflammatory cells, as well as in glandular and squamous components in adenocarcinomas and adenosquamous carcinomas.

Table 1.6. The semi-quantitative assessment of p53, Cyclin D1, EGFR, p16, and COX-2 in benign lesions

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
				PS	IS	Total	PS	IS	Total
1	0	2	0	0	0	0	0	0	0
2	1	1	0	0	0	0	0	0	0
3	0	0	0	2	1	3	3	2	5
4	0	2	1	0	0	0	2	1	3
5	0	1	0	0	0	0	0	0	0

PS – percentage of stained cells; IS – intensity of staining

Table 1.7. The semi-quantitative assessment of p53, Cyclin D1, EGFR, p16, and COX-2 in adenocarcinoma in situ (AIS)

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
				PS	IS	Total	PS	IS	Total
1	1	0	0	2	3	5	2	2	4
2	0	0	0	0	0	0	3	2	5
3	1	1	1	2	2	4	3	2	5
4	0	0	0	1	2	3	3	2	5
5	0	0	0	2	3	5	2	1	3
6	0	0	1	0	0	0	3	2	5
7	1	1	0	2	2	4	2	1	3

PS – percentage of stained cells; IS – intensity of staining

Table 1.8. The semi-quantitative Assessment of p53, Cyclin D1, EGFR, p16, and COX-2 in endocervical adenocarcinoma

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
				PS	IS	Total	PS	IS	Total
1	2	0	1	3	3	6	4	2	6
2	3	0	1	4	3	7	4	2	6
3	1	0	1	4	3	7	5	3	8
4	4	0	2	3	2	5	3	1	4
5	3	1	1	5	3	8	4	2	6
6	1	0	0	3	3	6	5	2	7
7	3	1	0	4	3	7	5	2	7
8	1	1	0	4	3	7	4	2	6
9	3	1	1	5	3	8	4	2	6

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
				PS	IS	Total	PS	IS	Total
10	1	1	0	4	3	7	3	2	5
11	2	1	0	5	3	8	4	1	5
12	0	0	0	5	3	8	4	3	7
13	1	1	0	3	2	5	4	2	6
14	3	1	1	5	3	8	5	2	7
15	3	0	0	5	3	8	4	2	6

PS – percentage of stained cells; IS – intensity of staining

Table 1.9. The semi-quantitative assessment of p53, Cyclin D1, EGFR, p16, and COX-2 in adenosquamous carcinoma

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
				PS	IS	Total	PS	IS	Total
1	3	0	0	3	3	6	4	2	6
2	4	1	2	4	3	7	5	2	7
3	4	0	1	4	2	6	4	2	6
4	3	1	2	5	3	8	4	2	6
5	2	0	1	5	3	8	3	1	4
6	0	0	0	5	3	8	3	2	5
7	2	0	0	4	2	8	4	2	6
8	3	1	1	5	3	8	4	1	5

PS – percentage of stained cells; IS – intensity of staining

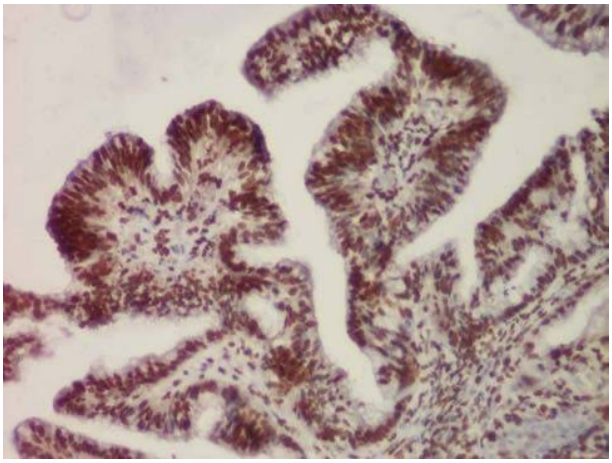


Figure 1.1. Endocervical adenocarcinoma, difusse strong nuclear p16 immunoexpression (anti-p16, x100).

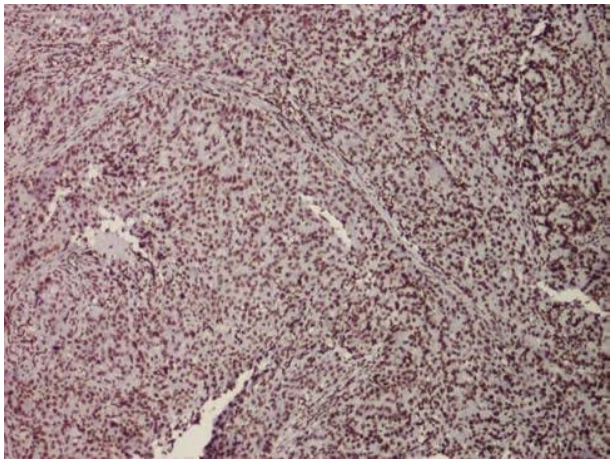


Figure 1.2. Adenosquamous carcinoma, diffuse strong nuclear p16 immunoexpression in both glandular and squamous components (anti-p16, x50).

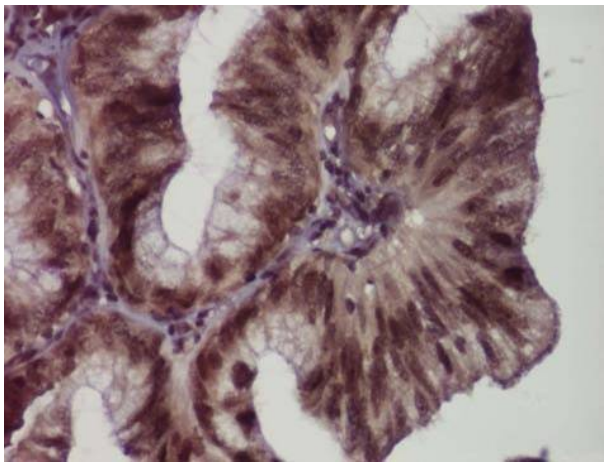


Figure 1.3. Endocervical adenocarcinoma, strong nuclear p53 immunopositivity (anti-p53, x400).

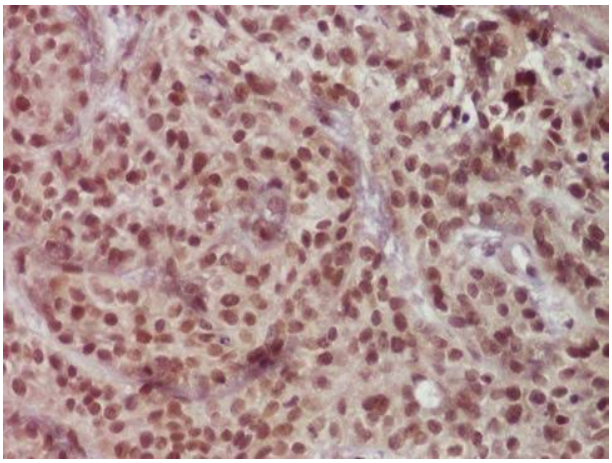


Figure 1.4. Adenosquamous carcinoma, homogenous strong nuclear p53 immunopositivity (anti-p53, x200).

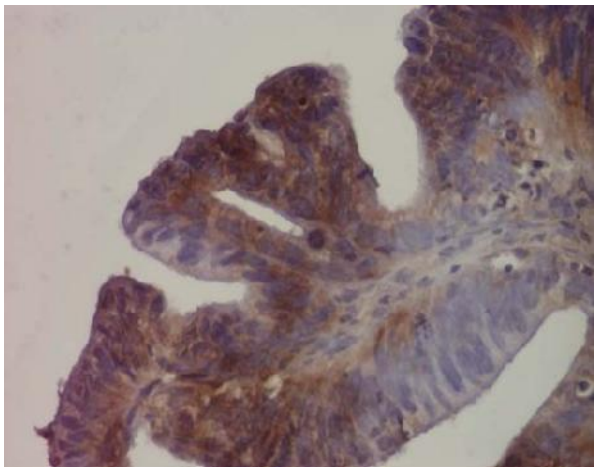


Figure 1.5. Endocervical adenocarcinoma, moderate focal cytoplasmic EGFR positivity (anti-EGFR, x200).

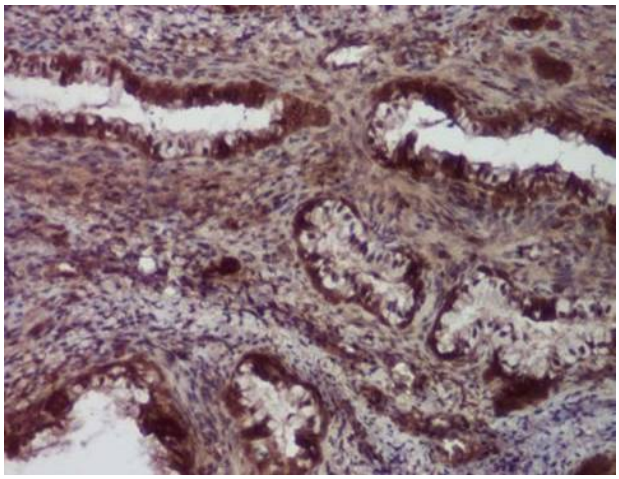


Figure 1.6. AIS, patchy cytoplasmic and nuclear Cyclin D1 immunoexpression (anti-cyclin D1, x100).

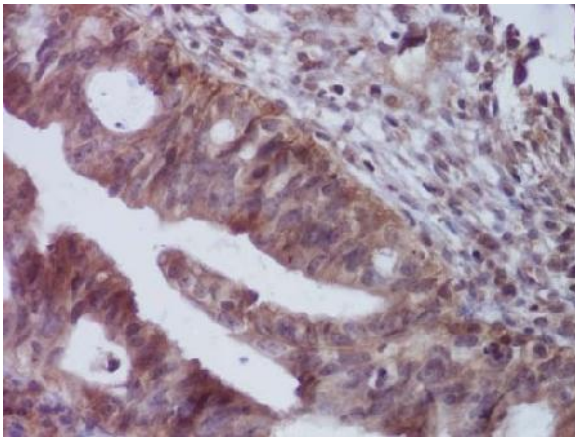


Figure 1.7. Endocervical adenocarcinoma, faint diffuse cytoplasmic cyclin D1 immunoreactivity, with focal nuclear expression (anti-cyclin D1, x200).

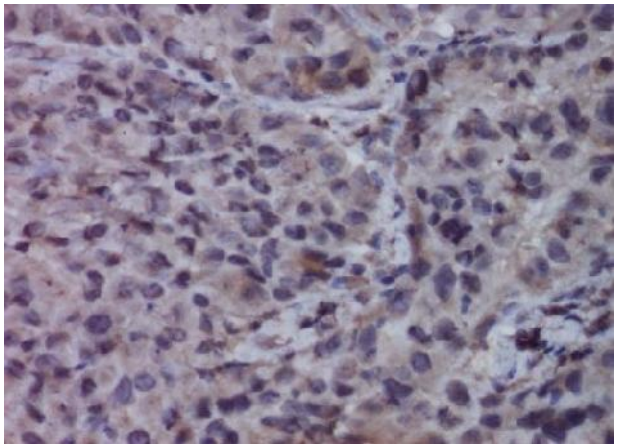


Figure 1.8. Adenosquamous carcinoma, homogenous cytoplasmic cyclin D1 immunoexpression (anti-cyclin D1, x200).

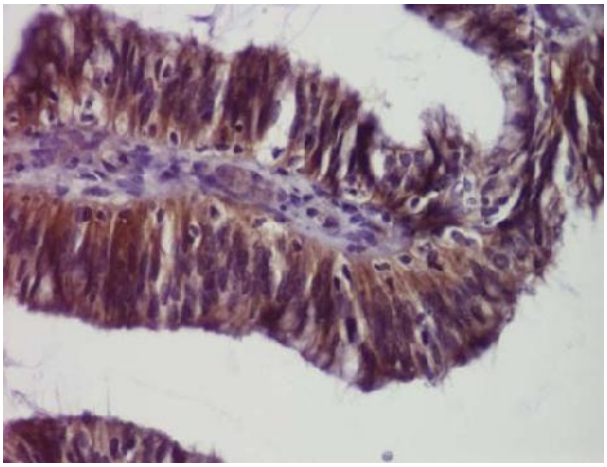


Figure 1.9. Endocervical adenocarcinoma, strong diffuse cytoplasmic COX-2 immunostaining (anti-COX-2, x200).

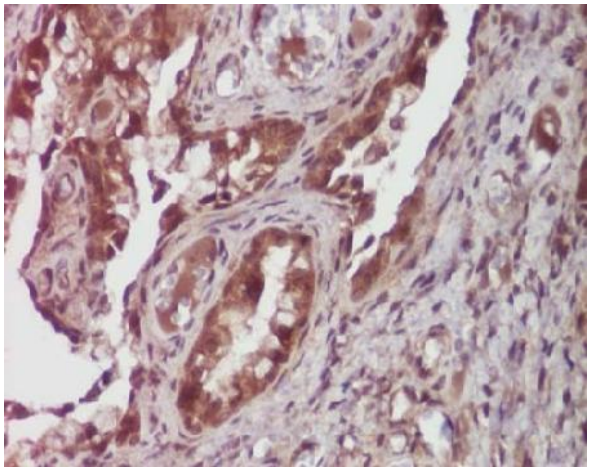


Figure 1.10. AIS, moderate cytoplasmic COX-2 immunoexpression (anti-COX-2, x200).

● ***Immunohistochemical assessment of p16, COX-2 and EGFR in HPV-associated cervical squamous intraepithelial lesions – second group***

Clinicopathological characteristics

In all 50 studied cervical biopsies, the cytological diagnosis was consistent with the histopathologic diagnosis (32 cases with LSIL (CIN1), 18 cases with HSIL (eight cases of CIN2 and 10 cases of CIN3/CIS). The HPV infection was morphologically confirmed by the presence of cytopatic HPV effect in the intermediate and superficial squamous cells (koilocytes) from the smears and biopsies.

Immunocytochemistry in cervical smears

From all cervical smears, the HPV L1 capsid protein was expressed in 52% of LSIL and 23% of HSIL. The expression of L1-capsid protein was significantly reduced for HPV-positive HSIL. In HPV-positive LSIL, no significant reduction of L1 capsid protein expression could be demonstrated. The strong staining of the nucleus surrounded by cytoplasm, with no background, confirmed the positive reaction. The reaction for HR–HPV L1 was positive in typical koilocytes or in dyskeratocytes, presenting nuclear morphological characteristics for HSIL (CIN 2 or CIN 3). In LSIL cases, the nuclei were positive only in typical koilocytes (Fig. 1.11).

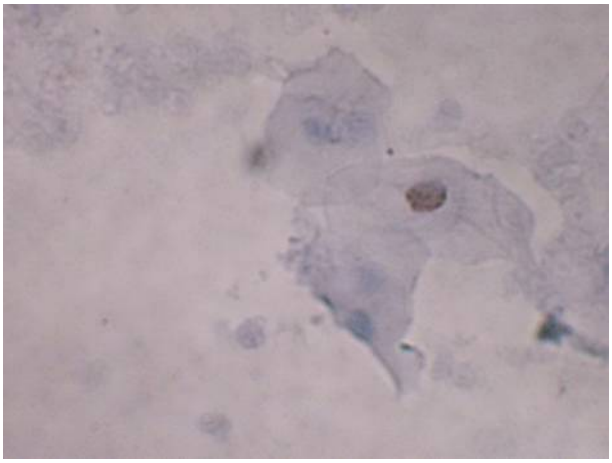


Figure 1.11. LSIL, superficial squamous cell with HPV cytopatic effect, positive nuclear staining, conventional smear (anti-HR HPV L1,×400).

Immunohistochemistry in cervical biopsies

From all cervical biopsies, p16 was positive in 62.5% of LSIL (two cases with score 1, six cases with score 2 and 12 cases with score 3) (Table 1.10), 88.88% of HSIL (two cases with score 1, eight cases with score 2 and six cases with score 3) (Table 1.11).

Table 1.10. The semi-quantitative assessment for p16, EGFR and COX-2 in LSIL cases

CASE	EGFR SCORE	p16 SCORE			COX-2 SCORE		
		PS	IS	Total	PS	IS	Total
1	0	1	0	1	2	2	4
2	0	0	0	0	2	1	3
3	0	2	3	5	3	3	6
4	0	0	0	0	2	2	4
5	0	2	2	4	3	3	6
6	2	3	3	6	4	3	7
7	1	1	2	3	2	3	5
8	0	1	2	3	3	3	6
9	2	2	2	4	4	3	7
10	0	1	2	3	3	2	5
11	1	0	0	0	2	2	4
12	0	2	1	3	2	3	5
13	0	3	2	5	4	2	6
14	2	2	3	5	3	4	7
15	0	0	0	0	3	2	5
16	0	0	0	0	2	1	3
17	2	3	3	6	4	3	7
18	0	2	3	5	3	2	5
19	0	3	3	6	4	2	6
20	3	2	3	5	4	3	7
21	0	0	0	0	2	2	4
22	0	0	0	0	2	1	3
23	0	2	1	3	3	2	5
24	1	0	0	0	3	2	5
25	2	3	3	6	4	3	7
26	0	0	0	0	2	1	3
27	0	0	0	0	3	2	5
28	2	2	3	5	4	2	6
29	0	0	0	0	2	2	4
30	0	0	0	0	2	1	3
31	0	3	3	6	3	3	6
32	0	4	3	7	3	2	5

Table 1.11. The semi-quantitative assessment for p16, EGFR and COX-2 in HSIL cases

CASE	EGFR SCORE	p16			COX-2		
		SCORE					
		PS	IS	Total	PS	IS	Total
1	0	0	0	0	3	3	6
2	3	4	3	7	4	3	7
3	3	4	3	7	5	3	8
4	3	3	2	5	5	3	8
5	2	2	1	3	4	3	7
6	0	0	0	0	3	2	5
7	1	2	3	5	4	2	6
8	3	4	3	7	5	3	8
9	3	2	3	5	5	3	8
10	0	2	2	4	5	2	7
11	3	5	3	8	5	3	8
12	3	3	3	6	5	2	7
13	0	2	2	4	4	3	7
14	0	2	3	5	4	3	7
15	3	2	3	5	5	3	8
16	0	1	2	3	3	3	6
17	3	2	2	4	5	2	7
18	3	3	3	6	5	3	8

The p16 staining pattern was predominantly nuclear with occasional cytoplasmic positivity. Most cases presented heterogeneity of staining, with positive cells admixed with negative cells. P16 presented a positive immune reaction in the precancerous lesions, with or without L1 HPV-positivity.

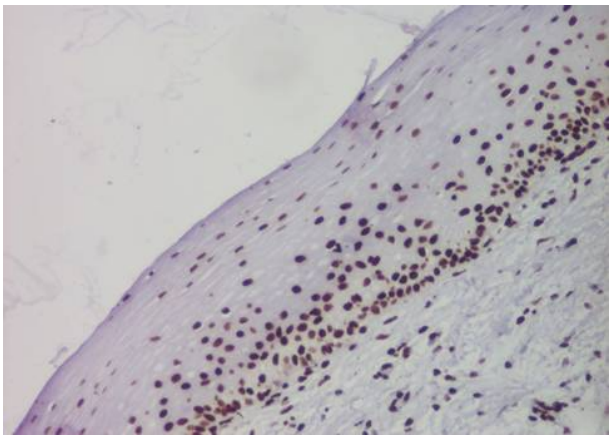


Figure 1.12. Exocervical epithelium, LSIL, strong nuclear staining in the basal third of the epithelium (anti-p16, x100).

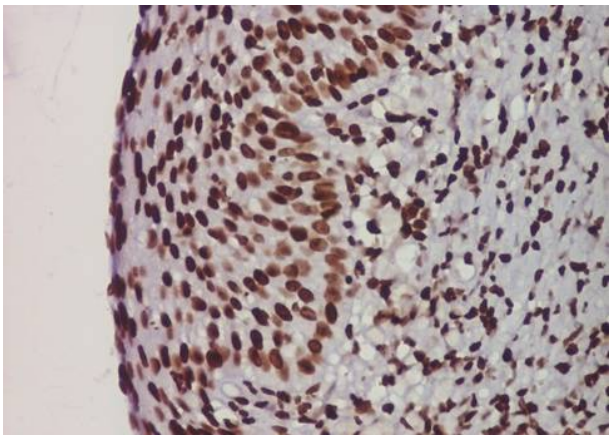


Figure 1.13. Exocervical epithelium, HSIL (CIN3), strong nuclear staining in the entire epithelial thickness (anti-p16, x200).

The proportion of biopsies with intense immunoexpression of p16 increased with severity of cytological abnormality. In LSIL cases, the staining distribution was basal in 72% of

cases (Fig. 1.12) and dispersed in 28%. The staining intensity of LSIL cases was strong in 26% of cases, moderate in 16% of cases, and weak in 58% of cases. Regarding HSIL category, the staining distribution was as follows: 62% – full thickness (Fig. 1.13), 38% – 1/3 and 2/3 of the epithelium thickness. The staining intensity for HSIL cases was strong in 75% of cases, moderate in 12% of cases and weak in 13% of cases.

From all biopsied cases, EGFR was overexpressed in 32% of LSIL (with score values: 1 – three cases, 2 – six cases, 3 – one case) (Table 1.10) and 67% of HSIL (with score values: 1 – one case, 2 – one case, 3 – 10 cases) (Table 1.11). The EGFR staining pattern was predominantly membranar with occasional cytoplasmic positivity. Most cases presented heterogeneity of staining, with positive cells admixed with negative cells. The proportion of biopsies with intense immunoexpression of EGFR increased with the severity of cytological abnormality. EGFR staining was observed in basal and parabasal cells, in koilocytes and in dysplastic squamous cells of the intraepithelial lesions.

Regarding LSIL category, the staining distribution was identified in basal and parabasal cells and in koilocytes (Figure 1.14), and was considered strong in 10% cases, moderate in 64% cases, and weak in 26% cases. In HSIL cases, the staining distribution was as follows: 72% full thickness, 28% in basal and intermediate layers, and the staining intensity was assessed as strong in 87% cases, moderate in 7% cases, and weak in 6% cases, being more intense in CIN2 lesions (Figure 1.15) than in CIN3.

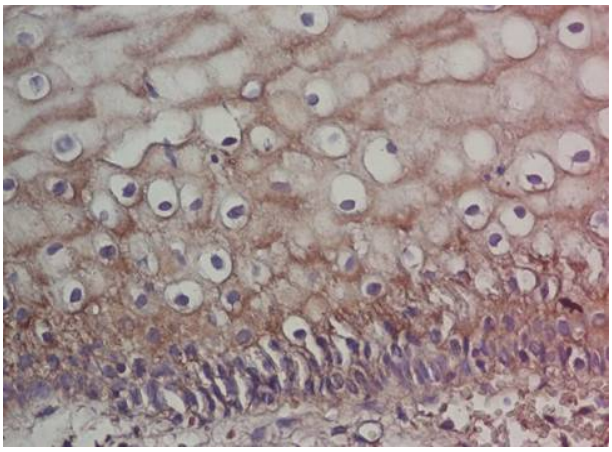


Figure 1.14. Exocervical epithelium, LSIL, strong membranar staining in koilocytes and dysplastic basal squamous cells (anti-EGFR, x200).

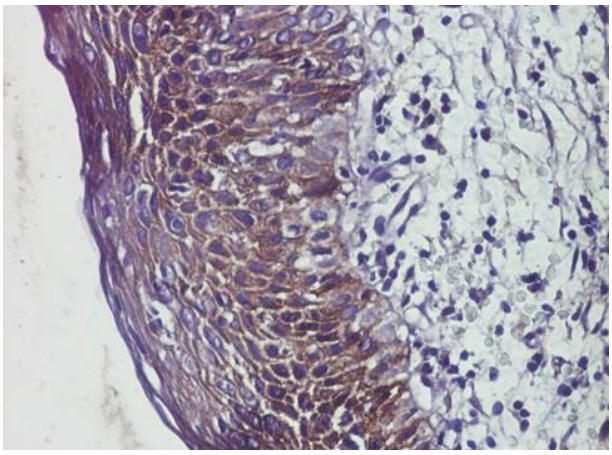


Figure 1.15. Exocervical epithelium, HSIL (CIN2), strong membranar and diffuse cytoplasmic staining (anti-EGFR, x200).

COX-2 expression showed finely granular cytoplasmic staining with occasional membrane staining, especially in koilocytes. The stromal inflammatory cells, in cases with associated chronic cervicitis, were also intense positive for COX-2.

The results of the semiquantitative exam, based on the Allred score applied to the immunohistochemical reactions, were summarized in Tables 1.10 and 1.11. The general score was higher in HSIL when compared to LSIL. Our data revealed 33 cases belonging to both LSIL and HSIL categories with the same Allred score, as follows: nine LSIL cases and one HSIL case with value 5, seven LSIL cases and three HSIL cases with value 6, six LSIL cases and seven HSIL cases with value 7. Regarding the intensity of cytoplasmic COX-2 immunostaining, a weaker expression was observed in specimens with LSIL (Figure 1.16) and a stronger one in those diagnosed with HSIL (Figure 1.17). The highest score was noted in HSIL corresponding to CIS lesions.

No correlation between the intensity of the COX-2 immunostaining and the presence of the koilocytes within the squamous dysplastic epithelium was found.

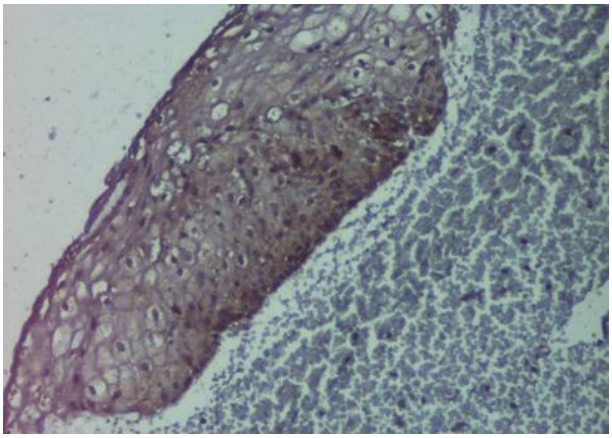


Figure 1.16. LSIL, strong, heterogeneous cytoplasmic staining in the basal third of the exocervical epithelium (anti-COX-2, x100).

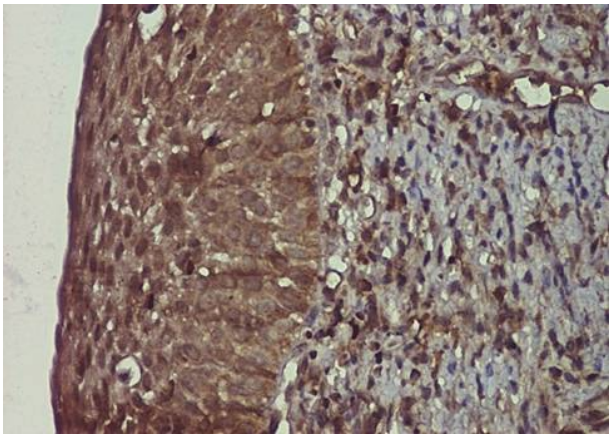


Figure 1.17. HSIL (CIN2), strong heterogenous cytoplasmic staining of the exocervical epithelium; stromal lymphocytes with moderate cytoplasmic staining (anti-COX-2, x200).

● ***Comparative assessment of p16, COX-2, and EGFR immunoexpression in HPV-associated adenocarcinoma and HPV- associated squamous intraepithelial lesions from study groups one and two***

The comparative evaluation of the immunoexpression of EGFR, p16, and COX-2 in both types of lesions from the two study groups shows a certain symmetry regarding the correspondence of the expression with the increased severity of the lesion, regardless of the squamous or glandular type (Table 1.12).

Table 1.12. Comparative assessment of p16, COX-2, and EGFR immunoexpression in HPV-associated adenocarcinoma and HPV- associated squamous intraepithelial lesions

DIAGNOSTIC CATEGORY (n)	EGFR		p16		COX-2	
	n	%	n	%	n	%
AIS (n=7)	2	28.57	5	71.42	7	100
Endocervical AK (n=15)	7	46.66	15	100	15	100
Adenosquamous Carcinoma (n=8)	5	62.5	8	100	8	100
LSIL (n=32)	10	31.25	20	62.5	22	68.75
HSIL (n=18)	12	67	16	88.88	11	61.11

n – number of cells; % - percentage; AIS – adenocarcinoma in situ; AK – adenocarcinoma; LSIL – low grade squamous intraepithelial lesion; HSIL – high grade squamous intraepithelial lesion

1.2.4. DISCUSSIONS

Currently, it is considered that cervical carcinogenesis is not rely on the presence of HPV oncogenes E6 and E7 [Olthof et al., 2015; Scarpini et al., 2014; Gray et al., 2010; Hu et al., 2015; Ojesina et al., 2013; Oyervides-Muñoz et al., 2018], as previously thought [zur Hausen, 1989]. There are now discussed several mechanisms involved in cancer development and progression, like viral integration causing genetic alterations [Badaracco et al., 2002; Hopman et al., 2004], without the E6 and E7 involvement [Hu et al., 2015; Groves and Coleman, 2015], or different

epigenetic mechanisms which alter the carcinogenesis key genes expression, but the latter mechanisms are still under study [Groves, Coleman, 2015; Oyervides-Muñoz et al., 2018].

There are described two types of viral genome integration in the cell host genome: (i) integration as a single genome; (ii) integration as a multiple tandem viral genome repeats [McBride, Warburton, 2017].

Regarding the possibility of HPV transformation into the cell host genome, Rusan et al. described several modifications and alterations initiated by viral integration associated with carcinogenesis, resumed in three main pathways: (i) tumor suppressor genes function loss; 2) oncogene overexpression; (iii) intra- and inter-chromosomal rearrangements [Rusan et al., 2015; Oyervides-Muñoz et al., 2018].

Another relevant finding is the description of HPV specific integration points, located inside or adjacent to fragile sites [Liu et al., 2016], known as "hot spots", through which genes and thus specific pathways can be altered, leading to carcinogenesis [Oyervides-Muñoz et al., 2018].

Although numerous researches regarding cervical adenocarcinoma have been recently performed, to our knowledge, there are few released papers focused on an extensive immunoprofile of the molecules involved in the HPV related precursor lesion progression and cervical adenocarcinoma development.

For this reason, our study aimed to assess the immunohistochemical pattern of p16, p53, cyclin D1, EGFR, and COX-2 in benign glandular lesions and HPV- associated cervical adenocarcinoma, as well as the immunohistochemical expression of p16, COX-2, and EGFR in HPV- associated squamous intraepithelial lesions, in order to identify possible correlations between these markers and the HPV infection status.

● *HPV L1 capsid protein immunoassessment in benign glandular lesions and HPV-associated adenocarcinoma*

The L1 capsid protein represents the main target of the cellular immune response to HPV infection and consequently it is expressed in the early productive phase of cervical carcinogenesis (LSIL) [Wu et al., 2011]. The studies performed on cervical squamous cell carcinoma and its precursor – namely intraepithelial neoplasia – revealed a progressively decrease of HPVL1 expression, in parallel with the increase of the cervical lesion severity [Griesser et al., 2004; Wu et al., 2011]. Our results showed that the HPVL1 capsid protein immunoreactivity was detected in the epithelial cells from a case belonging to the subgroup diagnosed with benign lesions, and absent in all cases of AIS, endocervical adenocarcinoma, and adenosquamous carcinoma. Although L1 expression was absent in AIS cases, it was detected in the nuclei of the LSIL component. These findings suggest that the expression of capsid protein occurs as an early event in endocervical carcinogenesis, despite the lack of specific HPV morphological features in glandular cells in contrast with infected squamous cells. Our findings are consistent with previous studies on cervical squamous precancerous lesions and carcinoma, which concluded that the deficiency of L1 expression has a predictive value for an increased risk of malignant transformation of cervical dysplasia [Yu et al., 2010; Wu et al., 2011].

● *p16 immunoassessment in benign glandular lesions and HPV-associated adenocarcinoma*

The p16 value as a biomarker of the E7-driven oncogenic activity of HPV has been already proven, in keeping with its expression in almost all high-grade squamous lesions, squamous carcinoma, high grade-dysplastic glandular lesions, and adenocarcinomas of the cervix [Negri et al., 2003; Ishikawa et al., 2003; Liang et al., 2007; Muller et al., 2008; Negri et al., 2011]. E7 is responsible for the main converting and immortalizing activity that characterizes the high-risk types of HPV. The binding site for retinoblastoma gene (Rb) provides E7 with the capacity of cell proliferation regulation by variable phosphorylation

degrees during the cell cycle [Fiedler et al, 2006]. Moreover, the retinoblastoma protein (pRb) intervenes in the cell cycle activation by inhibiting the transcription of the inhibitory gene of cyclin-dependent kinase p16(INK4A). Subsequently, the blocking of pRb function results in the overexpression of p16INK4A in the corresponding cells.

Regarding the localization pattern of p16 immunostaining, our data revealed a moderate or strong expression restricted to nucleus, without cytoplasmic staining described by other researchers [Koo et al., 2009; Negri et al., 2011]. p16 was positive in one case with immature squamous metaplasia of both the endocervical surface and the glandular epithelium, and strongly positive in five cases of AIS and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma.

These results are in agreement with the literature data, several papers showing that p16 expressed diffuse immunoreaction in most neoplastic cervical epithelia, and no or only focal positivity in nonneoplastic lesions [Riethdorf et al., 2002; Negri et al., 2003; Murphy et al., 2004; Negri et al., 2011]. Two cases of AIS were p16 negative, despite the fact that the patients were HPV 16/18 positive.

The lack of protein p16 expression can be the result of the hypermethylation of the p16INK4a promoter and it is not correlated to HPV status or to the lesion grade, as has been already mentioned in previous studies [Riethdorf et al., 2002; Negri et al., 2003; Ishikawa et al., 2003; Murphy et al., 2004; Lesnikova et al., 2009; Negri et al., 2011].

● *HPVL1 and p16 association in benign glandular lesions and HPV-associated adenocarcinoma*

Cervical adenocarcinomas of the cervix are now divided into HPV-associated and non-HPV-associated categories (Herrington et al., 2020). It was found that HPV-associated adenocarcinoma more rarely displays histological Silva C pattern, LVSI, or lymph node metastases, presenting a superior overall survival (OS) and disease-free survival (DFS), with lower recurrence risk (Stolnicu et al., 2019; Wang et al., 2022). The p16 immunoexpression is related to HPV-associated neoplastic transformation, being also proeminent in endometrial mucinous adenocarcinoma [Stewart et al., 2019]. This finding emphasizes the importance of HPV DNA testing, besides its role in differentiating endocervical adenocarcinoma from the endometrial correspondent [Wang et al., 2022].

Numerous studies reported the importance of association of p16 and HPVL1 immunodetection in the prediction of cervical disease progression [Wu et al., 2011; Yu et al., 2010; Negri et al., 2008]. As we have already mentioned, the protein capsid L1 is expressed in the early, productive phase of cervical carcinogenesis and is progressively lost in the later phases, when p16 gets overexpressed [Doorbar, 2005]. In our study, the association L1(+)/p16(+) was found only in one case of benign lesions, with immature squamous metaplasia and tunnel clusters. The significance of this finding is that the lesion is still productive (L1+), but the pRb pathway is probably inhibited (p16+). L1(-)/p16(+) pattern of expression was registered in five cases of AIS, and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma.

These lesions could be interpreted as proliferative entities (L1-), with an inhibited pRb pathway, with p16 positivity. They probably represent high-risk glandular lesions, as previously demonstrated for squamous precursor lesions of the cervix [Negri et al., 2008]. There were four cases of benign lesions and two cases of AIS with negative expression of both L1 and p16. This finding was expected in benign lesions, but a careful evaluation is mandatory in such AIS cases, mainly because the patients were positive for HPV 16/18. As already demonstrated for squamous intraepithelial lesions [Negri et al., 2008], we can assert that the combination of these two biomarkers can predict the progression risk of precursor lesion of endocervical adenocarcinomas.

● *p53 immunoassessment in benign glandular lesions and HPV-associated adenocarcinoma*

It has been already demonstrated that p53 regulates the cell proliferation in cervical neoplasia, through stimulation of other specific cell cycle control genes [Baalbergen et al., 2007]. The staining pattern of p53 was predominantly nuclear in our study. In the benign lesion subgroup, 4 cases were p53 negative and 1 case presented a p53 expression quantified as score 1, based on less than 25% of epithelial cells were stained positively for p53. In three of five cases of AIS a weak positive reaction of p53, also assessed with score 1, was noted. Within the malignant lesion subgroups, the p53 immunoexpression was present in the majority of cases (14 of 15 endocervical adenocarcinomas and 7 of 8 adenosquamous carcinomas). The score values range the entire scale, one case of endocervical adenocarcinoma and two cases of adenosquamous carcinoma exhibiting a strong reaction, corresponding to the maximum score value, namely 4. A consensus of our results with one of the previous published work was noticed, with 70% of adenocarcinoma and 20% of AIS p53 immunoexpression [McCluggage et al., 1997]. Oppositely, other studies reported lower or absent p53 protein expression in AIS or high-grade dysplastic glandular lesions [Cina et al., 1997; Yoon et al., 2001] or even a lack of correlation of p53 immunoexpression with the malignant development [Cina et al., 1997].

The p53 immunoexpresion, as a reflection of the protein conformational changes, reflects a key point in the late stages of carcinogenesis sequence, with evident p16 overexpression parallel features.

Although a controversial matter [Dimitrakakis et al., 2000; Lee et al., 2002; Saito et al., 2004], in our opinion, this parallelism can have clinical prognostic significance in the evaluation of HPV related cervical adenocarcinoma, revealing p53 as a supplementary biomarker of the association of protein capsid L1 and p16.

● *EGFR immunoassessment in benign glandular lesions and HPV-associated adenocarcinoma*

It has been already demonstrated the HPV proteins role in EGFR expression [Bosch et al., 2002], which is correlated with HPV infection status. The EGFR immunoreactivity has a parallel expression with the increase in severity of intraepithelial lesions, without an identifiable relation to the HPV type [Chapman et al., 1992; Hu et al, 1997; Akerman et al., 2001]. HPV E5 protein causes the overexpression of EGFR, through inhibition of internalized EGFR degradation [Zhang et al., 2005]. HPV E6/E7 protein complex may increase the EGFR levels and disrupt the growth rate of cervical carcinoma cell lines [Hu et al., 1997; Akerman et al., 2001]. Moreover, a possible difference between the squamous carcinomas and adenocarcinomas were identified based on their genetic alterations [Iida et al., 2011].

In our study, only two cases of adenosquamous carcinoma and one case of endocervical adenocarcinoma presented a moderate EGFR staining, quantified as score 2. A weak immunostaining, corresponding to the score 1, was also more frequently observed in carcinomas than in AIS and benign lesions (6 and 3 cases for endocervical and adenosquamous carcinomas respectively, compared with one and two cases for benign epithelium and AIS), as already reported in previous studies [Kersemaekers et al., 1999; Baltazar et al., 2007; Longatto-Filho et al., 2009] which revealed EGFR overexpression in adenosquamous carcinomas. In conclusion, our results demonstrate the EGFR immunoexpression increase according to the progression in lesions severity, based on the HPV 16/18 etiopathogenic context.

Although EGFR gene amplification was reported as an independent prognostic factor in cervical squamous cell carcinoma [Iida et al., 2011], this statement cannot be yet extended on adenocarcinomas. EGFR is a very useful tool that should be added to the panel of biomarkers used in monitoring the evolution of HPV related dysplastic glandular lesions and adenocarcinomas. Moreover, recent papers consider EGFR expression as beneficial in clinical

trials, which evaluate the efficacy of anti-EGFR therapies in advanced cervical cancers, including adenosquamous carcinomas [Del Campo et al., 2008; Longatto-Filho et al., 2009].

● *Cyclin D1 immunoassessment in benign glandular lesions and HPV-associated adenocarcinoma*

Cyclin D1 was evaluated in normal cervix and endocervical adenocarcinomas, the latter in relationship with the epithelial-mesenchymal transition and the tumoral growth pattern [Little, Stewart, 2010; Stewart et al., 2011]. The authors reported the presence of cyclin D1 in normal and parabasal squamous cells of non-neoplastic mucosa, its lack of expression in AIS, and a focal labelling in the infiltrative areas of the moderately differentiated adenocarcinomas. The positive reaction along the deep border of the malignant glands, in parallel with the negativity of the rest of the tumor, and the correlation with specific markers for EMT indicate that cyclin D1 is upregulated. This fact can be interpreted in the context of its role of mediator in the main biochemical pathways that regulate the cellular proliferation, apoptosis and invasive capacity. Consequently, cyclin D1 was associated with migration and invasion [Li et al., 2006].

In our study, cyclin D1 was diffusely positive in the cytoplasm and nuclei of only two cases with immature squamous metaplasia and in normal endocervical epithelium. The higher expression noticed in the squamous metaplasia is indicative of important basal/reserve cells reactivity. The immunoreactivity was observed also in two cases of AIS, 8 cases of endocervical adenocarcinoma and three cases of adenosquamous carcinoma, both in proper tumoral areas, and in tumoral invasion front. Because twelve of the total twenty-three cases of adenocarcinoma and adenosquamous carcinomas were completely unstained, we consider our results partially similar with the previous reports mentioned above, in which the cyclin D1 immunoexpression was negative in the main tumoral areas, being positive only at the invasion borders.

The interconnection between HPV infection and cyclin D1 activity has been already demonstrated [Nichols et al., 1996; Skomedal et al., 1999]. pRb is inhibited by HPV oncoprotein E7, and thus pRb avoids the normal requisite for cyclin D1-CDK complex to initiate the cell cycle. PRb inactivation down-regulates cyclin D1, additionally enhancing its activity in cellular proliferation, and upregulates p16, although this is functionally inactive [Little, Stewart, 2010]. These events are also useful in understanding and achieving a manageable dissimilarity of the mechanisms of both main types of cervical carcinogenesis [Iida et al., 2011], with the contrast increasing of a tumor suppressor protein (p16) and decreasing of a proliferation marker (cyclin D1). The relationship between HPV infection and the peculiar behaviour of these two biomarkers has been already reported in cervical adenocarcinoma and squamous cell carcinoma of the head and neck [Andl et al., 1998; Li et al., 2004; Little, Stewart, 2010]. These correlations emphasize the feasible usefulness of cyclin D1 in the clinical management of HPV-associated cervical adenocarcinomas.

It is widely accepted that, in cervical carcinoma, the expression of cyclin D1 represents another subsequent target of Notch signaling pathway [Shin et al., 2006; Ramdass et al., 2007; Maliekal et al., 2008; Myong et al., 2017], being activated by Notch-ICD domain [Ronchini et al., 2001]. This finding is reinforced by the observation that cyclin D1 expression is affected by the incidental dialogue between NF-κB and Notch signaling pathways [Yao et al., 2007]. Cyclin D1 induces cell proliferation by its association with CDK (cyclin-dependent kinase) 6 or CDK4 in G1 stage, connecting with retinoblastoma protein (pRb) in order to regulate G1/S phase check point [Lu et al., 2005]. The higher levels of cyclin D1 lead to G1 phase decrease and cell proliferation increase [Liang et al., 2013], emphasizing the importance in carcinogenesis by its involvement in cell adhesion, proliferation, motility, as well as stromal invasion [Ronchini et al., 2001; Myong et al., 2017]. Tripathi et al. found that cyclin D1 is associated with cervical adenocarcinoma in patients infected with HPV type16 [Tripathi et al., 2019]. Moreover, they noticed an important impact of associated Notch signaling proteins-

JAG1, Notch-3, and Cyclin D1 in adenocarcinoma, emphasizing the significant interrelation between these three Notch signaling proteins and validating cyclin D1 utility in cervical adenocarcinoma diagnosis [Tripathi et al., 2019].

● *COX-2 immunoassessment in benign glandular lesions and HPV*-associated *adenocarcinoma*

In our study, COX-2 was detected in all in situ and invasive carcinomas, with a higher total score and a more diffuse immunopattern in endocervical adenocarcinomas and adenosquamous carcinomas than in AIS. There were also two cases of benign lesions with a weak or moderate COX-2 positivity. COX-2 was expressed in both glandular and squamous epithelial tumor cells, and stromal inflammatory cells of adenocarcinomas. These results are in accordance with previous data, including also our report, in which COX-2 was overexpressed in squamous cervical carcinomas, adenocarcinomas, as well as intraepithelial lesions [Kulkarni et al., 2001; Balan et al., 2011].

Other studies confirmed the COX-2 overexpression in different cervical neoplasms, such as squamous intraepithelial lesions, squamous cell carcinoma, as well as adenocarcinoma, underlying the COX-2 association with cervical carcinogenesis [Kim et al., 2013; Ye et al., 2020]. The COX-2 immunoexpression is more often observed in endocervical adenocarcinoma comparative with cervical squamous cell carcinoma [Kim et al., 2004; Ye et al., 2020].

COX-2 levels increase along with the increase in severity of the glandular neoplastic epithelial lesion. This observation sustains the important role of COX-2 in tumor development and progression, correlated with the antitumor immunity [Chen et al., 2003; Chen et al., 2006].

Our findings raise also the possibility of a direct association between HPV status and COX-2. Thus, in the context of confirmed HPV 16/18 infection in patients with in situ and invasive adenocarcinoma, we observed a high correlation between COX-2 and p16 status, the immunoexpression of these two biomarkers showing an approximately parallel increase, especially in AIS and endocervical adenocarcinoma.

As we have already mentioned above, the HPV E-oncoproteins plays an essential role in the pathobiology of p53, EGFR, p16, in the cervical carcinogenesis pathogenic mechanism. It is widely accepted that L1 viral capsid protein is considered a major target of the cellular immune response [Melsheimer et al., 2003]. The loss of L1 immunoexpression indicates a poor local defense status, as a result of COX-2 involvement in host-antitumor immunity. For these reasons, COX-2 can be added to the protein panel, which already proved their role in the management of pathologic and clinical behavior of the HPV related adenocarcinomas.

● *HPVL1 capsid protein immunocytochemical assessment in HPV-associated intraepithelial squamous lesions*

The HPV infection of the cervical squamous epithelium occurs in the basal layer, this being the only region of the epithelium capable of mitotic activity necessary to induce epithelial transformation.

There are still many questions about the mechanism of HPV infection, regarding the presence of receptors on the target cells, the infection of the target epithelium by mature virions or sequences of viral DNA, or the pathway of viruses' travel across the cytoplasm to reach the nucleus [Koss, Melamed, 2006]. It is considered that the carcinogenic role of the HPV can only manifest under certain conditions that favor its persistence, one of them being the host immunodeficiency [Koss, Melamed, 2006]. A high frequency of viral infection and precancerous lesions was observed in immunosupressed women, particularly women infected with human immunodeficiency virus (HIV) and women with AIDS [Palefsky et al., 1999; Ellerbrock et al., 2000].

Because L1 capsid protein is expressed in the active phase of HPV infection, the viral

protein immunodetection is an evidence of the active HPV infection in the examined tissue [Gu et al., 2007]. It is widely accepted that L1 viral capsid protein represents a major target of the cellular immune response [Melsheimer et al, 2003]. The squamous intraepithelial lesions, without L1 HPV immunodetection are correlated, in more than 80% of cases, with dysplasia progression [Griesser et al., 2004]. Immunocytochemical detection of L1 capsid protein, on conventional Papanicolau smears, may correspond to a local defense status induced by HPV infection and may offer prognosis information, mainly in LSIL lesions.

Our current data, based also on our previous studies [Balan et al., 2010], sustain that L1 HPV positivity represent a favorable expression of the immune status and, consequently, ensure a relative protection against the progression of the lesions toward the malignant framework. In our group, HPV L1 capsid protein was expressed in 52% of LSIL and 23% of HSIL, diagnosed on the cervicovaginal smears. Expression of L1 capsid protein was significantly reduced for HPV-positive HSIL and less reduced in HPV-positive LSIL. Because of the low rate of HR-HPV L1 positivity found in LSIL cases in our study, we can suggest that HPV is not helpful in grading cervical SIL, which is in accordance with the literature data [Yildiz et al., 2007].

● *p16 immunoassessment in HPV-associated squamous intraepithelial lesions*

The tumor-suppressor protein p16 regulates transition from the G1 to the S-phase of the cell cycle [Ortega et al., 2002; Walts, Bose, 2009].

The viral oncoprotein E7 accounts for the major transforming and immortalizing activity in high-risk types of HPV. E7 contains also a binding site for retinoblastoma gene (Rb) [Fiedler et al., 2006], which is involved in regulation of cell proliferation, suffering various phosphorylation degrees during cell cycle. PRb inhibits also the transcription of an inhibitory gene of cyclin-dependent kinase p16(INK4A), with a role in cell cycle proliferation. The overexpression of p16(INK4A) in cells is the consequence of pRb blocked function.

Recent research highlight the role of p16, as an extremely sensitive marker for cervical epithelial dysplasia and high-risk HPV-type related neoplasia [Klaes et al., 2001; Bibbo et al., 2002; Volgareva et al., 2004; Gurrola-Diaz et al., 2008; Guo et al., 2011], and for predicting SIL progression [Guimaraes et al., 2005; Murphy et al., 2005].

Strong and full thickness staining of p16 in the cervical epithelium is supportive of HSIL, while weak and basal/rare staining favors LSIL [Walts, Bose, 2009].

In our study, from all cervical biopsies, p16 was positive in 62.5% of LSIL, 88.88% of HSIL. The proportion of biopsies with intense immunoexpression of p16 increased with the severity of cytological abnormality, which is consistent with our previous study [Balan et al., 2010] and with the data reported in the literature [Kurshumliu et al., 2009; Tsoumpou et al., 2009; Missaoui et al., 2010; Ungureanu et al., 2010].

● *p16 and HPVL1 capsid protein association in HPV-associated squamous intraepithelial lesions*

For the early diagnosis, p16 can contribute as an adjuvant tool in the follow-up of cervical intraepithelial lesions when the cytology sample is collected in the standard way [Rocha et al., 2009]. Moreover, the combination between L1 HPV capsid protein and p16 is more useful, having a higher accuracy than L1 or p16 alone [Huang et al., 2010]. There are also opinions for the relevance of p16 expression in cervical squamous and glandular epithelium, as a marker of dysplasia or malignancy irrespective of the HPV infection status [Lin et al., 2005]. Our results reveal an overexpression of p16 in the precancerous lesions, with or without L1 HPV positivity, as follows: p16-positive with HPV L1-positive in 28% of LSIL and 6% of HSIL; p16 positive with HPV L1-negative in 36% of LSIL and 17% of HSIL.

These data could be interpreted in the context of the HPV immune behavior, translated by the avoidance of the host immune response, through late expression of antigenic protein

responsible for antibodies production.

HPV induces local immune dysfunction characterized by the decrease of the intraepithelial antigen presenting cells and of the cytotoxic T-lymphocytes. It is possible that in the case of a low secretion of the protein L1 below the limit of its immunodetection, there is no antibodies production and no lymphocytes activation. This will increase the chance for persistent infection and for the the progression of intraepithelial squamous lesion, statement in concordance with our results presented above, in which the lack of immunoexpression of the HPV capsid protein L1 was better correlated with the overexpression of p16. Therefore, our results support the idea that the squamous intraepithelial lesions from the patients with an altered or disturbed immune status are more likely to progress toward a high-grade dysplasia or carcinoma, than those from the patients with an active HPV infection, in which the cellular immune response manifests through its particular mechanisms.

● *EGFR immunoassessment in HPV-associated intraepithelial squamous lesions*

In the cervix, EGFR is present in normal status and malignant conditions with varying degrees of expression. In normal cervical mucosa, EGFR is expressed in the cytoplasm and the membrane of the cells within the basal layer, and as cells differentiate, there is a shift toward the cytoplasm [Soonthornthum et al., 2011]. It is admitted that the high expression of EGFR, in collaboration with viral oncoproteins (E6 and E7) and the activation and overexpression of mTOR pathway (mammalian target of rapamycin inhibitor), play a key role in both highgrade squamous intraepithelial lesions and invasive squamous cell carcinoma [Feng et al., 2009]. HPV infection may change the biology of EGFR expression by preventing EGFR degradation [Chapman et al., 1992]. Recently, controversial data are published regarding the association of EGFR overexpression with the poor prognosis of the squamous precancerous lesions [Nicholson et al., 2001; Eijsink et al., 2010]. The exact biological mechanisms, which promote cell growth and the relationship between viral proteins, gene amplification, decreased levels of phosphatase, and coexpression of EGF, TGF-α, amphiregulin are not completely understood and still represent subjects of investigation [West et al., 2008].

In our study, from all cervical biopsies, EGFR was overexpressed in 67% of HSIL and 32% LSIL. The staining pattern was predominantly membranar, with occasional cytoplasmic positivity. Most cases presented heterogeneity of staining, with positive cells admixed with negative cells. The number of biopsies with intense immunoexpression of EGFR increased with the severity of cytological abnormality.

Regarding high-grade squamous lesions, the immunostaining was more intense in CIN2 lesions than in CIN3. According to our findings, the expression of EGFR can be associated with HPV infection, as the high EGFR expression parallels the increasing grade of the intraepithelial squamous lesion, but not the HPV type.

● *COX-2 immunoassessment in HPV-associated intraepithelial squamous lesions*

It is widely accepted that the inflammatory COX–2 prostaglandins axis, represented by COX-2-EPs-PGE2 (cyclooxygenase 2-prostaglandin E2 receptors-prostaglandin E2) signaling pathway is elevated in ovarian, endometrial and cervical cancers [Jabbour et al., 2001; Munkarah, Ali-Fehmi, 2005; Mitchell et al., 2007; Jabbour et al., 2009; Khunamornpong et al., 2009; Ye et al., 2020]. This proinflammatory pathway is induced by a variety of stimuli (cytokines, growth factors and tumor-promoting chemical carcinogens) [Modugno et al., 2005; Goswami et al., 2008]. The overexpression of COX-2 could impair host immune responses, as suggested by the ability of COX-2 inhibitors to revert tumor-induced immunosuppression [Hawk et al., 2002].

Opposite opinions are published, some papers showing that the expression of COX-2 increases with the severity of the grade of cervical dysplasia [Kulkarni et al., 2001; Farley et al., 2004], other considering no correlations with the disease severity [Sarian et al., 2006]. As

we mentioned above, there are few information on the COX-2 expression induced by the HPV infection, in preinvasive cervical lesions and cervical cancers [Ferrandina et al., 2002; Subbaramaiah, Dannenberg, 2007; Balan et al., 2011].

In the present study, we investigated the immunoexpression of COX-2 in cervical precancerous lesions of low grade and high-grade. Our data show that COX-2 levels are increased with the progression of the squamous intraepithelial lesions, with different degrees of overexpression from LSIL (CIN1) to HSIL (CIN2, CIN3/CIS). Furthermore, the stromal inflammatory cells of associated chronic cervicitis were also intense positive for COX-2. Consequently, we consider our results as supplementary evidences in order to sustain that COX-2 induction begins in the premalignant phase of cervical carcinogenesis and is correlated with inflammation [Saldivar et al., 2007]. Additionally, in the context of the confirmed HPV infection, we must stress the COX-2 expression of the cases presenting p16 positivity.

Nevertheless, our general results suggest that COX-2 and EGFR are closely related to each other and this interaction play an important role in the tumor development and progression of the squamous precancerous lesions induced by HPV infection. Definitely, there are other factors involved in regulation of EGFR expression and activity, thus COX-2 alone cannot determine the absolute expression level of EGFR in neoplastic cells [Adimi et al., 2011]. Other studies support our results [Dannenberg et al., 2005; Half et al, 2007], despite convergent data that suggest the possibility of a specifically downregulated EGFR expression through the COX-2 overexpression [Kim et al., 2009]. Moreover, when compared to the efficiency of a single therapy agent, it was demonstrated that the therapeutical association of COX-2 inhibitor (celecoxib) with EGFR tyrosine kinase inhibitor (erlotinib) lead to the inhibition of the tumor cells proliferation in head and neck carcinoma cell lines as well as the tumor growth in xenograft models of nude mouse [Shin et al., 2013; Ye et al., 2020].

Cervical carcinoma characterizes by elevated expressions of PGE2 and COX-2 [Sales et al., 2001; Sales et al., 2002]. Many studies confirm the COX-2 contribution in cervical carcinogenesis and progression. In this regard, it was found that an elevated COX-2 immunoexpression in cervical cancer is linked with poor overall survival (OS) and also with poor disease-free survival (DFS) [Huang et al., 2013; Ye et al., 2020].

Moreover, a poor 5-year DFS and OS was associated with the coexpression of thymidine phosphorylase (TP) and COX-2, which is thus consider a predictor for cervical squamous cell carcinoma [Pyo et al., 2005; Ye et al., 2020].

Poor DFS was also found to be linked with COX-2 expression in cervical cancer patients with chemo-radiation therapy, suggesting the role of COX-2 as a predictive factor of chemo-radiation resistance [Huang et al., 2013]. An important synthase involved in PGE2 synthesis, microsomal PGE synthase-1 (mPGES-1), has a higher expression in cervical carcinoma and its related squamous intraepithelial lesions than the normal uterine cervix epithelium [Herfs et al., 2009; Ye et al., 2020].

Another study reported the activated syntheses of cyclic adenosine monophosphate (cAMP), PGE2, EP2, EP4, and COX-2 in the malignant uterine cervix compared to the healthy cervical tissue, indicating the PGE2 role in neoplastic cell function regulation through the EP2/EP4 receptors [Sales et al., 2001; Ye et al., 2020].

● *The comparative assessment of the immunoexpression of EGFR, p16, and COX-2 in first and second study groups*

The comparative assessment of EGFR, p16, and COX-2 immunoexpression in HPV-associated cervical adenocarcinomas and different HPV-associated squamous intraepithelial lesions showed a parallel increase expression pattern with the lesion severity, regardless of the squamous or glandular type. Thereby, EGFR presented a higher expression in adenosquamous carcinoma and HSIL, comparative with the other adenocarcinomas and LSIL. This may suggest

a certain affinity of EGFR for squamous lesions compared to glandular lesions, a possibility also highlighted by a previous study [Iida et al., 2011]. P16 was found to be strong overexpressed in adenocarcinomas and HSIL, closely followed by adenocarcinoma in situ and LSIL. This finding is in agreement with literature data [Negri et al., 2011; Guo et al., 2011; Kalyani et al., 2020], which indicates that the p16 immunoexpression is related to HPV-associated neoplastic transformation. In this reagard, we may assume that p16 overexpression is more related to the aggressiveness of the lesion associated with HPV infection than to the epithelial type. COX-2 presented an increased expression in all adenocarcinomas, and in more than 2/3 of squamous intraepithelial lesions, being consistent with previous studies [Kim et al., 2013; Ye et al., 2020], the expression of stromal inflammatory cells emphasizing the importance of COX-2-PGE2-Pes signaling pathway in cervical carcinogenesis [Ye et al., 2020].

1.2.5. FINAL REMARKS

In the HPV-associated adenocarcinoma, the concomitant evaluation of L1 capsid protein and p16 can predict the progression risk of precursor lesions of endocervical adenocarcinomas. The co-assessment of p53-COX-2-p16 antibodies represents a usufull panel for HPV L1 – p16 association. EGFR expression increases according to the progression in lesions severity, and cyclin D1 is a reliable marker for the invasive capacity of cervical neoplasia.

Immunocytochemical detection of L1 capsid protein, on cervicovaginal smears, indicates a specific immune status induced by the HPV infection and may offer prognosis information, mainly in LSIL lesions. The assessment of p16, EGFR, and COX-2 allows for an integrative approach of the both cervical adenocarcinomas and squamous intraepithelial lesion progression, associated or not with HPV infection.

Further studies are necessary to identify other mechanisms and the concurrent effects of these markers in the precursor lesions, onset, and development of cervical cancer.

1.3. ENDOMETRIOSIS AND THE RELATIONSHIP WITH CANCER

1.3.1. INTRODUCTION

Endometriosis, defined as ectopic implantation of endometrial-like tissue, composed of both glands and stroma, has an incidence of about 2% in general population [Machairiotis et al., 2013], with numerous incidental occurrences, approximately 70% of cases developing pelvic inflammatory disease, and 25-30% of cases being associated with infertility [De Ceglie et al., 2008].

Endometriosis mainly involves the reproductive tract components (in about 75% of cases), such as ovaries, fallopian tubes, large, round, and uterosacral ligaments, uterine cervix, vagina, and recto-vaginal septum [de Ceglie et al., 2008]. In about 25% of cases, this process occurs in extra-reproductive organs, especially with intraperitoneal locations (peritoneum, Douglas pouch, appendix, gastro-intestinal tract, and lymph nodes) [Cacciato Insilla, 2014]. Rare extraperitoneal locations are also reported in literature, such as liver [Fluegen et al., 2013], lung [Jablonski et al., 2009], pleura or diaphragm [van der Linden, van der Linden, 1996], urinary tract [Cheng et al., 2015], tegument, mainly post-surgical abdominal scars, i.e. post cesarean-section, nasal cavity [De Ceglie et al., 2008], iliac vein wall [Zamurovic, 2014], and hernial sac wall [Albutt et al., 2014].

Although considered a benign disease, the endometrium acquires aggressive pathological characteristics, being able to migrate, implant, proliferate, and grow in other sites than those genetically established, during endometriosis development.

Endometriosis is a highly heterogeneous entity due to a broad spectrum of clinical manifestations, from incidentally discovered, asymptomatic cases to symptomatic cases, manifested mainly with characteristic cystic lesions and adherences or without evident aspects.

The gold standard for endometriosis diagnosis is the histopathological examination, although transvaginal ultrasonography and magnetic resonance represent also ordinary diagnostic tools [Malvezzi et al., 2020].

Gross findings are also highly variable, from small dispersed lesions, such as superficial or "gunpowder" appearance, to cystic, red implants, or petechiae, vesicles, nodules, and, sometimes, polyps, depending on their location, duration, and association with fibrous adhesions [Vang, Wheeler, 2011].

A combined anatomical and histopathological classification recognizes three main types of endometriosis, as follows: endometrioma, peritoneal, and deep infiltrative lesions [van der Linden, van der Linden, 1996].

Histopathological findings have been based on the identification of endometrial glands surrounded by characteristic cellular stroma, registering an analogous pattern to the eutopic endometrial cycle.

Although histopathological diagnosis can be relatively simply reached, the microscopic differentials with ovarian endosalpingiosis [Vang, Wheeler, 2011], a lesion frequently overdiagnosed as endometriosis, has to be made. Rarely, benign cystic lesions or even well differentiated adenocarcinomas may be included in the differential diagnosis. Currently, immunohistochemical profile may be useful, by CD10 strong stromal positivity, ERβ and PR-A variable epithelial and stromal positivity, along with Bcl-2 and Ki-67 epithelial and stromal positivity correlated to the size of the implant, supplementing the histological characteristics exhibited in routine staining [Bratila et al., 2015].

Clinicopathological, molecular, and genetic evidences support the hypothesis of endometriosis as a neoplastic process, with a potential to malignant transformation. The first evidence is that of the common features shared by these entities that are both able to disseminate, to invade, and to form distant implants [Kobayashi et al., 2007]. Moreover, a significant association of endometriosis with clear cell and endometrioid ovarian carcinomas has been reported [Sato et al., 2000; Körner et al., 2006; Kuo et al., 2009; Kajihara et al., 2010; Jones et al., 2010; Keita et al., 2010; Stewart et al., 2012; Matsumoto et al., 2015; Chene et al., 2015; Worley et al., 2015], e.g. 30-55% and 30-40%, respectively [Kobayashi et al., 2007]. Furthermore, an increased incidence of concurrent primary malignancy, i.e. endometrial carcinoma, in endometriosis-associated ovarian malignancies suggests common molecular pathways in both locations [Zhao et al., 2011]. A plethora of phenomena are common in sporadic cancer and endometriosis, being determined by complex interactions between hereditary polygenic alleles of low penetrance (polymorphism), acquired genetic alterations, leading to a "signature" of mutations, and microenvironment "permissive" factors, expressed by hormonal influences and chronic inflammation [Keita et al., 2010; Keita et al., 2011; Zhao et al., 2011]. The current molecular techniques of genetic, transcriptomic and proteomic profiling allow for better characterization of correlations between genetic and local milieu alterations and transition process from normal endometrium to malignant transformation.

The current knowledge is that two pathways seem to be involved in endometriosis and its potential progression toward neoplasia: either malignant transformation, probably by an atypical transition stage, either the common precursor mechanism or predisposing factors are shared by both processes, with a consecutive molecular divergence [Varma et al., 2004]. Thereby, the malignant progression of endometriosis involves oxidative stress, hyperestrogenism, and inflammation related networks, all the microenvironmental alterations inducing tumor invasion and promoting metastases [Paulino et al., 2020].

Relatively recent histopathological data confirm the possibility of transition due to identification of an atypical stage and frequent association of ovarian cancer to atypical endometriosis. The significance of borderline tumors is currently regarded as a part of endometriosis-associated ovarian carcinoma spectrum [Kobayashi et al., 2007; Amemiya et al., 2004; Otsuka et al., 2004]. Moreover, recent evidences indicate that patients with endometriosis have a significant risk for endometrial cancer, mainly endometrioid and clear cell subtypes [Yu et al., 2015].

In general, in the mechanism of implantation, three phenomena are succeeding, namely, apposition, adhesion, and invasion [Matsuzaki et al., 2010]. During these phenomena dynamics, several adhesion molecules are involved, such as cadherins, selectins, integrins, galectins, heparan-sulphate, and trophinin-tastin-bystin complex [Matsuzaki et al., 2010]. Considering the role of epithelial-mesenchymal transition (EMT) and its reverse mesenchymal-epithelial transition (MET) in endometriosis, E-cadherin, β-catenin, and CK18 are known to be useful markers in the study of endometriosis, demonstrating the initiation of early E-cadherin-β-catenin complex mutations in the EMT process. It is estimated that β-catenin mutations represent an early event in endometriotic-dependent ovarian carcinogenesis and CK18 expression is correlated with EOC stage progression along with EMT, as the malignant process is extending [Matsumoto et al., 2015].

Apoptosis represents a component of tumor growth, considering its antagonism to cellular proliferation [Zeren et al., 2014] and that it is frequently inhibited in variable types of tumors [Gross et al., 1999]. This process is prevented in malignant cells and is correlated to carcinogenesis [Hanahan, Weinberg, 2000; Fischer et al., 2007], being associated to malignant transformation in ovarian tumors [Fischer et al., 2007], to high-grade tumors, and with a poor prognosis [Yamasaki et al., 1997; Brustmann et al., 2006]. Moreover, this process is associated to high-grade tumors, and with a poor prognosis in ovarian tumors [Yamasaki et al., 1997; Brustmann et al., 2006]. Bcl-2 family maintains the balance between apoptosis and its inhibition, by molecules pro- and anti-apoptotic, in functional antagonism [Schorr et al., 1999; Correia-da-Silva et al., 2005; Zeren et al., 2014]. An apoptotic evasion mechanism has been proven, allowing the development of ectopic implants, in endometriosis. Furthermore, Bcl-2/Bax ratio is progressively increasing in EOC compared to endometriosis [Vatansever et al., 2009].

Steroid hormones and hormone-like substances play an important role in endometrium physiology and their unbalance is involved in endometrial pathology, including in endometriosis. Numerous studies have demonstrated that estrogen (ER) and progesterone receptors (PR) expressions are associated with endometriosis and EOC [Pearce et al., 2012; Yu et al., 2015]. In this regard, there is described the association between hyperestrogenism and the endometriotic cysts malignant transformation, due to altered microenvironment, with increased aromatase activity, which promotes excessive estrogen accumulation [Paulino et al., 2020].

BMI-1 protein, a stem-like marker, represents a homologue of the Drosophila polycomb group of proteins, and its role is the regulation of homeotic genes expression by transcription repression [Honig et al., 2010]. The BMI-1 gene has been initially isolated as an oncogene, which cooperates with c-Myc in lymphoma experimental models [Qin et al., 2009]. It belongs to the Polycomb-group (PcG) of proteins, which are involved in axial pattern establishment, hematopoiesis, cementogenesis, and senescence [Qin et al., 2009].

Considering BMI-1's involvement in cellular proliferation and tumor progression, this gene has been identified, as expected, in a large variety of human tumors, such as: lymphoma [Beà et al., 2001; van Kemenade et al., 2001; Raaphorst et al., 2005], brain [Cui et al., 2007], prostate [Crea et al., 2011], oropharynx and nasopharynx [Song et al., 2006; Honig et al., 2010; Huber et al., 2011], breast [Kim et al., 2004; Guo et al., 2011], bladder [Qin et al., 2009], gastric [Lu et al., 2010], pancreas [Proctor et al., 2013], esophagus [Yoshikawa et al., 2012], lungs

[Vonlanthen et al., 2001; Breuer et al., 2005], head and neck cancers [Elkashty et al., 2019], malignant melanoma [Bachmann et al., 2008], pleomorphic adenoma [Sadassari et al., 2015], and also displaying a prognosis value in mielodysplastic syndromes [Mihara et al., 2006] and in gallbladder cancer [Jiao et al., 2019]. Although its action has been initially thought to be achieved by p16 suppressor gene repression, subsequent studies have demonstrated another specific mechanism of action by intercellular adhesion pathway modulation [Douglas et al., 2008].

Limited information is available about BMI-1 in OC, as few studies address this topic, mainly providing experimental evidences [Zhang et al., 2008; Honig et al., 2010; Wang et al., 2011; Vathipadiekal et al., 2012; Xin et al., 2012; He et al., 2014; Dey et al., 2018; Shishido et al., 2018; Zhang et al., 2019]. BMI-1 increased expression mirrors an early and maybe reversible event in carcinogenesis [Honig et al., 2010], suggestive for an invasive and aggressive phenotype during tumor development [Honig et al., 2010; Abd El Hafez et al., 2014]. It is demonstrated that BMI-1 regulates cell cycle and promotes cell proliferation, which has self-renewal and differentiation potential [Muinao et al., 2018], acts as a potential modulator of cellular adhesion in endometriotic tumor cells, and alters endometrial stromal cells by changing microenvironment interactions in OC [Horie et al., 2020]. Several results support its potential value as an independent predictor for poor outcomes [Yang et al., 2010] and as a possible new therapeutic target in chemoresistant ovarian cancer [Bhattacharya et al., 2009; Wang et al., 2011; Muinao et al., 2018; Zhang et al., 2019; Motohara et al., 2021].

Aim

Currently, there is a high interest in a better understanding and characterization of EOC, in an attempt to provide a different clinical and therapeutic management compared to that of NEOC. In this regard, our research comprises two studies.

The first study compared a group of cases with endometriosis and a group with ovarian malignancies associated with endometriosis in order to evaluate the expression of some molecules of the EMT process (E-cadherin, β-catenin, and CK18) associated to apoptotic markers (Bcl-2, Bax) and hormonal profile (ER, PR) providing correlations with molecular changes sequence during the pathogenic mechanism of these diseases.

The second study aimed to evaluate the immunohistochemical (IHC) BMI-1 expression in two different groups of OC (associated or not with endometriosis), in order to identify the differences in its tissue profile. The novelty of this research consisted in a double assessment of BMI-1, in tumor epithelial cells and stromal cells, following the potentiation relationship of these two cell types in tumor progression. Nevertheless, the BMI-1 expression was correlated with clinicopathological data that offer a solid functional image of the tumor progression.

1.3.2. MATERIALS AND METHODS

● **Study 1**

Patients

This was a retrospective study on 50 patients diagnosed with endometriosis and endometriosis related ovarian carcinoma (EOC). Patients were divided in 2 groups. The first group comprised of 31 cases with endometriosis, which were diagnosed in the Department of Histopathology of the "Elena Doamna" Clinical Hospital in Iași, between January 2005 and April 2017. The second group consisted of 19 cases of ovarian carcinomas associated with endometriotic lesions, diagnosed between February 2013 and January 2016, in the Regional Institute of Oncology Iasi, which have been treated by total radical hysterectomy and pelvic lymphadenectomy.

The relevant clinicopathological data have been collected from the medical records in both groups (Table 1.13). Accordingly, age, parity, menopausal status, type (unifocal versus multifocal and cystic lesions), site (ovarian, cervical, tubal, and cutaneous), and associations with other gynecological pathologies have been recorded in endometriosis group. Supplementary to age, parity, menopausal status, data regarding tumor size, ovarian capsular invasion, and histological types of EOC, FIGO, TNM stages, and CA 125 serum values have been recorded in EOC group.

Table 1.13. The main clinicopathological features in the study groups

ENDOMETRIOSIS GROUP (n=31 cases) (median age - 36.61 years)								
ENDOMETRIOTIC FOCI			**PARITY**	**MENSTRUAL STATUS**	**ASSOCIATED LESIONS**			
Parietal/mural	Ovarian uni/bilateral	Mixed-multifocal	Multiparous	Menopausal status	ULM	AD	OC	CC
3	22	6	22	9	7	10	5	9

EPITHELIAL OVARIAN TUMORS ASSOCIATED WITH ENDOMETRIOSIS (n=19 cases) (median age - 59.10 years)										
HISTOLOGICAL TYPES		**FIGO STAGES**			**HISTOLOGICAL GRADING**			**TNM SYSTEM**		
Endometrioid carcinoma	Non-endometrioid carcinoma	I	II	III	GI	GII	GIII	T1	T2	T3
8	11	4	6	9	1	6	12	5	6	8

ULM - uterine leiomyomas; AD - adenomyosis; OC - ovarian cysts; CC - chronic cervicitis

Methods

All cases were investigated by applying the immunohistochemical method, using a panel of primary antibodies with the appropriate dilutions (Table 1.14).

Immunohistochemistry was performed on representative tissues samples of each type of lesion. Serial sections from the corresponding paraffin blocks, of 3-4 µm thickness were cut and placed on slides SuperFrostPlus. The immunostaining was performed using the automated BenchMark XT system (Ventana Medical System, Inc., Tucson, AZ), following standardization. Negative controls, obtained by replacement of primary antibodies with distilled water, along with positive controls have been simultaneously run.

Table 1.14. Antibodies types, clones, dilutions, and staining pattern used in the immunohistochemical technique

Antibody	**Type**	**Clone**	**Dilution**	**Staining pattern**
E-cadherin	Mouse Monoclonal	36B5/ Novocastra	1/50	membrane
β-catenin	Mouse Monoclonal	β-Catenin-1/Dako	1/200	membrane and cytoplasm
CK18	Mouse Monoclonal	DC-10/Novocastra	1/100	membrane
Bax	Rabbit Polyclonal	Polyclonal /Dako	1/1500	cytoplasm
Bcl-2	Mouse Monoclonal	Bcl-2/100/D5/Novocastra	1/80	cytoplasm
ER	Mouse Monoclonal	6F11/Novocastra	RTU	nuclear
PR	Mouse Monoclonal	PGR-312/16/Novocastra	RTU	nuclear

Semi-quantitative analysis

Semi-quantitative assessment of immunohistochemical reactions was performed using the score systems reported in the literature, for each marker. The differences in quantification

methods applied are justified by markers specific patterns of expression which require or not a double measurement (intensity and the percentage of positive cells).

The score systems took into consideration the staining intensity multiplied with the percent of positive cells with a cut-off of 4 (<4 - negative and low score and ≥4 – high score). The immunostaining intensity for all investigated markers was considered as 0 – negative, 1 – weak, 2 – moderate, and 3 – strong intensity.

The percentage of positive cells for E-cadherin was classified as 1 for ≤10%, 2 for 11-50%, and 3 for ≥50% positive cells. The percentage of β-catenin positive cells was scored as 1 for <10%, 2 for 10-30%, 3 for 30-50%, and 4 for >50% positive cells. The percentage of CK18 positive cells, was evaluated as 0 for 0-5% positive cells, 1 for 6-25%, 2 for 26-50%, 3 for 51-75%, and 4 for >76-100% positive cells [7, 24, 25]. For Bcl-2 analysis, the percentage of positive cells was scored as 0 for none positive cells, 1 for 1-25%, 2 for 26-50%, 3 for 51-75%, and 4 > 75%. Score 0 has been considered as negative, and scores 1-4 have been considered as positive [Suzuki et al., 2002].

A score which was exclusively based on the staining intensity has been used for Bax and accordingly, the cases have been divided into three categories, as follows: weak intensity (+), moderate intensity (++), and strong positivity (+++) [Zlobec et al., 2007].

Allred score [Allred et al., 1998; Esmaili et al., 2016], based on index of positive cells, has been used for ER and PR immunostaining epithelial quantification, with the score considered as 0 – none, 1<1/100 (<1%), 2=1/100-1/10 (1%-10%), 3>1/10-1/3 (10%-33%), 4>1/3-2/3 (33%-66%), 5>2/3 (>66%), added to immunostaining intensity. ER and PR were also qualitatively assessed in stromal cells.

Statistical analysis

Statistical data processing was performed using SPSS v. 19.0 (IBM SPSS Statistics). Continuous variables were expressed as the mean and standard deviation and the categorical variables as number (%). The level of association between the expression of markers studied and the clinicopathological characteristics was achieved using non-parametric specific tests (Chi- square test or Fisher's exact test). The Student t test or the Wilcoxon rank-sum tests were used for continuous variables. The correlation was considered significant when $p < 0.05$.

- **Study 2**

Patients

The second study group included 47 cases of ovarian carcinomas (OC), diagnosed between 2006 and 2017 and treated in several hospitals of Iasi, Romania: "Sf. Spiridon" County Clinical Emergency Hospital, "Cuza Vodă" and "Elena Doamna" Obstetrics and Gynecological Hospitals, and Oncology Regional Institute. All cases were histopathologically reassessed by two pathologists to ascertain the OC histological subtype and then divided into two groups: endometriosis related ovarian carcinomas (EOC) and non-endometriotic ovarian carcinomas (NEOC). The study has been approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, based on the patients' informed consent (12378/June 2015). All subjects who provided ovarian tissue had given written and informed consent prior the surgery.

Clinicopathological and tumor serum marker profile of the study cohort

At the time of the diagnosis, the age of the patients ranged between 37 and 76 years old: 22 patients were younger (<55 years old) and 25 patients were older (≥55 years old). Based on the standards of the FIGO staging, 17 cases were staged as FIGO stage I, 11 cases as FIGO stage II, 18 cases as FIGO stage III, and 1 case FIGO stage IV. According to tumor grade, 13 cases were graded as G1 (well differentiated), 9 cases as G2 (moderately differentiated), and 25 cases as G3 (poorly differentiated or undifferentiated). The distribution of OC histological

variants was as follows: LGSC—4 cases; LGEC—5 cases; COC—9 cases; MOC—5 cases; HGSC—8 cases; HGEC—11 cases; undifferentiated—1 case; and mixed tumor (serous, endometrioid, and clear cells phenotypes)—4 cases. According to the pathogenic classification, the cases have been divided in low-grade (type I; 23 cases) and high-grade (type II; 24 cases). The histopathological exam revealed the tumor extension (residual tumor after primary surgery) in 25 cases (residual tumor ≥ 1 cm), with 13 patients diagnosed with a residual tumor < 1 cm and 9 cases without evident data about a residual tumor. Preoperatory CA125 levels higher than 35 U/mL were found in all cases comprised in the study group, ranging between 46–4163 U/mL.

Clinicopathological and tumor serum marker profile of the EOC and NEOC groups

In the whole group, 19 of 47 (40%) patients belonged to the EOC group and 28 patients (60%) belonged to the NEOC group. Cases included in the EOC group were characterized by the presence of associate endometriotic lesions consisting of the endometriosis area in the form of an endometriotic cyst lined with endometrial epithelium and endometrial stroma, as well as evidence of hemosiderin deposits and chronic hemorrhage or proliferative endometriosis foci with a well-developed glandular profile.

The mean age of patients was 59.10 ± 8.66 years in the EOC group and 56.57 ± 2.64 years in the NEOC group. The EOC group comprised the following histological types: COC - 4 cases; HGSC - 3 cases; HGEC - 8 cases; mixed tumors - 4 cases; and none of LGSC, LGEC, MOC, or undifferentiated carcinoma cases were classified as EOC. The histological types in NEOC group were: LGSC - 4 cases; LGEC - 5 cases; COC - 5 cases; HGEC - 3 cases; MOC -5 cases; HGSC - 5 cases; and undifferentiated - 1 case. The median value of the preoperatory CA 125 level in the EOC group was 1201.5 U/mL, while a median value of 101 U/mL was found in the NEOC group.

Immunohistochemical exam

The immunohistochemical staining for the identification of Antigens has been achieved using BenchMark XT automatic system (Ventana Medical System, Inc., Tucson, AZ, USA), according to protocols that needed standardization for different types of antibodies. The sections obtained from the selected paraffin-embedded blocks were dewaxed in xylene, rehydrated in ethanol, and rinsed in distillated water. The antigen retrieval was made by using the Heat-Induced Epitope Retrieval (HIER) procedure, with an antigen retrieval solution of pH 9 using CC1 solution (Ventana Medical System, Tucson, AZ, USA), consisting of a combination of ethylenediaminetetraacetic and boric acid diluted in Tris buffer for 30—60 minutes. After the endogenous peroxidase blocking with 3% hydrogen peroxide and treatment with normal goat serum 10%, used to block the non-specific protein bonds, the sections were incubated with the primary antibody BMI-1 (clone F6/ABCAM, 1/50 dilution, Abcam, Cambridge, MA, USA). Consequently, the incubation with UltraVision Quanto Detection System Horseradish peroxidase (HRP) (Igs; Ventana Medical Systems) has been performed. Antigen-antibody reaction has been visualized using 3,30-Diaminobenzidine as a chromogen (UltraView, Ventana Medical Systems, Tucson, AZ, USA). The counterstaining of the sections was done with Mayer's Hematoxylin. After counterstaining, the slides have been washed with liquid soap in order to eliminate the oily film, they have been rinsed with taping water and have been also bathed twice in distilled water. Negative controls have been used for results interpretation, in which primary antibodies have been skipped and replaced with distilled water and positive controls have been considered as endothelial cells and stromal fibroblasts immunostaining.

Semi-quantitative assessment

BMI-1 expression has been individually quantified in the epithelial and in the stromal components. The semi-quantitative assessment of the BMI-1 in tumor cells was done by using adapted scores based on literature reports [Bachmann et al., 2006; Bachmann et al., 2008] that

took into account the staining intensity (I) and the percentage of positive cells (P). BMI-1 showed a double immunostaining, nuclear, and cytoplasmic/membrane [Bachmann et al., 2006; Bachmann et al., 2008]. The intensity of BMI-1 immunoreaction was scored as: 0 - absent, 1 - weak, 2 - moderate, and 3 - strong. The percentage of BMI-1 positive cells was scored as follows: 1< 10%, 2 - 10–50%, 3 > 50%. The final BMI-1 score was obtained by multiplying P by I. BMI-1 score values < 3 were considered as a low score, and score values ≥ 3 were considered as a high score. For the semi-quantitative assessment of stromal BMI-1, we used a standard 2-point scale scoring system. The immunoreaction was considered negative when ≤ 10% of the tumor stromal area had a positive immunostaining of BMI-1, and positive when > 10% of the stromal area showed BMI-1 immunostaining, regardless of the level of staining intensity.

BMI-1 expression has been independently evaluated and scored by three histopathologists with experience in immunohistochemistry interpretation and scoring differences have been revised in the evaluation panel in order to reach a consensus.

Statistical Analysis

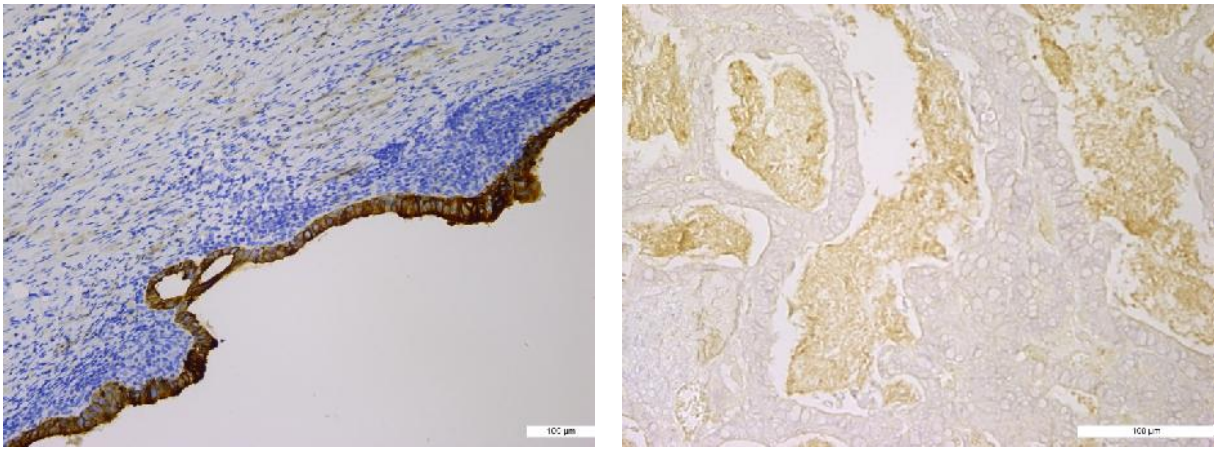
Statistical analysis was carried out with Statistical Package for the Social Sciences (SPSS) v. 19 program (SPSS Inc., IBM Corporation, Chicago, IL, USA). A Chi-square ($\chi 2$) test was performed to analyze the differences in BMI-1 epithelial and stromal profile in each group and between groups, and its relationship with classical clinicopathological characteristics (age, tumor stage, grade, histological subtype, tumorigenic dualistic tumor types, residual disease, and preoperatory CA 125 level). Yates' correction was applied when the number of cases in a subgroup was lower than five. Statistical significance was considered when $p < 0.05$.

1.3.3. RESULTS

- **Study 1**

Results in endometriosis

E-cadherin expression had a homogeneous, membrane distribution throughout the entire endometriosis foci in glandular epithelium, and had been also noticed in few stromal cells. The assessment of E-cadherin showed positive immune expression in all cases, with moderate intensity in 18 cases (58.06%) and strong in 13 cases (41.93%) (Fig. 1.17 - a). The percentage of positive cells was higher than 50% in 23 cases (77.41%) (Table 1.15).



a **b**

Figure 1.17. **a.** Positive homogeneous, apical membrane E-cadherin expression in glandular epithelium of endometriosis (anti-E-cadherin, x200). **b**. Negative E-cadherin expression in EOC, with weak positive luminal secretion (anti-E-cadherin, x200).

In endometriotic area, glandular epithelium showed homogeneous, membrane and cytoplasmic β-catenin expression (Fig. 1.18). β-catenin staining intensity was moderate in 14 cases (45.16%) and strong in 17 cases (54.83%). The percentage of positive cells was over 50% in 14 cases (45.16%). An increased β-catenin expression could be observed as well in the periglandular stromal cells.

Most of epithelial cells in the endometriotic foci were strongly positive for CK 18 and only a small proportion of cells, stained moderate or weak, CK18 intensity being moderate and weak in 9 cases (29.03%) and strong in 22 cases (70.96%).

Expression of CK18 was found to be homogeneous, with a higher intensity than E-cadherin and β-catenin expression. The percentage of CK18 positive cells was higher than 50% in almost all cases (29 cases - 93.54%) (Fig. 1.19).

The correlation analysis between EMT markers expression (E-cadherin, β-catenin, and CK18) did not reveal any statistically significant associations.

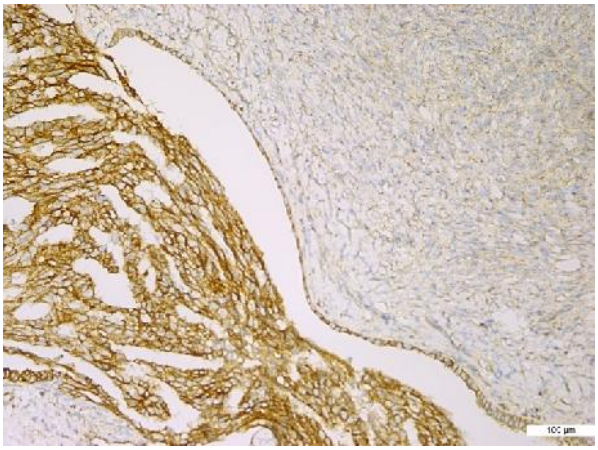


Figure 1.18 Strong, homogeneous membrane and cytoplasmic β-catenin expression in EOC (left) and surface epithelium in endometriosis (right) (anti-β-catenin, x100).

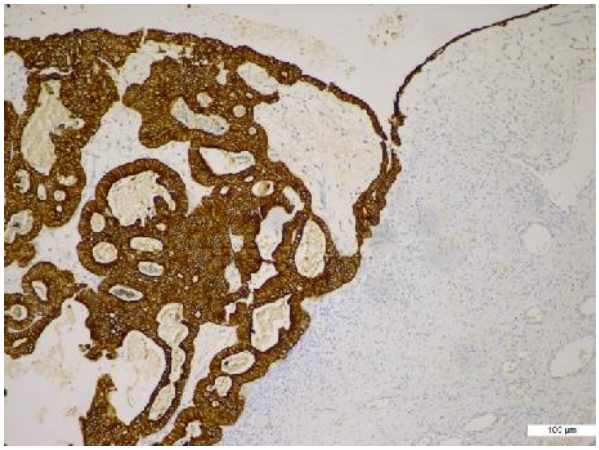


Figure 1.19 Limited CK18 expression in surface epithelium in endometriosis (right) compared to strong, homogenous, diffuse, membrane expression in EOC area (anti-CK18, x100).

Bcl-2 and Bax had a variable cytoplasmic distribution, in the endometriosis foci, though some heterogenous areas were focally identified. Bcl-2 expression has been negative in 15 cases (48.38%) and positive in 16 cases (51.61%) of endometriosis (Fig. 1.20), while Bax showed negative or weak expression in 14 cases (45.16%) of endometriosis and moderate or high expression in 17 cases (54.83%) (Fig. 1.21). The percentage of Bcl-2 positive cells was over 50% in 2 cases (6.45%) (Table 1.15).

A negative Bcl-2 expression associated a negative Bax expression in 2 cases (13.33%) and Bax positivity in 13 cases (86.66%), while cases with positive Bcl-2 expression associated a negative Bax expression only in 1 case (6.25%) and Bax positivity in 15 cases (93.75%).

The statistical correlation analysis revealed statistically significant differences between Bax and Bcl-2 expression in the endometriosis group ($p = 0.020$). The statistical correlation analysis between Bcl-2 and Bax and clinicopathological factors did not reveal any statistically significant associations.

The immunoexpression of ER and PR was diffusely nuclear and homogeneous through all endometriotic area. ER expression in endometriotic areas had negative score in 9 (29.03%) cases and positive score in 22 (70.96%) cases (Fig. 1.22), while PR revealed negative score in 12 (38.70%) cases and positive score in 19 (61.30%) cases (Fig. 1.23). The statistical correlation analysis between ER expression and clinicopathological characteristics has not shown any

statistically significant associations. The statistical correlation analysis between the percentage of ER positive endometriotic cells and percentage of PR positive cells and clinicopathological factors revealed statistically significant differences only in the ovarian location of endometriosis (p = 0.004) (Table 1.15).

We identified also a particular expression pattern of ER and PR in the stromal cells. According to immunohistochemical evaluation of stroma component for both hormonal markers, the percentage revealed a moderate to strong, heterogeneous expression, with the following aspects: 10 (32.25%) cases were positive and 21 (67.74%) cases had negative ER immunoreaction, while 11 (35.48%) cases were PR positive and 20 (64.51%) cases were PR negative.

The statistical correlation analysis between stromal ER/PR expression and clinicopathological factors (age, parity, menopausal status, lesional type and site, association with other gynecological disease) did not reveal any statistically significant associations.

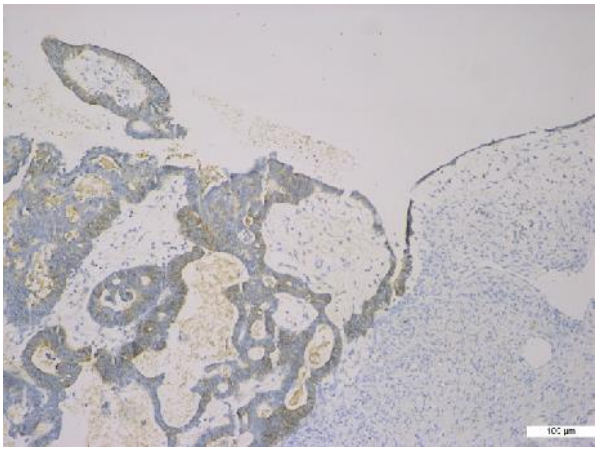


Figure 1.20. Moderate intensity, heterogeneous cytoplasmic Bcl-2 expression in endometriosis (right) and weak to moderate expression in EOC (left) (anti-Bcl-2, x100).

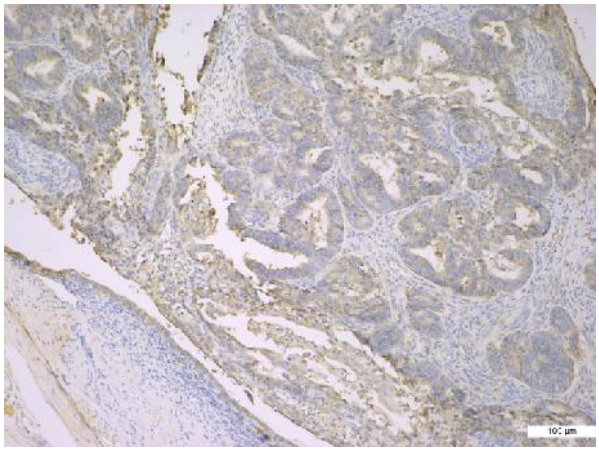


Figure 1.21. Bax cytoplasmic weak expression in EOC (right) compared to moderate cytoplasmic expression in endometriosis (left) (anti-Bax, x100).

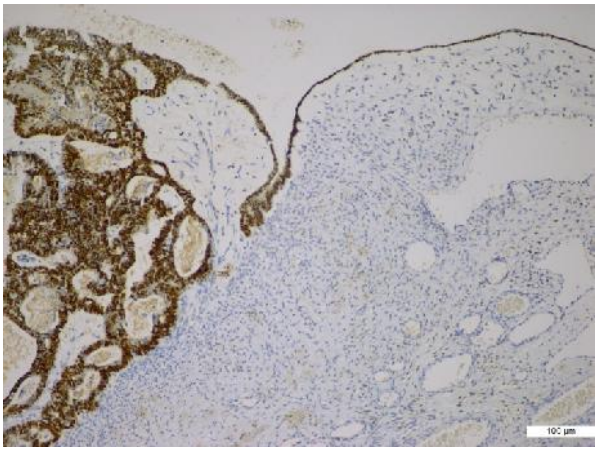


Figure 1.22. ER epithelial expression in endometriosis (right) and contiguous area of EOC (left) with homogeneous, diffuse, nuclear ER expression (anti-ER, x100)

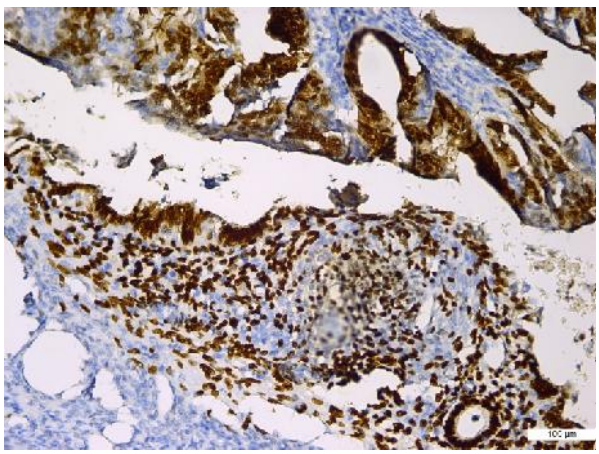


Figure 1.23. Diffuse, strong, heterogeneous epithelial and stromal PR expression in an endometriotic focus (inferior) and in neighboring EOC (superior) (anti-PR, x200)

Results in endometriosis related ovarian carcinoma (EOC)

E-cadherin distribution was heterogeneous throughout the entire tumor cells, the immunoexpression being weak in 13 cases (68.42%) and moderate in 4 cases (21.05%) (Fig.

1.17 - b). Although immunohistochemistry revealed a lower percentage of positive cells (less than 40%) in 13 cases (68 %) and a higher one (more than 45%) in 6 cases (32%), all cases have been considered as E-cadherin negative, as their scores have ranged between 0 and 4. In 5 cases (26.31%), we noticed a very low positivity percentage (<10% of E-cadherin-positive tumor cells), and these cases associated high β-catenin staining, in more than 50% of the tumor cells (Table 1.15).

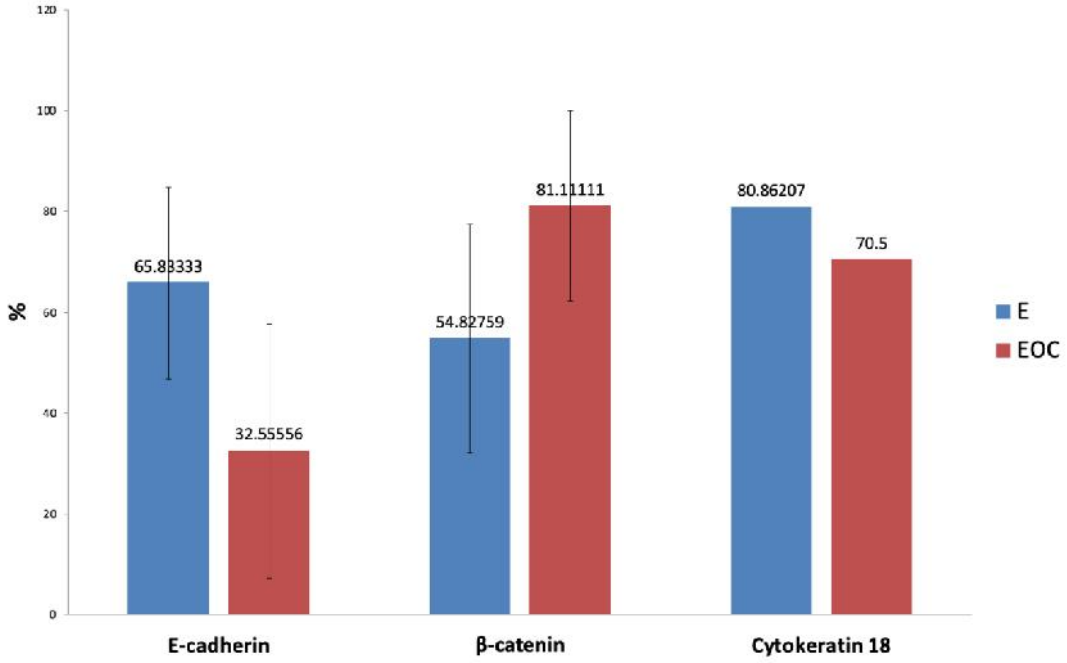
β-catenin expression was positive in all cases with moderate and weak intensity in 3 (15.78%) cases and strong in 16 (84.21%) of cases (Fig. 1.18). The percentage of positive cells was over 50% in 17 cases (89.47%) with scores ranging from 4 to 12. In tumor cells β-catenin showed homogeneous, diffuse expression with a more intense staining compared with the E-cadherin (Table 1.15).

CK18 expression in all EOC cases had a moderate (5 cases - 26.31%) or strong intensity (14 cases - 73.68%) (Fig. 1.19). For most of the tumor area, the percentage of positive cells was higher than 70%, with homogeneous expression pattern and higher intensity than E-cadherin and β-catenin expression.

The statistical analysis between EMT markers revealed significant differences only between E-cadherin and β-catenin (p=0.0001) and between E-cadherin and CK18 expression (p=0.0001), in EOC group.

The correlation analysis between EMT markers (E-cadherin, β-catenin, and CK18) expression in EOC and clinicopathological characteristics (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values) did not reveal any statistical significant associations (Table 1.3.3).

The statistical analysis between the two studied groups showed significant differences for EMT markers: E-cadherin (p=0.001), β-catenin (p=0.000112) and CK18 (p=0.032468) in endometriosis versus EOC derived from endometriotic foci (Fig. 1.24).



E – endometriosis; EOC - endometriosis related ovarian carcinoma

Figure 1.24. CK18, E-cadherin, and β-catenin comparative scores in the study groups.

Bcl-2 expression has been negative in 12 cases (63.15%) and weak or moderate positive in 7 cases (36.84%) of EOC (Fig. 1.20), while Bax showed negative or weak expression in 7 cases (36.84%) of EOC and moderate or high expression in 12 cases (63.75%) (Fig. 1.21). Bcl-

2 and Bax expression exhibited a heterogeneous, cytoplasmic, finely granular pattern, in tumor cells.

We observed an interesting pattern of expression of Bcl-2 and Bax, namely groups of tumor cells that were positive for Bcl-2 (7 cases) were negative for Bax (7 cases), while groups of tumor cells that were negative for Bcl-2 (12 cases) were positive for Bax (12 cases). Furthermore, negative Bcl-2 cases associated moderately and increased Bax expression, whereas Bcl-2 positive cases associated negative or low Bax expression in half of the cases (Table 1.15).

Cases with negative Bcl-2 expression associated Bax negativity in 6 cases (31.57%) and Bax positivity in 6 cases (31.57%), while cases with positive Bcl-2 expression associated a negative Bax expression in 1 case (5.26%) and positive one in 7 cases (36.84%). No statistically significant differences have been registered between the expression of Bax and Bcl-2 in EOC. The statistical correlation analysis between Bcl-2 and Bax and clinicopathological factors did not reveal any statistical significant associations from a statistical point of view.

ER and PR immunopositivity has been noticed in both tumor cells and stroma. ER and PR expression has been positive, exhibiting a nuclear staining in the tumor cells. The distribution of ER was predominantly homogenous, while PR showed a predominantly heterogeneous expression (Table 1.15). ER expression in epithelial tumor areas had negative score in 4 (21.05%) cases and positive score in 15 (78.94%) cases (Fig. 1.22), while PR revealed negative score in 9 (47.36%) cases and a positive score in 10 (52.63%) cases (Fig. 1.23).

For both markers, ER and PR, we identified a moderate to strong, heterogeneous expression in tumor stroma. ER/PR ratio in stromal cells have been positive in more than 50% of cases (n= 11; 57.89%). Negative expression has been registered in 8 cases (42.10%).

There have been no statistically significant differences that have been registered between the expression of ER in EOC and clinicopathological factors (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values).

Regarding PR expression, significant differences have been registered between more differentiated tumors compared to less differentiated tumors (p=0.027) and accordingly, the statistical analysis revealed significant associations between PR tumor cells expression and tumor grading.

For both steroid markers (ER and PR) stromal expression in EOC has been significantly higher compared to endometriosis stroma (p=0.001) and (p=0.000), respectively. Moreover, the statistical analysis between stromal PR and tumor grading revealed significant associations (p=0.005).

Table 1.15. Distribution of the investigated molecules in endometriotic and EOC group.

Markers	ENDOMETRIOTIC GROUP n=31 cases			CP features	EOC GROUP n=19 cases			CP features
	Distribution	**Intensity (%)**	**p <0.05**	**p <0.05**	**Distribution**	**Intensity (%)**	**p <0.05**	**p <0.05**
E-cadherin	Homogeneous, membrane glandular epithelium (all) & stromal cells (rare)	Positive (all cases) Moderate (18 cases) Strong (13 cases) Positive cells (23 cases/>50%)	NSSV	NSSV	Heterogeneous, membrane	Negative (all cases)	p= 0.0001	NSSV

Markers	ENDOMETRIOTIC GROUP n=31 cases			CP features	EOC GROUP n=19 cases			CP features
	Distribution	Intensity (%)	p <0.05	p <0.05	Distribution	Intensity (%)	p <0.05	p <0.05
β-catenin	Homogeneous, membrane and cytoplasmic epithelium (all) & stromal cells (rare)	Positive (all cases) Moderate (14 cases) Strong (17 cases) Positive cells (14 cases/>50%)			Homogeneous, membrane and cytoplasmic epithelium (all) & stromal cells	Moderate to weak (3 cases) Strong (16 cases) Positive cells (17 cases/>50%)		
CK18	Homogeneous membrane glandular epithelium (all) & stromal cells (rare)	Positive (all cases) Moderate to weak (9 cases) Strong (22 cases) Positive cells (29cases/>50 %)			Homogeneous membrane glandular epithelium (all) & stromal cells (rare)	Moderate (5 cases) Strong (14 cases) Positive cells (14 cases/>70%)	NSSV	
Bcl-2	Heterogeneous cytoplasmic glandular epithelium (all) stromal cells (rare)	Positive (16 cases) Weak (11 cases) Moderate (3 cases) Strong (2 cases/>50%) Negative (15 cases)	p= 0.020	NSSV	Heterogeneous, cytoplasmic, finely granular pattern	Weak to moderate (7 cases) Negative (12 cases)	NSSV	NSSV
Bax		Weak/negative (14 cases) Moderate to strong (17 cases)				Strong to moderate (12 cases) Negative/ weak (7 cases)		
ER	Homogeneous diffusely nuclear (glandular epithelium)	Positive (22 cases) Negative (9 cases)	NSSV	NSSV	Homogeneous (ER)/ heterogeneous (PR) diffusely nuclear (glandular epithelium)	Strong to moderate (15 cases) Negative/ weak (4 cases)	NSSV	NSSV
PR		Positive (19 cases) Negative (12 cases)		p=0.004 (OE)		Strong (10 cases) Negative/ weak (9 cases)		p=0.027 (TG)

† T student test or Wilcoxon rank-sum test for continuous variables; ‡ Chi square test or exact Fisher test;
(*) p <0.05; CP - clinicopathological features; EOC - endometriosis related ovarian carcinoma; ER - estrogen receptor; OE - ovarian endometriosis; PR - progesterone receptor; TG - tumor grade; NSSV - non-statistically significant values.

- **Study 2**

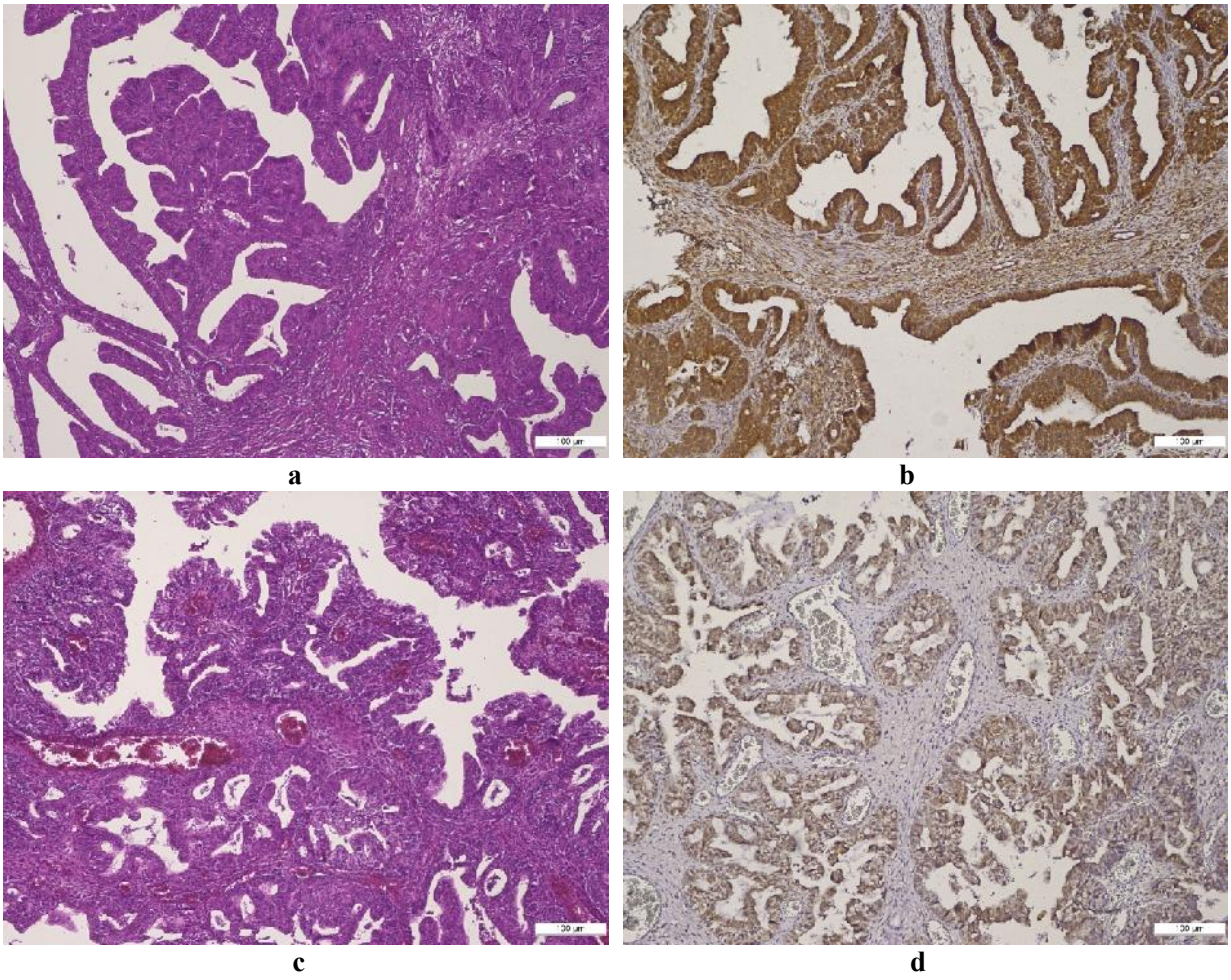
BMI-1 expression - qualitative assessment

The qualitative evaluation showed, at a glance, a heterogeneous expression in both groups, without a specific pattern for each group.

A double BMI-1 staining was found: a nuclear and cytoplasmic/membrane immunoexpression in EOC group. Strong expression of epithelial cells was observed in cases with poor prognosis, such as high-grade serous and endometrioid carcinomas (HGSCs and HGECs), as well in clear cell ovarian carcinomas (COCs). A negative BMI-1 stroma expression in the endometrioid phenotype of EOC group was found, while positive stroma was dominant in the serous phenotype, clear cell and mixed subtypes. Relevant aspects of BMI-1 expression in EOC are presented in Fig. 1.25.

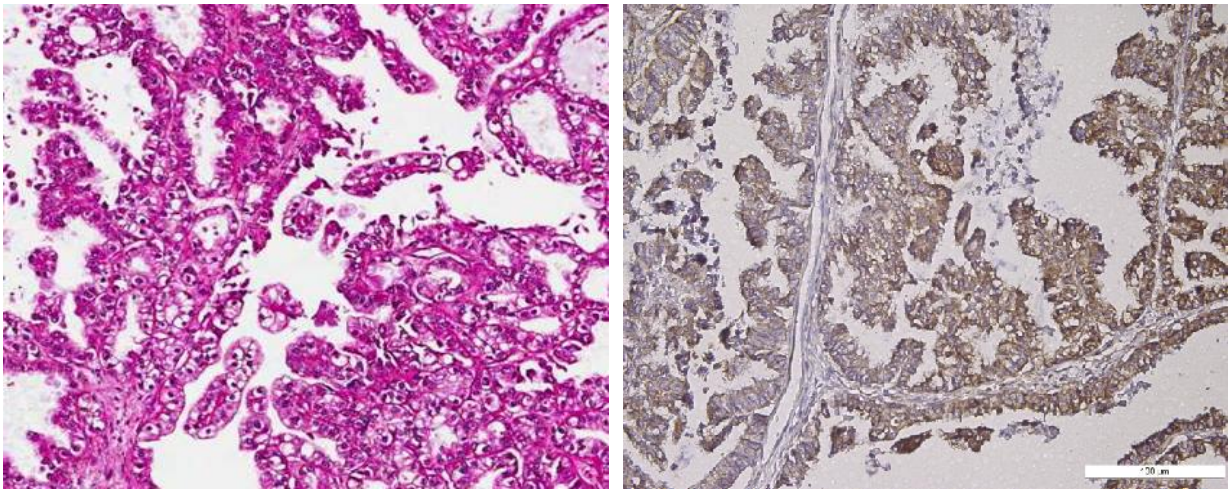
In the NEOC group, the intensity of BMI-1 was predominantly moderate or strong in epithelial (nuclear or cytoplasmic/membrane immunoexpression) and stromal cells. Moderate and strong nuclear expression and weaker cytoplasmic expression was observed in cases with a serous phenotype and a more aggressive course, such as HGSC, while the endometrioid phenotype preserved a strong, diffuse, membrane BMI-1 staining. In undifferentiated carcinomas, BMI-1 expression was heterogeneous, displaying a weak cytoplasmic staining. Differences between BMI-1 expression in variable types of NEOC are illustrated in Fig. 1.26.

We also noted the lack of BMI-1 expression in normal ovary or ovarian surface, and its positivity in the normal tubal surface epithelium.



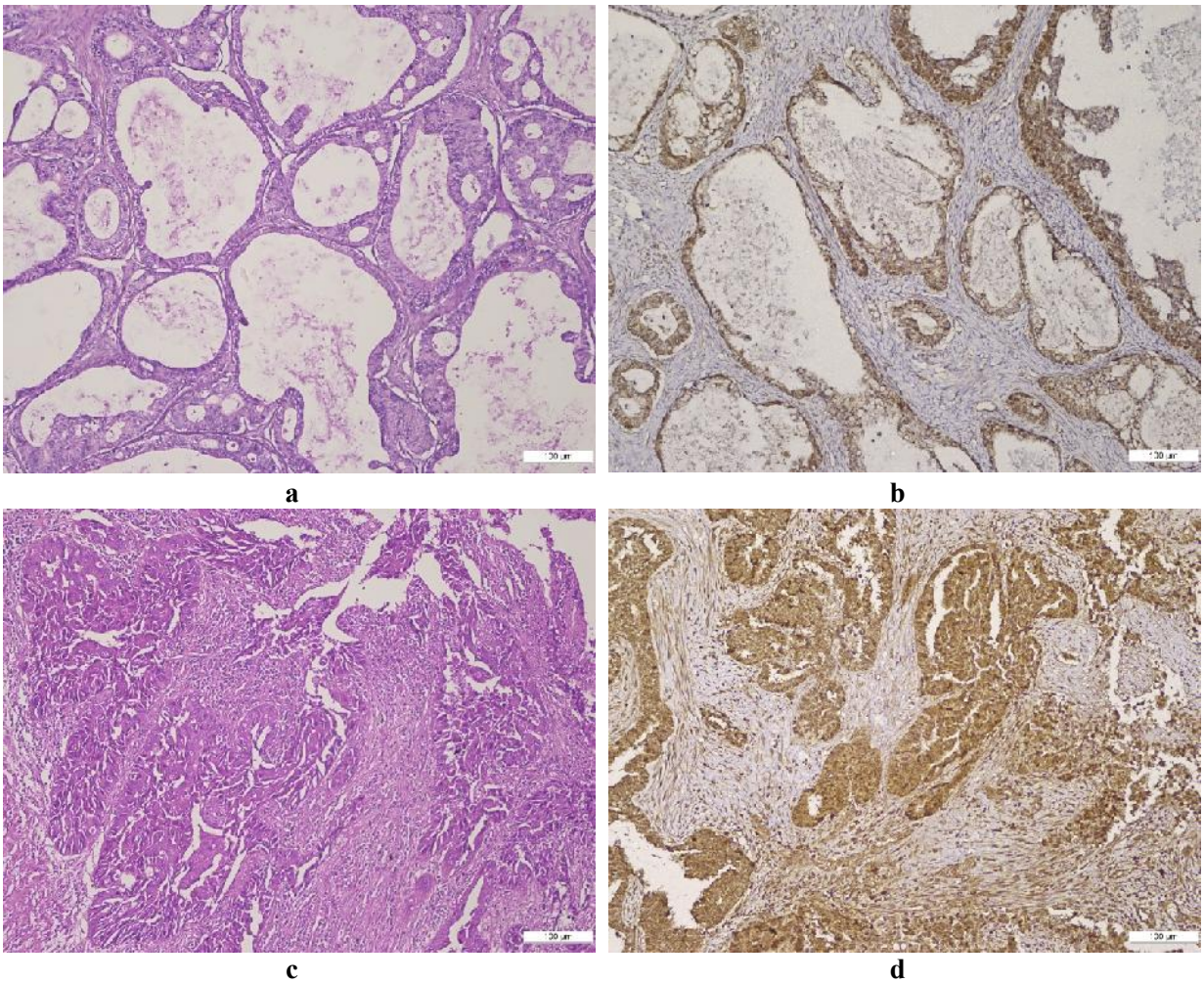
a **b**

c **d**



e f

Figure 1.25. (a–f) Histologic features and BMI-1 expression in EOC group in different ovarian tumor subtypes: (a,b) HGSC: (a) papillary growth, enlarged and irregular nuclei, prominent nucleoli, high cellular size and shape (H&E, x 100), (b) strong BMI-1 nuclear staining in epithelial tumor cells of HGSC (H&E, x100); (c,d) HGEC: (c) crowded back-to-back glands, lined by atypical columnar epithelium, and smooth luminal borders (H&E, x100), (d) weak BMI-1 cytoplasmic staining in epithelial tumor cells of HGEC (H&E, x 100); (e,f) COC: (e) papillary and tubulocystic pattern, combined with clear and eosinophilic cells and stromal hyalinization (H&E, x100), (f) strong BMI-1 cytoplasmic staining of tumor cells and stroma in COC (H&E, x100).



a b

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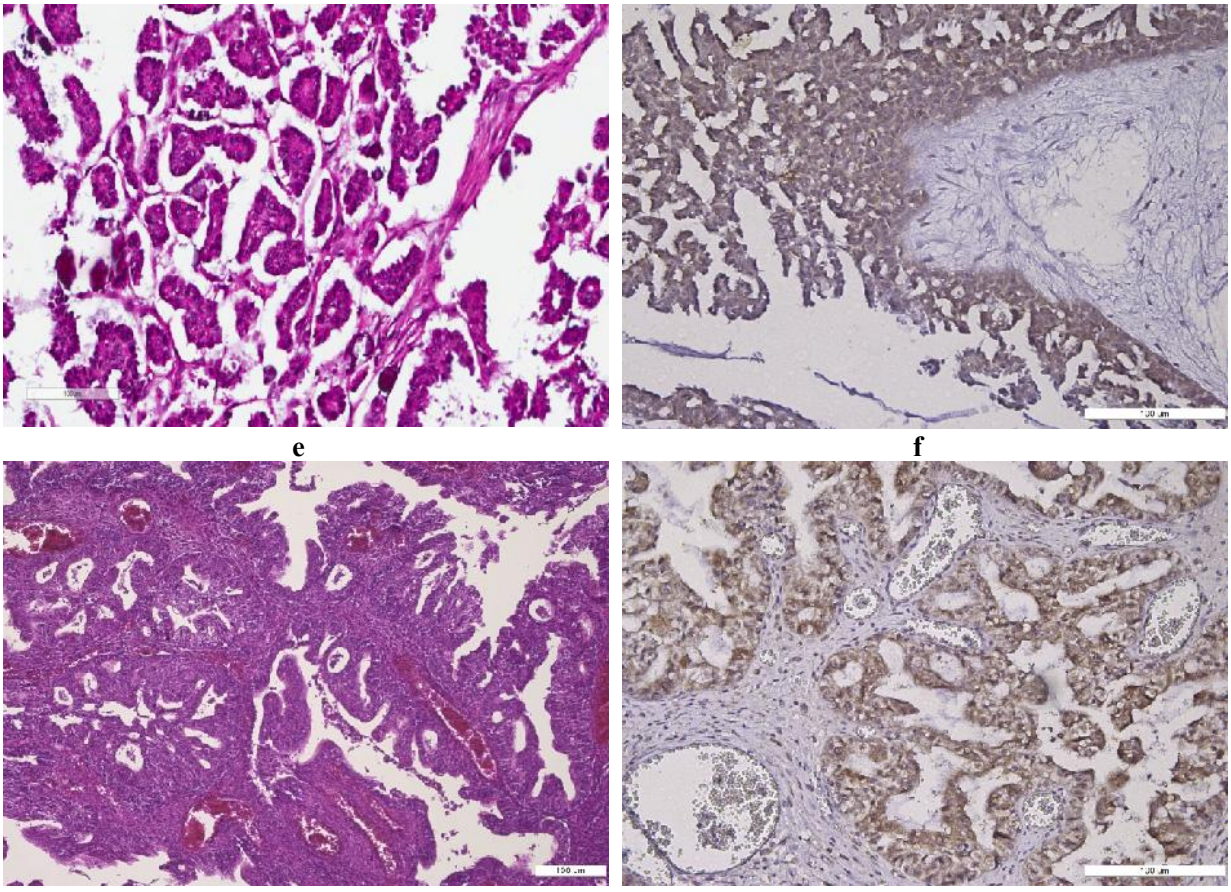


Figure 1.26. (a–h) Histologic features and BMI-1 expression in NEOC group in different ovarian tumor subtypes: (a,b) MOC: (a) atypical mucin-producing tumor cells with an infiltrative pattern of invasion (H&E, x100), (b) negative BMI-1 staining in tumor stroma of MOC (H&E, x100); (c,d) HGSC: (c) variation in cellular size and shape, marked nuclear atypia, dense fibrous stroma, and inflammation around the tumor nests (H&E, x100), (d) strong BMI-1 cytoplasmic staining of tumor stroma in HGSC (H&E, x100); (e,f) LGSC: (e) micropapillary growth with minimal nuclear atypia in LGSC (H&E, x100), (f) moderate BMI-1 cytoplasmic staining of tumor cells and stroma in LGSC (H&E, x100); (g,h) LGEC: (g) papillary and glandular differentiation in LGEC (H&E, x100), (h) strong BMI-1 cytoplasmic staining of tumor cells and stroma in LGEC (H&E, x100).

BMI expression – semi-quantitative assessment

In the whole group of study, without division into EOC and NEOC categories, the BMI1 semi-quantitative assessment showed the following: a high expression in 31 cases (65.96%) and a low expression in 16 cases (34.04%), in tumor cells, along with immunopositivity in 34 cases (72.34%), and immunonegativity in 13 cases (27.65%) in tumor stroma. The statistical analysis revealed significant correlations between BMI-1 expression in epithelial tumor cells (low/high) versus tumor stroma (negative/positive) ($p = 0.01$).

The semi-quantitative expression of BMI-1 showed a different profile in the two groups. BMI-1 expression in epithelial tumor cells was mostly low or negative in the EOC group and predominantly positive in NEOC group. On the other hand, the cases of the EOC group expressed positive and negative stromal BMI-1 immunoreactions approximately equally, whereas the stromal BMI-1 expression was mainly strong in the NEOC group (Table 1.15). We noted statistically significant differences between the BMI-1 epithelial and stromal profiles in each group (Table 1.16). Comparing the epithelial and stromal BMI-1 expressions between the EOC and NEOC groups, we obtained statistically significant differences only for the epithelial component ($p = 0.0002$), not for the stromal one ($p = 0.06$).

Table 1.16. Correlations between the epithelial and stromal BMI-1 expression in the EOC and the NEOC groups

BMI-1	EOC			NEOC		
	High Score/ Positive Reaction	Low Score/ Negative Reaction	*p* value	High Score/ Positive Reaction	Low Score/ Negative Reaction	*p* value
Epithelial tumor cells	5 (26.31%)	14 (73.68%)	0.04	26 (92.85%)	2 (7.14%)	0.001
Stromal cells	11 (57.89%)	8 (42.10%)		23 (82.14%)	5 (17.85%)	

Relationship between BMI-1 epithelial and stromal expression, and clinicopathological parameters in eoc

The results of the statistical analysis revealed a significant relationship between BMI-1 expression in tumor cells (low/high) and tumor grade (well and moderately differentiated versus poorly differentiated) ($p = 0.04$). On the other hand, stromal BMI-1 expression was significantly correlated with the median value of cancer antigen 125 (CA 125) ($p = 0.03$). No other significant differences were registered (Table 1.17).

Table 1.17. Correlations between BMI-1 expression in tumoral cells and clinicopathological parameters – EOC group

Clinicopathological Characteristics	#	Tumor cells BMI-1				*p* value	Stromal BMI-1				*p* value
		Low Score		High Score			Negative Reaction		Positive Reaction		
		#	%	#	%		#	%	#	%	
Age											
<55 age	8	5	62.5	3	37.5	**0.34**	4	50	4	50	**0.55**
≥55 age	11	9	81.82	2	18.18		4	36.36	7	63.64	
Tumor stage											
1	4	4	100	0	0	**0.48**	1	25	3	75	**0.55**
2	6	4	66.66	2	33.33		3	50	3	50	
3	8	5	62.50	3	37.50		3	37.50	5	62.50	
4	1	1	100	0	0		1	100	0	0	
Tumor grade											
I/II	7	7	100	0	0	**0.04**	4	57.14	3	42.85	**0.31**
III	12	7	58.33	5	41.66		4	33.33	8	66.66	
Histological subtype											
LGSC	0	0	0	0	0	**0.78**	0	0	0	0	**0.93**
LGEC	0	0	0	0	0		0	0	0	0	
COC	4	3	75	1	25		1	25	3	75	
MOC	0	0	0	0	0		0	0	0	0	
HGSC	3	1	33.33	2	66.66		1	33.33	2	66.66	
HGEC	8	6	75	2	25		5	62.50	3	37.50	
Undifferentiated	0	0	0	0	0		0	0	0	0	
Mixed	4	4	100	0	0		1	25	3	75	
Type											
Type I	4	3	75	1	25	**0.94**	1	25	3	75	**0.57**
Type II	15	11	73.33	4	26.67		7	46.67	8	53.33	

Residual disease											
NED/ < 1cm	6	5	83.33	1	16.67	**0.51**	2	33.33	4	66.67	**0.59**
≥1cm	13	9	69.23	4	30.77		6	46.15	7	53.85	
CA 125—median value											
< 1201.5 U/mL	10	8	80	2	20	**0.51**	2	20	8	80	**0.03**
≥1201.5 U/mL	9	6	66.67	3	33.33		6	66.67	3	33.33	

LGSC (low-grade serous carcinoma); LGEC (low-grade endometrioid carcinoma); COC (clear cell ovarian carcinoma); MOC (mu-cinous ovarian carcinoma); HGSC (high-grade serous carcinoma); HGEC (high-grade endometrioid carcinoma); NED (no evident data about residual tumor).

Relationship between BMI-1 epithelial and stromal expression, and clinicopathological parameters in neoc

The statistical analysis showed significant correlations between BMI-1 expression in the tumor cells (low/high), the stroma (negative/positive), and the tumor histological subtypes ($p = 0.002$ and $p = 0.04$, respectively) (Table 1.18). No associations were found for the other clinical clinicopathological parameters.

Table 1.18. Correlations between BMI-1 expression in tumoral cells and clinicopathological parameters – EOC group

Clinicopathological Characteristics	**#**	**Tumor Cells BMI-1**				***p* value**	**Stromal BMI-1**				***p* value**
		Low Score		**High Score**			**Negative Reaction**		**Positive Reaction**		
		#	**%**	**#**	**%**		**#**	**%**	**#**	**%**	
Age											
<55 age	14	1	7.14	13	92.86	**0.30**	2	14.29	12	85.71	**0.62**
≥55 age	14	1	7.14	13	92.86		3	21.43	11	78.57	
Tumor stage											
1	13	1	7.69	12	92.30	**0.91**	3	23.07	10	76.92	**0.71**
2	5	0	0	5	100		0	0	5	100	
3	10	1	10	9	90		2	20	8	80	
4	0	0	0	0	0		0	0	0	0	
Tumor grade											
I/II	15	0	0	15	100	**0.11**	3	20	12	80	**0.75**
III	13	2	15.38	11	84.61		2	15.38	11	84.61	
Histological subtype											
LGSC	4	0	0	4	100	**0.002**	0	0	4	100	**0.04**
LGEC	5	0	0	5	100		3	60	2	40	
COC	5	0	0	5	100		0	0	5	100	
MOC	5	1	20	4	80		1	20	4	80	
HGSC	5	0	0	5	100		0	0	5	100	
HGEC	3	0	0	3	100		0	0	3	100	
Undifferentiated	1	1	100	0	0		1	100	0	0	
Mixed	0	0	0	0	0		0	0	0	0	
Type											
Type I	19	1	5.26	18	94.74	**0.57**	4	21.05	15	78.95	**0.43**
Type II	9	1	11.11	8	88.89		0	0	9	100	

Residual disease											
NED/ < 1cm	16	1	6.25	15	93.75	**0.83**	2	12.5	14	87.5	**0.72**
≥1cm	12	1	8.33	11	91.67		1	8.33	11	91.67	
CA 125 - median value											
< 101	14	1	7.14	13	92.86	**1**	4	28.57	10	71.43	0.13
≥ 101	14	1	7.14	13	92.30		1	7.14	13	92.86	

LGSC (low-grade serous carcinoma); LGEC (low-grade endometrioid carcinoma); COC (clear cell ovarian carcinoma); MOC (mu-cinous ovarian carcinoma); HGSC (high-grade serous carcinoma); HGEC (high-grade endometrioid carcinoma); NED (no evident data about residual tumor).

1.3.4. DISCUSSIONS

- **Study 1**

Among the multitude of risk factors incriminated in endometriosis development, such as early menarche, nulliparity, aberrant estrogen levels, dysfunctional uterine bleeding, [Darrow et al., 1993; Signorello et al., 1997; Cramer et al., 2002], and low BMI (body mass index) [Signorello et al., 1997], there are notable risk factors which include circumstances for retrograde menstruation and genetic factors [Dawson et al., 2018], being known the much higher incidence of endometriosis in women who have first-degree relatives diagnosticated with this disease [Treloar et al., 1999; matalliotakis et al., 2008; Dawson et al., 2018].

To demonstrate the presumed multifactorial genetic susceptibility for endometriosis, numerous genome-wide association studies detected various profiles of single-nucleotide polymorphism (SNP) likely to increase the endometriosis risk [Rahmioglu et al., 2014], as follows: WNT4 gene significant anomalies [Jaaskelainen et al., 2010; Boyer et al., 2010; Albertsen et al., 2013], 18 genomic regions with 38 supposed endometriosis-associated SNPs [Nyholt et al., 2012], VEZT (Vezatin, Adherens Junctions Transmembrane Protein) [Guo et al., 2011] and GREB1 (Growth Regulation by Estrogen in Breast cancer 1) [Ghosh et al., 2000; Rae et al., 2005] abberations, MAPK (mitogen-activated protein kinase)-related pathways [Uimari et al., 2017], five new loci of sex shormone pathways (FSHB, FN1, ESR1, CCDC170, and SYNE1) [Sapkota et al., 2017; Dawson et al., 2018].

The mechanisms underlying the development of endometriosis are retrograde menstruation [Sampson, 1927a], coelomic metaplasia [Ferguson et al., 1969], haematogenous or lymphatic spread of endometrial tissue [Sampson, 1927b], as well as more recent theories regarding stem cell proliferation and differentiation [Lagana et al., 2017], or endometrial cells implantation following retrograde menstruation [Dawson et al., 2018].

A peculiar endometriosis subtype which is also under genomic evaluation is the deep infiltrating endometriosis, with a tendency to invade the adjacent bowel, bladder, or ureter, but with rare metastases, and with demonstrated somatic mutation in several oncogenes such as PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), KRAS (Kirsten rat sarcoma virus), PPP2R1A (Protein Phosphatase 2 Scaffold Subunit Aalpha), and ARID1A (AT-rich interacting domain 1A) [Anglesio et al; Dawson et al., 2018; Samartzis et al., 2020]. Besides these somatic mutations, other endometriosis genomic anomalies involved in carcinogenesis are described: TP53 [Bischoff et al., 2002; Sainz de la Cuesta et al., 2004] and PTEN (Phosphatase and tensin homolog) [Sato et al., 2000] mutations, microsatellite instability [Fuseya et al., 2012], or mismatch repair enzymes expression loss [Grassi et al., 2015; Dawson et al., 2018].

The association between endometriosis and cancers is supported by epidemiological factors, such as: common risk factors (early menarche, short menstrual periods, nulliparity, late menopause), on one hand and by factors with potential protective effect for both diseases (oral

contraceptives, high parity, tubal ligature, and hysterectomy), on the other hand [Van Gorp et al., 2004].

The current state of knowledge shows that there are two pathways that appear to be involved in the endometriosis potential to progress towards neoplasia: either malignant transformation, perhaps through an atypical transitional stage, or a common precursor mechanism or predisposing factors is common in both processes, with a consecutive molecular divergence [Varma et al.,2004].

Endometriosis related ovarian carcinoma (EOC) occurs in 60-80% of cases in association with atypical endometriosis. Numerous studies have tried to identify a common model for endometriosis and EOC. The association of these two diseases has been identified most frequently in endometrioid ovarian carcinoma (OC), clear cell carcinoma, seromucinous carcinoma, müllerian adenosarcoma, and endometrioid stromal sarcoma [Taniguchi, 2017].

Most of these tumors (70%) are developed during the first 10 years from endometriosis diagnosis, being associated in most cases (60%) with an intermediary stage of atypical endometriosis [Taniguchi, 2017]. Moreover, considering the intrinsic invasive and metastatic capacity of endometriosis, its behavior has a high degree of homology with malignancies [Zeitvogel et al., 2001].

Since the multitude of etiopathogenic mechanisms was proposed for endometriosis, a variable involvement of multiple processes may be related to location and lesional type, with the possibility that some of the phenomena may represent, in fact, consequences of an initial lesion.

One mechanism that has aroused the interest of researchers in the last decade is that of EMT, and its reverse process, MET, both mechanisms that raise researchers' interest in the last decade, these being studied mainly in the carcinogenesis and metastasis process. EMT is a strictly controlled, reversible process characteristic for the embryogenesis period and can be also found during adulthood in epithelia with fast cell regeneration, such as epidermis and intestinal villi, in wound healing and in pathological processes, such as fibrosis and inflammation [Sethi et al., 2011]. EMT also occurs during carcinogenesis, being characterized by changes that give malignant cells a high potential for invasion and metastasis, respectively [Sethi et al., 2011].

As E-cadherin is expressed in all epithelial cells, being involved in the maintenance of polarization and of cellular integrity [Zhou et al., 2017], its loss represents a marker of EMT process [Zhou et al., 2017]. An ablation of E-cadherin expression is noticed in EMT and, therefore, cells gain an increased mobility, allowing them invasion and metastasis [Zhou et al., 2017]. Thus, E-cadherin decreased expression is involved in a decisive way in the pathogenic mechanism of endometriosis, cells that lose expression having the same invasive and metastatic phenotype as carcinomatous cells [Zhou et al., 2017].

Because ovarian endometriosis represents a clonal proliferation of cells with genetic alterations, this disease is considered a true neoplasm, a precursor of OC [Matsumoto et al., 2015]. It is estimated that β-catenin mutations represent an early event in the endometriotic-dependent ovarian carcinogenesis sequence [Matsumoto et al., 2015].

Considering the correlation between endometrioid or clear cell carcinoma (CCC) and endometriosis, as a precursor lesion in 20-40% of cases and 40-55% of cases, respectively [Matsumoto et al., 2015], it has been demonstrated that they are associated in 5-10% of cases of endometriosis, showing an intermediary phase of atypical endometriosis identified in 0.7-1.6% of cases, mainly in cases with long-standing endometriosis [Matsumoto et al., 2015].

Oncogenic mutations of phosphorylation site of β-catenin (gene CTNNB1) results in a stable protein formation, detected in 40%-60% of EOC and in 52.4% of associated endometriosis and in 73.3% of associated atypical endometriosis [Matsumoto et al., 2015].

The alteration of E-cadherin, β-catenin, and CK18 are correlated to the progression along EMT process and to stages progression of EOC. An increased level of E-cadherin and β-catenin has been registered in the endometriosis group of our study and gradually decreased

with the staged evolution of cases diagnosed with malignancy. This data suggests that its loss occurs late during the EMT process, being evident in the carcinogenesis process associated with endometriosis. Furthermore, the negative E-cadherin score in our EOC group demonstrates the existence of the cadherinic switch, characteristic of the EMT process. Statistical analysis revealed in the investigated cases a significant difference between the E-cadherin staining score in endometriosis versus EOC ($p=0.001$). We found that β-catenin showed a marked immunointensity in the endometriosis group, showing a slight decrease in the EOC group. β-catenin percentage of positive tumor cells registered a slight decrease in EOC group. Statistical analysis revealed significant differences between the group of cases diagnosed with endometriosis and EOC ($p=0.000112$). This suggests the gradual loss of epithelial features as the EMT process extends. Moreover, β-catenin registered 94.73% positivity in endometrioid versus 100% in CCC, suggesting its value as a poor prognosis factor in EOC.

CK18, a cytokeratin type I belonging to the cytoskeleton, expressed both in glandular endometriotic cells [Klemmt et al., 2006] and in carcinomatous cells, is considered a marker of morphological heterogeneity and of neoplastic changes [Trisdale et al., 2016], including those of ovarian carcinomas [Duncan et al., 2012]. CK18 maintain its expression in endometrioid carcinomas or in those with an endometrioid component.

CK18 showed a high staining index and percentage of positive cells as a specific marker of endometriotic epithelial cells, demonstrating its involvement in apoptosis in the investigated cases [Moll et al., 2008], with statistically significant differences between the endometriotic and EOC groups ($p=0.032468$). In our study both components: intensity and positive tumor cells have registered evident changes, in malignant group. Moreover, the malignant group showed a progressive reduction of the staining index with the stage. This demonstrates the loss of epithelial phenotypic characteristics and the acquisition of a stromal phenotype, gradually according to the malignant process extension.

Although alterations of E-cadherin, β-catenin, and CK18 have been recorded, the correlation analysis between EMT markers expression in endometriosis did not revealed significant differences but revealed statistically significant differences between E-cadherin and β-catenin ($p=0.0001$) and between E-cadherin and CK18 expression ($p=0.0001$), in EOC group, in our study.

The correlation analysis between EMT markers (E-cadherin, β-catenin, and CK18) expression in EOC and clinicopathological characteristics (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values) did not reveal any statistical significant associations, suggestive of other factors interventions in this process.

Apoptosis, as a pivotal mechanism of regulation of variable cellular populations, normal and pathological, is mainly based on the antagonism between Bcl-2 and Bax [Petros et al., 2004]. Bcl-2 is preventing apoptosis without any effect on proliferation and protects against DNA-induced apoptosis [Simonin et al., 2009]. It is considered that Bcl-2 has a relevant anti-apoptotic activity, depending on Bax involvement [Korsmeyer et al., 2000; Lozneanu et al., 2015]. Due to apoptosis involvement in carcinogenesis, tumor promoter or suppressor roles are attributed to Bcl-2. Due to its pro-apoptotic role, Bax has the characteristics of tumor suppressor role [Cartron et al., 2002]. The endometriotic cells show a weak Bax expression, associated to a high expression of Bcl-2 [Meresman et al., 2000]. An enhanced rate Bcl-2/Bax has been demonstrated in EOC, representing a possible prognosis marker and, at the same time, creates the premises for Bcl-2 antagonists and/or Bax agonists therapeutic use [Zeren et al., 2014]. Furthermore the Bcl-2/Bax ratio expression in EOC compared to endometriosis reflects the decreased ovarian cell sensitivity to apoptotic stimuli. In agreement with this data, it has been demonstrated that spontaneous endometriosis may be induced by Bax and may be prevented by Bcl-2 [Vatanseven et al., 2009].

The expression of pro- and anti-apoptotic markers revealed low levels of Bax, associated with a slight increase of Bcl-2 in the epithelial component of the investigated cases. The data obtained are consistent with literature reports showing that the relationship between anti-apoptotic and pro-apoptotic factors appears to be involved in the etiopathogenesis of endometriosis [Meresman et al., 2000; Goumenou et al., 2001]. It has been also demonstrated that Bcl-2/Bax high rate in endometriosis is correlated to a high malignant potential [Zeren et al., 2014]. The statistical correlation analysis revealed statistically significant differences between Bax and Bcl-2 expression in the endometriosis group ($p=0.020$). Amplification of Bcl-2/Bax ratio demonstrates the progressive decrease in ovarian cell sensitivity to apoptotic signaling and contributes, along with the alteration of steroid receptor, to the mechanism of endometriosis transition to EOC, opening new perspectives for prognostic evaluation and therapeutic modulation.

Although a significant difference has been identified between Bax and Bcl-2 expression in endometriosis, no statistically significant differences have been registered between the expressions of Bax and Bcl-2 in EOC. Furthermore, no statistically significant differences have been registered between Bax and Bcl-2 expressions and clinicopathological characteristics in both groups, suggesting the intervention of other factors.

Estrogens action, modulated by specific receptors, ERs, has effects on the production of cytokines and of apoptotic phenomena, in endometriosis [Han et al., 2015], counterbalanced by progesterone action, modulated by their counterpart specific receptors, PRs [Veillat et al., 2010; Lee et al., 2012; Monsivais et al., 2014; Khan et al., 2015]. Different from eutopic endometrium, where steroid receptors level may be correlated with endometrial cycle phases, ER being strongly expressed in proliferative stage and decreasing its expression in both epithelial and stromal components in secretory phase, no cyclical changes have been noticed in endometriosis [Calcagno et al., 2011]. Moreover, a generally more reduced ER expression has been observed in endometriosis [Suzuki et al., 2010; Calcagno et al., 2011]. Estrogen is recognized as a stimulator of ovarian cells proliferation, of malignant cells mobility, and of intercellular adhesion inhibition [Park et al., 2008; Chen et al., 2017]. Although in the secretory phase of eutopic endometrium, PR are strongly expressed, mainly in the stromal component, no cyclical changes of these receptors have been noticed in endometriosis [Calcagno et al., 2011], showing a weak expression [Suzuki et al., 2010; Calcagno et al., 2011]. Relatively new data have demonstrated the correlation between ER or PR expression and their clinical impact in OC [Tkalia et al., 2014; Chen et al., 2017].

It is widely accepted that the loss of ER and PR expression in endometriosis-associated ovarian carcinoma may represent a carcinogenesis stage, suggesting the cell dedifferentiation [Bartiromo et al., 2022]. Several studies also assessed the hormonal receptors expression in atypical endometriosis, some of them revealing important decreased ER and PR expression in atypical endometriosis as well as in endometriosis-associated ovarian carcinoma [Del Carmen et al., 2003; Akahane et al., 2005; Xiao et al., 2012; Lai et al., 2013; Lin et al., 2014; Andersen et al., 2018; Jiao et al., 2019; Penciu et al., 2020; Lenz et al., 2021].

Another conclusion of these studies was that hormonal receptors expression was higher in atypical endometriosis compared to endometriosis-associated ovarian carcinomas, but lower than in endometriosis. Thus, it is assumed that the progressive decreased expression of ER and PR from endometriosis to endometriosis-associated carcinogenesis may represent a useful marker for endometriotic atypical or malignant lesions [Bartiromo et al., 2022].

In the current study, the hormonal receptors expression has been noticed in both components of endometriosis, epithelial and stromal. In comparative terms with their expression in eutopic and normal endometrium, a degree of difficulty in their interpretation occurs, considering that the hormones are responsible for the cyclical stimulation of proliferation and regeneration and of secretory function, in the secretory phase, respectively.

Moreover, another recent theory, based on comparative studies between the different endometriotic locations and eutopic endometrium, are focused on paracrine inhibition of steroid receptors expression in ovarian endometriosis [Calcagno et al., 2011].

As a consequence, although the steroid receptors levels are high in ovarian endometriosis, a moderately-increased expression has been observed in our group of study, without any statistical significant correlations with clinicopathological characteristics.

Supplementary, PR expression has shown statistical significant differences according to ovarian endometriosis ($p=0.04$). This finding is validating PR role in endometriosis mechanism, on one hand, and on the other hand, supports the influence of paracrine factors in ovarian microenvironment, responsible for differences observed from other locations, demonstrating a high susceptibility of ovarian endometriosis to malignant transformation.

ER score in tumor cells was high in most cases of our study with strong nuclear expression, supporting the role of hormonal mediation in the pathogenesis of ovarian malignancies. ER epithelial expression was also high in endometriosis, but there were large variations in expression. Although we would expect a concordance with literature data, no significant difference has been noticed between ER expressions in tumor cells of endometrioid OC compared to non-endometrioid type in the investigated cases. These discrepancies are attributed probably to the limited number of cases available for our study or to the cut-off value.

Stromal ER expression in endometriosis has been significantly lower compared to tumor stroma ($p=0.001$), possibly as an indicator of a less responsive component to the hormonal stimulation or to a reduced expression of one of the ER isoforms.

Regarding FIGO staging, no significant difference has been observed between PR expression in EOC epithelial and stromal cells in early stages compared to late stages in the studied group, although literature data show a higher PR expression in endometrioid types without peritoneal metastases, being correlated to tumor cells proliferation inhibition and of metastases development [Sieh et al., 2013; Worley et al., 2013; Chen et al., 2017]. However, significant differences have been noticed between the epithelial ($p=0.027$) and stromal PR expression ($p=0.005$) in EOC with more differentiated histological types, suggesting a partial protective role of progesterone in carcinogenesis.

PR stromal expression in endometriosis has been significantly lower compared to that of tumor stroma ($p=0.000$), possible as an indicator of unbalanced estrogenic stimulation involvement in endometriosis pathogenesis, but with a less important role in malignancy development. The identification of a weaker PR expression both in epithelial and stromal endometriotic cells suggest that loss of PR expression has been attributed to early genetic changes prior to morphological atypia, as an important immunohistochemical marker in endometriosis malignant transformation risk [Xiao et al., 2012]. The imunohistochemical expression of ER, PR, corroborated to clinicopathological features supports the mechanism of transition of ovarian endometriosis to EOC; provide tools for prognosis evaluation and opens new perspectives of therapy for these types of tumors.

- **Study 2**

Numerous hypotheses regarding the mechanisms involved in OC etiopathogenesis have been proposed over time as attempts to explain the multiple tumor phenotypes, poor prognosis, and chemoresistance. Endometriosis represents a precursor lesion for certain types of epithelial OC, since the identification of the same genetic alterations in both diseases are demonstrated [Douglas et al., 2008; Laganà et al., 2017; Herreros-Villanueva et al., 2019]. Accordingly, the corroboration of specific clinicopathological findings with specific mutations led to the EOC and NEOC categories distinction [Silva et al., 2007].

The BMI-1 protein, involved in homeotic genes regulation by transcription inhibition [Honig et al., 2010], represents a survival factor of malignant stem cells [Honig et al., 2010], and is correlated to hormonal receptor expression, and is considered as a prognosis factor surrogate [Silva et al., 2007; Choi et al., 2009]. Thereby, BMI-1overexpression was reported in different malignant tumors, with impact on carcinogenesis, metastasis, and radiation therapy resistance [Song et al., 2006; Vormittag et al., 2009; Chen et al., 2011; Allegra et al., 2012], as well as in nasopharyngeal, salivary, gastric, adenoid cystic, and breast carcinomas, as a prognosis predictor [Bachmann et al., 2006; Song et al., 2006; Mihic-Probst et al., 2007; Silva et al., 2007; Vrzalikova et al., 2008; Liu et al., 2008; dos Santos et al., 2019].

For normal tissues, BMI-1 represents an epigenetic regulator and a transcription factor necessary for maintaining the suppression of cell proliferation genes. It is demonstrated that the BMI-1 effect on cell proliferation is partially mediated through inhibition of the locus encoding p16 [Huber et al., 2011]. The p16 protein, implicated in G1-S transition regulation, inhibits the cyclin D1/cdk4/6 complexes formation, repressing the cell cycle progression [Li et al., 2006; Fecher et al., 2009; Mitra, Fisher, 2009]. Therefore, p16 suppression by BMI-1 generates cell cycle progression [dos Santos et al., 2019].

In neoplastic tissues, BMI-1 expression is not necessarily related to expression of p16, having no consequences on cell cycle progress, such in different malignancies from colon/rectum, lung, and brain cancers, as well as in head and neck carcinomas [Vonlanthen et al., 2001; Hemmati et al., 2003; Breuer et al., 2004; Kim et al., 2004; Lundberg et al., 2016; dos Santos et al., 2019].

BMI-1 has been identified in experimental studies of OC (cell lines, clone derivation, and animal experiments) [Zhanf et al., 2008; Wang et al., 2011; Vathipadiekal et al., 2012; Xin et al., 2012; He et al., 2014; Dey et al., 2018; Shishido et al., 2018], both in protein and the protein-coding gene [Yang et al., 2010], and in human ovarian tumors or ascites fluid samples [Zhang et al., 2008; Zhang et al., 2008; Honig et al., 2010; Yang et al., 2010; Vathipadiekal et al., 2012; He et al., 2014]. Despite these reported results, BMI-1 expression is not fully established in OC. The review of the literature shows that less than 10 studies have addressed BMI-1's involvement in OC, most of them highlighting the molecular action and potential therapeutic value of this protein.

A positive correlation between BMI-1 positive expression in human epithelial OC and elevated telomerase activity was demonstrated [Zhang et al., 2008]. Another study, based on human specimens and ovarian cancer cells, showed that BMI-1 expression is downregulated by MiR-15a or MiR-16 underexpression, with subsequent significant decreases in cell proliferation and clonal growth [Bhattacharya et al., 2009]. Therefore, BMI-1 seems to be a potential target in OC therapy. Eloquent evidences in this direction are provided in recent papers that have demonstrated the therapeutic activity of PTC-028 as a novel inhibitor of BMI-1 function in OC [Dey et al., 2018] and the role of MiR-132 in cisplatin resistance and OC metastasis by the targeted regulation of BMI-1 [Zhang et al., 2019]. In terms of the number of human OC samples, the studies on BMI-1 have been generally performed on small groups, with a median number of research sample of 41 (range 5–179) [Zhang et al., 2008; Zhang et al., 2008; Bhattacharya et al., 2009; Honig et al., 2010; Vathipadiekal et al., 2012; He et al., 2014]. These samples were collected from tumor tissue [Zhang et al., 2008; Zhang et al., 2008; Bhattacharya et al., 2009; Yang et al., 2010; Vathipadiekal et al., 2012; Honig et al., 2010; He et al., 2014], fresh ascites [Vathipadiekal et al., 2012], and frozen ovarian tissues [Zhang et al., 2008].

One recent study reported a significant cell proliferation inhibition from BMI-1 repressing, with epithelial ovarian carcinoma cells arrested in G1 phase [Zhao et al., 2018]. Another recent work, related to breast cancer, reported the influence of ERα (estrogen receptor α)-BMI1 couple upon cyclin D1 and p16INK4a status, which is associate with cancer molecular subtype and its biologic behavior [Wang et al., 2014]. Moreover, knocking down BMI-1

decreases the abilities of epithelial ovarian carcinoma cells for invasion and migration, but increasing their platinum sensitivity [Bracken et al., 2007; Huber et al., 2011; Siddique et al., 2012; Wang et al., 2014; Torre et al., 2015; Zhao et al., 2018]. Correspondingly, it was shown that BMI-1 suppression enhanced the reactive oxygen species (ROS), increased the DNA repair pathway, stimulating apoptosis induced by cisplatin [Wang et al., 2011; Zhao et al., 2018].

Consistent with these results, other studies demonstrated the involvement of BMI-1 in epithelial- mesenchymal transition, inducing invasion and metastasis [Koren et al., 2016]. Moreover, overexpression of BMI-1 in epithelial ovarian carcinoma cells stimulates Bcl-2, cyclin D1, and CDK4 expression, enhancing cell proliferation and suppressing apoptosis [Kim et al., 2017; Zhao et al., 2018].

The reported data target only BMI-1 in epithelial tumor cells, showing a high expression in 80.9% of OC and its relationship with tumor aggressiveness [Zhang et al., 2008]. Moreover, a positive correlation between BMI-1 expression and advanced International Federation of Gynecology and Obstetrics (FIGO) stages, bilaterality, higher tumor grades, and serous morphology [Zhang et al., 2008; Abd El Hafez et al., 2014], and a progressive incremental number of BMI-1-positive cases in accordance with the increase of tumor grade and stage were demonstrated, while increased BMI-1 expression was associated with reduced patient survival [Yang et al., 2010].

This short review of data concerning the correlation between BMI-1 and OC shows that the current knowledge is predominantly based on experimental data as the first level of evidence regarding its role in carcinogenesis, while the results obtained by the investigation of BMI-1 in human tissues is very scarce. Within this general context, our study complements the knowledge on BMI-1 in OC by doing research that translates the evidences level from the experimental area to the clinical domain by reference to the clinicopathological characteristics of OC with different parameters for EOC and NEOC.

Our work has demonstrated high BMI-1 expression levels in the epithelial tumor cells in 66% of OC (26% in EOC and 93% in NEOC). Moreover, our study provides valuable data on BMI-1 profile in OC, bringing to the foreground the relationship of OC with endometriosis, and the differences between the epithelial and stromal expression. This endeavor was possible by consistent differences in the design of the patient's cohort, comprising 47 cases of OC separated in two different tumor groups: EOC and NEOC. Thus, we have demonstrated, for the first time, the possible correlations between epithelial and stromal BMI-1 profiles in EOC and NEOC and several classical clinicopathological parameters.

The segregation into EOC and NEOC has been justified by the findings that certain histological types of EOC, mainly endometrioid and clear cell carcinomas, have different clinical features, such as younger age at diagnosis, unilaterality, identification at an earlier stage, and a better survival rate, compared to the counterpart entities of NEOC [Brilhante et al., 2017]. Our study supports the hypothesis of EOC development within endometriosis, showing mostly an endometrioid (42% in EOC versus 28.57% in NEOC) or clear cell phenotype (21% in EOC versus 18% in NEOC), and, implicitly, the quality of precursor lesion of ovarian endometriosis. Endometriosis and EOC represent two entities with the same target organ (ovary), the same tissue of origin (endometrial-like), and the same pathogenic mechanism which progresses from benign to atypical and malignant phenotypes. Having these in mind, tubal ligature or salpingectomy may be used as preventative maneuvers which may be applied within a screening and early therapy algorithm.

An original finding in our research is the dual staining pattern, nuclear and cytoplasmic/membrane in both study groups, although only a nuclear staining is reported in literature [Bachmann et al., 2008; Abd El Hafez et al., 2014; Zhang et al., 2015; Zhang et al., 2019]. This immunostaining pattern may indicate a possible relocation of protein during the transition to tumor phenotype. Moreover, it may suggest the involvement of additional factors

as a possible reflection of adhesion molecules interrelationship in the context of epithelial mesenchymal transition (EMT) [Bachmann et al., 2008; Zhang et al., 2015] or of the involvement of variable ovarian microenvironmental factors in both EOC and NEOC.

Our study confirms the relationship between BMI-1 in epithelial tumor cells and stroma in three instances: (i) in the general OC group ($p = 0.01$), (ii) in the NEOC group ($p = 0.001$), and (iii) in the EOC group ($p = 0.04$). In parallel, the comparative analysis of BMI1 expression in EOC and NEOC showed a statistically significant higher expression of BMI-1 in the epithelial tumor component than in the stroma ($p = 0.0002$). Our results clearly show EOC's association with BMI-1 low expression in epithelial tumor cells without a dominant expression profile in stromal cells, while NEOC is characterized by high BMI-1 expression in both the epithelial and stromal types of cells. However, stromal BMI-1 expression is reflecting EMT involvement in tumor progression and the interrelationship between the two cellular components, which result in BMI-1 synthesis as a stromal-dependent mechanism. Therefore, if present, stromal BMI-1 could be considered as a valuable marker for poor survival.

To the best of our knowledge, our study provides for the first time evidence for BMI-1 expression in human EOC. Differently from NEOC group findings, a progressive gain of BMI-1 expression in epithelial tumor cells has been noticed in the EOC group along with tumor grade, with statistically significant differences when we compared well and moderately differentiated with poorly differentiated tumors. This finding indicates a relationship between BMI-1 epithelial overexpression and a poorer prognosis in the selected EOC cases. Currently, CA125, expressed in the embryonic development of ovaries and re-expressed in endometriosis and ovarian neoplasms, can be used as a prognostic and predictive biomarker related to patient survival, independent of OC treatment [Zhang et al., 2015].

CA 125 shows significant different values in the two major types of OC, suggesting that they occur as a result of different factors, following specific pathway initiations and progressions [Charkhchi et al., 2020]. Many studies have shown that the CA125 profiles of HGSC and HGEC are different from other subtypes [Köbel et al., 2008]. We also found a statistically significant correlation between stromal BMI-1 and CA 125 level, suggesting that EOC may be influenced by a microenvironment modulation specific for endometriosis-based ovarian carcinomas, supporting the rapid growth pattern and the unfavorable prognosis in a subcategory of cases. Thus, we may conclude that the interrelationship and reciprocal stimulation between a tumor's epithelial and stromal components occurs latter during the endometriosis-related carcinogenic process, with a subsequent uptake of BMI-1 expression by stromal component, which may be reflected in an increased CA-125 level. The aggressive behavior of these EOC cases has a different significance from that of aggressive type I OC, probably originating from fallopian tube epithelium. It is worth mentioning that BMI-1 was absent in the normal ovaries or ovarian surface in the study groups, while BMI-1 expression has been identified in the normal tubal surface epithelium; this finding comports with the hypothesis of some OCs development from the fallopian tube, providing another support for this pathogenic mechanism.

On the other hand, in the NEOC group, we have shown statistically significant differences between BMI-1 immunopositivity in the tumor's epithelial cells, stromal cells, and histological subtypes. In our opinion, these results may be considered as solid evidence for the association of BMI-1 with high grade OC phenotypes and, consequently, with tumor aggressiveness.

Overall, our study reveals a different BMI-1 profile in the EOC an NEOC groups, thus underlying the differences in their etiopathogeny. We are aware of the limitations of our study due to the small size of the study groups and their heterogeneity in histological types, as the selection criteria have been strictly applied. Despite these limitations, our results open promising perspectives for differentiation of EOC from NEOC that need to be further validated

in a larger and homogenous cohort of study. An interesting research item can be directed to the high-grade serous phenotype of OC that may be further subdivided into subcategories according to their affiliation to the EOC or to NEOC groups.

Nevertheless, the reported differences in BMI-1 expression in EOC and NEOC need to be further validated in a larger and homogenous cohort of study.

1.3.5. FINAL REMARKS

Study 1

Endometriosis can be considered a precursor lesion of the EOC, as demonstrated in this study, and immunohistochemical expression of E-cadherin, β-catenin, Bcl-2, Bax, ER, PR in corroboration with clinicopathological features supports the mechanism of transition of ovarian endometriosis into EOC, provides tools for prognosis evaluation, and opens new perspectives of therapy for these types of tumors.

Considering the value of ovarian endometriosis as a precursor lesion for a large spectrum of ovarian carcinomas, the understanding of mechanisms which are involved in these diseases has also a prevention value for ovarian malignancies, with a beneficial effect for populations at high- risk.

Study 2

This study provides solid evidence for a different BMI-1 expression in EOC and NEOC, corresponding to the differences in their etiopathogeny. The EOCs were largely characterized by a low BMI-1 expression in epithelial tumor cells, without a dominant expression profile in stromal cells. Epithelial BMI-1 is progressively increased alongside the tumor grade and strong stromal BMI-1 may be correlated to microenvironment modulation, supporting the rapid growth pattern and the recognized poor prognosis in a subcategory of EOC cases. The NEOCs were characterized by high BMI-1 expression in both the epithelial and stromal types of cells; therefore, BMI-1 expression could be regarded as an indicator of aggressiveness of this type of malignancies in general, and for HGSC in particular.

Additionally, BMI-1 expression limited to the normal tubal surface epithelium and its lack in normal germinal/surface ovarian epithelium may support the hypothesis that many OCs are originating from the fallopian tube epithelium.

1.4. AGGRESSIVE BEHAVIOR IN ENDOMETRIAL ENDOMETRIOID CARCINOMA – PARTICULAR ASPECTS

1.4.1. INTRODUCTION

Endometrial carcinoma is the most frequent gynecological neoplasia in women, which bears an overall good prognosis, with a relative five-year survival rate of 84.5% for all stages and histological types [Genestie et al., 2017]. Endometrial carcinomas are sporadic in 95% of cases, while less than 5% of cases is associated with a hereditary predisposition, especially when presenting in younger patients [Daniilidou et al., 2013; Stavropoulos et al., 2020].

As classified for the first time by Bokhman, in 1983, there are two types of endometrial carcinoma, with distinct epidemiological, clinical and histopathological features [Bokhman, 1983]. Type 1 comprises the majority (70–80%) of cases. It frequently affects women at perimenopausal age and develops on a background of endometrial hyperplasia, initiated by estrogen stimulation unopposed by progesterone, in conditions such as obesity, anovulation, nulliparity or exogenous hormone use. Type 1 endometrial carcinoma has low grade, endometrioid histological type, it is usually early diagnosed, and has a favorable prognosis.

Type 2 includes the rest of 20–30% of cases, it is more frequent in post-menopausal women, and appears on a background of atrophic endometrium, in the absence of estrogenic stimulation. The histological type in this category is high grade, non-endometrioid (mainly serous, mucinous, and clear cell). Commonly, it is diagnosed in an advanced stage and has an unfavorable prognosis [Weiderpass, Labrèche, 2012; Visser et al., 2015].

The surgical treatment for endometrial cancer is total abdominal hysterectomy with bilateral oophorectomy [Stavropoulos et al., 2020]. Moreover, the treatment of endometrial carcinoma is related to tumor stage, type I early stages benefiting from adjuvant radiotherapy, whereas advanced stages from both types usually needing chemotherapy [Cancer Genome Atlas Research Network et al., 2013; Bilyk et al., 2017].

Immunohistochemical (IHC) and molecular profile differs among these two types of carcinomas, as follows: (i) estrogen receptor (ER) positive, along with variable mutations of tumor protein 53 (TP53), CTNNB1 (β-catenin), AT-rich interaction domain 1A (ARID1A), phosphatase and tensin homolog (PTEN), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and Kirsten rat sarcoma virus (KRAS) genes, in type 1; (ii) p53 positive, p16 positive, along with variable mutations of PIK3CA, F-box and WD repeat domain containing 7 (FBXW7), and protein phosphatase 2 scaffold subunit alpha (PPP2R1A) genes in type 2 [Hedrick Ellenson et al., 2011; Goebel et al., 2018; Bilyk et al., 2017] (Table 1.19.)

Table 1.19. Immunohistochemical and molecular profile of endometrial carcinomas

EC TYPE	IHC PROFILE	GENES MUTATIONS
Type I	ER +	TP53 CTNNB1 ARID1A PTEN PIK3CA KRAS
Type II	p53 + p16 +	PIK3CA FBXW7 PPP2R1A

ER – estrogen receptor; TP53 – tumor protein 53; CTNNB1 - β-catenin; ARID1A - AT-rich interaction domain 1A; PTEN - phosphatase and tensin homolog; PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; KRAS - Kirsten rat sarcoma virus; FBXW7 - F-box and WD repeat domain containing 7; PPP2R1A - protein phosphatase 2 scaffold subunit alpha.

The high survival rate is due to the predominance of endometrioid histological type (80%), correspondent to anatomoclinical type 1 endometrial carcinoma, which generally has a favorable evolution [Genestie et al., 2017]. However, survival rates are variable among International Federation of Gynecologists and Obstetricians (FIGO – Fédération Internationale de Gynécologie et d'Obstétrique) stages [Ballester et al., 2017], defined by the depth of myometrial invasion, cervical stromal invasion, loco-regional spread, regional lymph node metastasis, involvement of adjacent organs and distant metastases [Lewin, 2011]. In FIGO stage I tumors (limited to the uterine body), five-year survival rates can vary between 92% and 42% [Ballester et al., 2017], demonstrating the heterogeneity of this neoplasia and suggesting that there may be other factors affecting the tumor's biological behavior.

1.4.2. EPITHELIAL–MESENCHYMAL TRANSITION IN ENDOMETRIAL CARCINOMA

Epithelial–mesenchymal transition (EMT) is a key process in embryo development, which is reactivated during neoplastic progression, having a crucial role in tumor invasion and metastasis [Weinberg, 2008]. EMT refers to the switch from an epithelial cell phenotype

to a mesenchymal one, by losing cell polarity, changing the cell's shape to fusiform and acquiring motility. These features reflect in a change of cell markers expression, respectively, loss of apical and basolateral junction proteins (E-cadherin), simultaneously with the expression of mesenchymal markers, such as smooth muscle actin (SMA), vimentin, fibronectin, and the increase in matrix metalloproteinases (MMP2, MMP3 and MMP9) activity.

A complex interaction between various microenvironment components such as hormones, hypoxia, tumor-infiltrating immune cells, cytokines, and growth factors are responsible for the EMT induction [Bilyk et al., 2017]. EMT is induced by various extracellular impulses, that activate signaling pathways common with carcinogenesis pathways, and it is regulated by transcription factors, such as TWIST, SNAIL, SLUG, and ZEB1. A significant hallmark of EMT is the process called "cadherin switch", that assumes the progressive loss of E-cadherin expression, because of intercellular junction disassembly, and its replacement by mesenchymal-type cadherins, such as N-cadherin and cadherin-11 [Makker, Goel, 2016]. Estrogen and progesterone receptors (ER, PR) status is reversely correlated with EMT status, such that progressive loss of ER and PR is associated with EMT markers expression in high-grade endometrioid endometrial carcinoma. Concomitantly, in low-grade endometrioid endometrial carcinoma and in premalignant lesions, hormone expression is preserved, showing that EMT is a late event during carcinogenesis [Berg et al., 2015]. EMT mediated tumor progression and invasion is associated with activation of Ras/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase (MEK)/ mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway in lowgrade endometrioid endometrial carcinoma, demonstrated by a high expression of phosphorylated ERK (p-ERK) and EMT regulatory factors [SLUG, zinc finger E-boxbinding homeobox 1 (ZEB1), at high-mobility group AThook 2 (HMGA2)] at the myoinvasive front [Montserrat et al., 2012].

It was proven that EMT is reversible during carcinogenesis, so that the inverse process, of mesenchymal–epithelial transition (MET) is essential for the growth of distant metastasis. MET could be triggered by the absence of EMT inducing-signals, but also by additional signals from the metastatic niche. More studies could further elucidate the molecular regulators of MET [Tsai, Yang, 2013].

Metabolic processes are also involved in EMT regulation. In this regard, it is considered that enolase (ENO1), a glycolytic pathway enzyme, participates in carcinogenesis, as aerobic glycolysis and increased glucose uptake represent main features of fast-growing cells [Altenberg, Greulich, 2004]. The mechanisms of suppressive ENO1 activity in EC implicate the upregulation of E-cadherin levels, the downregulation of PI3K/Akt pathway proteins, as well as decrease of N-cadherin expression and SNAIL [Zhao et al., 2015]. Thus, it is assumed that ENO1 could work as an oncogene in EC, through activating the PI3K/Akt pathway and initiating EMT signaling cascades [Bilyk et al., 2017].

Moreover, it was shown that MiRNAs are upregulated frequently in many cancers, including endometrial carcinoma, functioning as tumor suppressor genes or oncogenes [Castilla et al., 2011; Li et al., 2015; Bilyk et al., 2017].

Recent research has identified a particular type of stem cells, which has been considered as responsible for invasion, metastasis, and the development of resistance to conventional therapy, called cancer stem cells (CSCs) [Park et al., 2017], having its counterpart in endometrium. Moreover, tumor milieu has reciprocal interactions with malignant cells. Therefore, stromal matrix is inducing CSCs proliferation, while a specific epithelial cells phenotype induces EMT, followed by invasion, metastasis, along with hormonal, chemo-, and radiotherapy resistance acquisition in different tumors, including endometrial carcinoma [Park et al., 2017].

Several markers have emerged as useful for identification of CSCs, such as CD133 (human prominin-1), CD44, Nanog1, Sal-like protein 4 (Sall4) [Park et al., 2017], along with CXC motif chemokine receptor 4 (CXCR4), c-Myc, sex determining region Y-box 2 (Sox-2), octamer-binding transcription factor 4A (Oct4A), ATP-binding cassette subfamily G member 2 (ABCG2), B lymphoma Mo-MLV insertion region 1 homolog (BMI1), cytokeratin (CK) 18, Nestin, and β-actin [Sun et al., 2017]. Moreover, CD133, CD44, Sall4, CXCR4 may be associated with a higher aggressiveness of endometrial cancers [Sun et al., 2017]. The downregulation of hormone receptors expression in endometrial cancers may be significant for invasion and metastasis and, added to the expression of CSCs markers and loss of E-cadherin expression, led to the hypothesis that CSCs possess the capability of EMT [Thiery, 2002]. However, if the markers of EMT are permanently expressed, a correlation with the development of carcinosarcomas has been demonstrated [Mirantes et al., 2013].

1.4.3. NEW MOLECULAR SUBTYPES OF ENDOMETRIAL CARCINOMA

Early prompt diagnosis and introduction of new-targeted therapies assume a better understanding of molecular and cellular EC pathogenesis, through the identification of various gene expression, genetic mutations, or apoptosis down-regulation [Stavropoulous et al., 2020].

The main change related to endometrial carcinoma is the introduction of a molecular classification according to The Cancer Genome Atlas (TCGA), which includes four molecular subtypes - POLEmut, MMRd, p53abn and non-specific molecular profile (NSMP), identified by genomic architecture (ultramutate, hypermutated, high copy number, low copy number) [Cancer Genome Atlas Research Network et al., 2013; Kim et al, 2020; McCluggage et al., 2022].

This molecular subclassification of endometrial carcinoma is more reproducible than the diagnosis of the histotype, and can be certified on the biopsy specimen at the time of initial diagnosis, thus providing additional data for the therapeutic and prognostic plan, as well as predictions of the therapeutic response.

Molecular groups are heterogeneous compared to histotypes, with the largest variation being observed in p53abn endometrial carcinomas [McCluggage et al., 2022].

This molecular classification is particularly useful in assessing the prognosis of high grade endometrioid carcinoma (grade 3), but may be applied to other histotypes, as these new four molecular classes want to replace the morphological subtypes of endometrioid carcinoma (Table 1.20).

1. ***POLEmut*** represents a pathogenic mutation in the gene encoding the exonuclease domain of epsilon DNA polymerase (POLE), involved in DNA replication and repair [Shevelev, Hübscher, 2002; Church et al., 2013; Henninger, Pursell, 2014; Rayner et al., 2016]. These are one of the most common somatic mutations in solid tumors. POLEmut endometrial carcinomas (EC) are the most common endometrioid histotype and may have intratumoral morphological heterogeneity and ambiguous morphology, with aspects of endometrioid and serous carcinoma. An intratumoral lymphoid infiltrate is frequently seen. Patients with EC POLEmut are relatively young with a normal body mass index (BMI). The prognosis of these tumors is favorable, despite the high-risk pathologies frequently encountered, such as high tumor grade and extensive lymphovascular invasion (LVSI) [Church et al., 2015; McAlpine et al., 2021]. Clinical trials are underway to simplify the treatment of these patients by excluding chemotherapy or adjuvant radiotherapy [McCluggage et al., 2022].

2. ***MMRd*** – these tumors have a high mutational load due to loss of function of one or more mismatch repair proteins (MMRs) (MLH1, PMS2, MSH2, MSH6). Epigenetic extinction / attenuation of MLH1 is responsible for most tumors in this subgroup. These ECs occur in patients of various ages (younger for patients with Lynch syndrome than in sporadic cases) and are not associated with elevated BMI. They have predominantly high grade endometrioid morphology. The prognosis of CE MMRd associated with Lynch syndrome is more favorable than in sporadic cases.

For this type of tumors, there is FDA approval for PDL1 inhibitors, in recurrent or advanced disease, when there are no other treatment options [McConechy et al., 2015; Le et al., 2015; Vernij et al., 2020; Post et al., 2021].

3. ***NSMP*** - these no specific molecular profile ECs are generally genomically stable, with low levels of alteration in the copy number [Cancer Genome Atlas Research Network et al., 2013; Proctor et al., 2017] and are, by definition, MMR-competent, wild-type POLE with wild-type pattern p53 immunoreactivity or sequencing. This group includes especially endometrioid tumors with elevated levels of ER and PR expression. Mutations in exon 3 of CTNNB1 are associated with a poor prognosis of EC NSMP [Kurnit et al., 2017]. This molecular subtype is associated with an increased BMI [McCluggage et al., 2022].

4. ***p53abn*** - ECs in this group have elevated levels of somatic copies alterations, similar to tubo-ovarian HGSC, but in contrast, where the histotype correlates with molecular pathology, the EC subtype does not correlate very well with molecular pathology, especially in terms of genomic architecture. Similar to tubo-ovarian HGSC, these ECs show p53 mutant-type immunostaining / p53 mutations, but in contrast, some of the p53abn ECs have recurrent mutations in PIK3CA, FBXW7, and PPP2R1A [Cancer Genome Atlas Research Network et al., 2013; Plotkin et al., 2020]. It is unlikely to have BRCA1 or BRCA2 mutations compared to tubo-ovarian HGSC [de Jonge et al., 2019; Ashley et al., 2019]. HER2 amplification occurs in approximately 20% of tumors, with others showing homologous recombination deficiency [McCluggage et al., 2022].

Most serous carcinomas and carcinosarcomas belong to this molecular subcategory, with less frequency for mixed histology EC, high-grade endometrioid carcinomas, and clear cell carcinomas. Although it represents only 15% of the EC, this molecular subtype has the highest mortality (50-70%) [Talhouk et al., 2015; Stelloo et al., 2016; Talhouk et al., 2017; Kommoss et al., 2018]. Patients are older without a high BMI. PORTEC 3 clinical trials suggest that patients with EC p53abn perform better when receiving radiotherapy-associated chemotherapy than those undergoing radiotherapy alone [Leon-Castillo et al., 2020]. These patients may also benefit from therapy aimed at enhancing HER2, polymerase inhibition (ADPribose) or immunomodulators [McCluggage et al., 2022].

Table 1.20. New molecular subtypes of EC, histological types correlation, and characteristic features

TCGA MOLECULAR CLASSES	MAIN EC HISTOLOGICAL TYPES	MAIN FEATURES
POLEmut	Endometrioid, low-grade ↑ Endometrioid, high-grade ↓	• POLE mutation • young women • normal BMI • favorable prognosis
MMRd	Endometrioid, high-grade	• MMR (MLH1, PMS2, MSH2, MSH6) mutations • associated with Lynch syndrome/sporadic • normal BMI • younger women, favorable prognosis (associated with Lynch syndrome)
NSMP	Endometrioid with high ER, PR expression	• MMR-competent • wild-type POLE with wild-type pattern p53 immunoreactivity/sequencing • exon 3 CTNNB1 mutations (poor prognosis) • increased BMI

TCGA MOLECULAR CLASSES	MAIN EC HISTOLOGICAL TYPES	MAIN FEATURES
P53abn	Serous ↑ Carcinosarcomas ↑ Mixed ↓ Endometrioid, high-grade ↓ Clear cell ↓	• p53 mutant-type immunostaining / p53 mutations • recurrent mutations in PIK3CA, FBXW7, and PPP2R1A • HER2 amplification • poor prognosis • older women • high BMI

TCGA – The Cancer Genome Atlas; EC- endometrial carcinoma; POLE - exonuclease domain of DNA polymerase epsilon; BMI – body mass index; MMR – mismatch repair; NSMP – no specific molecular profile; CTNNB1 - catenin Beta 1; p53 – tumoral protein 53; PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; FBXW7 - F-Box and WD repeat domain containing 7; PP2R1A - protein phosphatase 2 scaffold subunit alpha; HER2 - receptor tyrosine-protein kinase erbB-2.

1.4.4. PROGNOSTIC PARAMETERS ASSOCIATED WITH POOR OUTCOME

Prognostic factors proven to have an impact on evolution and tumor recurrence are age, histological type and grade, depth of myometrial invasion (MI), lymphovascular tumor emboli, tumor size (>2 cm), lower uterine segment/surface cervical glandular tumor involvement, and metastasis in pelvic and lumbo-aortic lymph nodes (LN) [Ballester et al., 2017]. Among the most important is regional lymph node involvement, which represents an indication for adjuvant therapy [Lewin, 2011; Han et al., 2014; Benedetti et al., 2014; Lakhwani et al., 2019].

Although the TCGA molecular classification of EC contributes, besides histological grade and type, to the completion of tumor prognostic information, these new subtypes can not replaced the other clinicopathologic risk factors (Talhouk et al., 2015; Stelloo et al., 2016; Talhouk et al., 2017; Singh et al., 2018].

In this regard, several parameters, unrelated to tumor stage, must be rigorously reported, as they provide useful data for adjuvant therapy assessment in EC. [Singh et al., 2018].

Depth of MI represents an autonomous predictor of lymph node metastasis and overall prognosis in endometrial carcinoma that is why it is included in the FIGO staging system [Singh et al., 2018]. However, the existence of MELF or other aggressive myometrial invasion pattern should be reported, because it is associated with a possible lymphovascular space invasion (LVSI), despite the fact that, alone, it is not a definite factor of poor prognosis [Singh et al., 2018].

Signaling cervical stromal invasion is mandatory in EC, as this parameter upstages the tumor and these patients have a worse prognosis, recommending them adjuvant treatment [Colombo et al., 2016].

Another factor whose presence raises the EC stage is represented by adnexal involvement. In this regard, the histological aspects must be carefully evaluated, because endometrial carcinomas with adnexal involvement have a higher stage and a more severe evolution than synchronous tumors developed in the endometrium and ovaries or fallopian tubes, which show an indolent outcome [Connell et al., 1999].

Parametrial invasion is another parameter whose presence needs to be certified, as it also upstages the endometrial carcinoma [Singh et al., 2018].

Also unrelated to tumor stage, tumor size is another prognostic factor, which should be specified in the histopathological report, based on macroscopic and/or microscopic evaluation, in the case of small tumors [Singh et al., 2018].

The pathology report must also specify if LVSI is present or absent, being recommended to include the sites of lymphovascular invasion. However, the prognostic factor is represented

by LVSI extent, as more numerous myometrial vessels, located at a certain distance from the tumor invasive front are compatible with lymph node metastasis and survival [Kuroki et al., 2003], while rae vessels interested by LVSI at the invasive front have an uncertain prognostic value [Singh et al., 2018].

Although the presence of LN metastasis larger than 0.2 mm should be registered, as it upstages the endometrial carcinoma, the value of the pattern or the size of LN metastatic deposits in EC prognosis is limited reported [Singh et al., 2018].

1.4.5. PATTERNS OF MYOMETRIAL INVASION CORRELATED WITH AGGRESSIVE BEHAVIOR IN ENDOMETRIOID ENDOMETRIAL CARCINOMA

Recent evidence highlights the pattern of myoinvasion in low-grade, low-stage endometrioid endometrial carcinoma (EEC), as a possible predictor for tumor evolution [Quick et al., 2012].

There have been described five myoinvasive patterns, respectively diffusely infiltrating, broad front, adenomyosis (AM)-like, microcystic, elongated, and fragmented (MELF) glands, single-cell invasion (SCI), and adenoma malignum [Cole, Quick, 2013], each having morphological and prognostic particularities. Also, a new pattern of myoinvasion has been identified, respectively the nodular fasciitis-like stroma and large cystic growth pattern (phyllodes tumor-like), adding to the diagnostic challenge [Švajdler et al., 2016; Mateva et al., 2021].

Frequently, more than one pattern of myoinvasion may coexist, with a predominant type which associates a minor component of other pattern. In these cases, the prognosis is given by the most aggressive pattern [Quick et al., 2012; Longacre, Hendrickson, 1999].

In the following sections, we will discuss only the patterns of myoinvasion in endometrial endometrioid carcinoma, aiming to emphasize the importance of their recognition, in relation to the existing proof on their poor prognostic significance, on the controversial tumor behavior, or to unevaluated significance, due to their rarity (Table 1.21).

Table 1.21. Myoinvasive pattern associated with aggressive behavior EEC

MYOINVASIVE PATTERN	HISTOLOGICAL FEATURES	EEC PROGNOSIS ASSOCIATION
Diffusely infiltrating	• scattered individually or small groups (<3) of neoplastic glands within the myometrium, with irregular contours • desmoplastic stromal response +/-	Poor prognosis
MELF	• cystic-dilated/slit-like glands, lined by flattened, endothelial-like epithelium • squamoid tumor cells with eosinophilic cytoplasm, often with intraluminal tufts or fragmented • small groups or isolated tumor cells • lymphocytic or fibromyxoid stromal reaction	Poor prognosis
SCI	• isolated or cell groups with eosinophilic cytoplasm, without a precise structure • myxoid/edematous stroma	Poor prognosis

MYOINVASIVE PATTERN	HISTOLOGICAL FEATURES	EEC PROGNOSIS ASSOCIATION
Adenoma malignum	• glands lined by mucinous epithelium, with clear pale eosinophilic cells, with pleomorphic nuclei, and mitotic figures • dilated, fused or cribriform dilated glands • various amount of desmoplastic stroma	Controversy
Nodular fasciitis-like stroma and large cystic growth	• elongated/slit-like/cystic neoplastic glands, lined by flattened cells, with variable degrees of squamous differentiation • vaguely nodular and fibromyxoid stroma, with variable cellularity, without nuclear atypia, pleomorphism or mitotic activity and collagen deposition (similar to nodular fasciitis)	Unevaluated (rare new entity)

EEC – endometrial endometrioid carcinoma; MELF - microcystic, elongated, and fragmented; SCI – single-cell invasion

Diffusely infiltrating

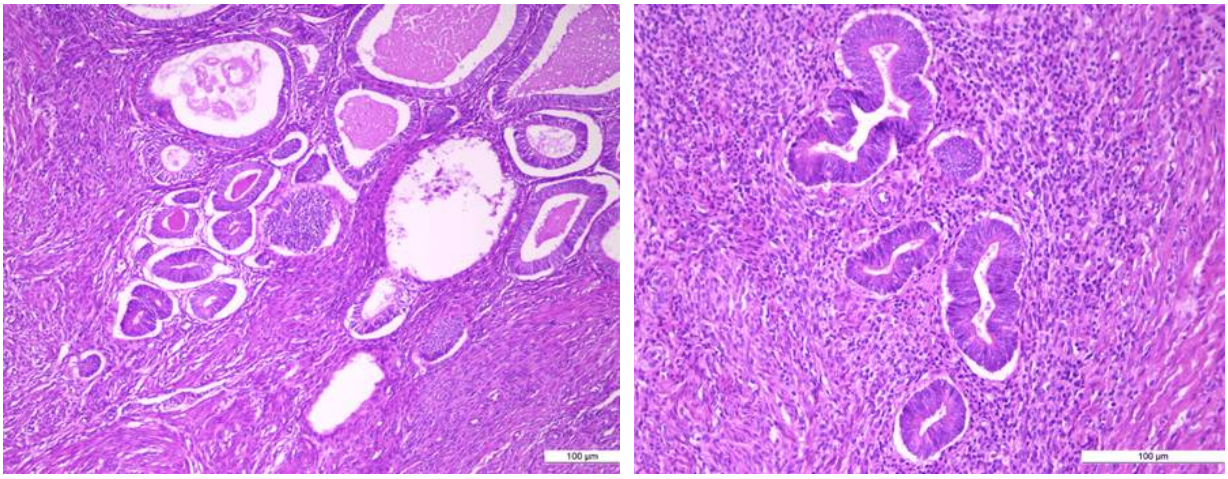
The diffusely infiltrating or single gland pattern [Cole, Quick, 2013] is the most common morphological aspect at the myoinvasive front in endometrioid endometrial carcinoma, observed with a frequency of 49–89% [Ali et al., 2007; Quick et al., 2012; Park et al., 2017]. It is defined as scattered neoplastic glands within the myometrium, arranged individually or in small groups (less than three), having irregular contours and possibly, but not necessarily accompanied by desmoplastic stromal response [Quick et al., 2012] (Fig. 1.27 – a, b).

The infiltrative pattern is indicative of a poor prognosis, since it was associated with a higher FIGO grade, lymphovascular invasion and tumor recurrence [Suzuki et al., 2003; Quick et al., 2012]. These findings could be justified by the molecular alterations identified in these areas. Respectively, in these zones, it was shown a high expression of cancer stem cell markers (CD44, CD133) and the loss of hormone receptors (ER, PR) [Park et al., 2017]. Both of these changes in the molecular phenotype contribute to the induction of EMT (marked by the loss of E-cadherin and the aberrant expression of β-catenin), a phenomenon highly involved in tumor invasion and metastasis [Park et al., 2017].

This pattern of invasion was observed in many other types of malignancies. Its direct association with EMT markers and poor prognosis was so far proven in squamous cell carcinoma of the head and neck [Basu et al., 2013], of the vulva [Holthoff et al., 2016], in endocervical adenocarcinoma [Stewart et al., 2011] and urothelial carcinoma of the bladder [Otto et al., 2017], serving as an evidence of how molecular changes associated with tumor progression imprint on the tumor's histology, including in endometrial carcinomas.

Additionally, another issue, which arises in practice, is represented by the difficulty of delimitation between the invasion of the basal endometrium and the superficial myometrium invasion in endometrioid endometrial tumors.

However, it is considered that there is no difference of survival rates between superficial invasive endometrial carcinoma and that which invades less than 1/2 of the myometrium thickness (90% versus 91%) [Creasman et al., 2003].



a b

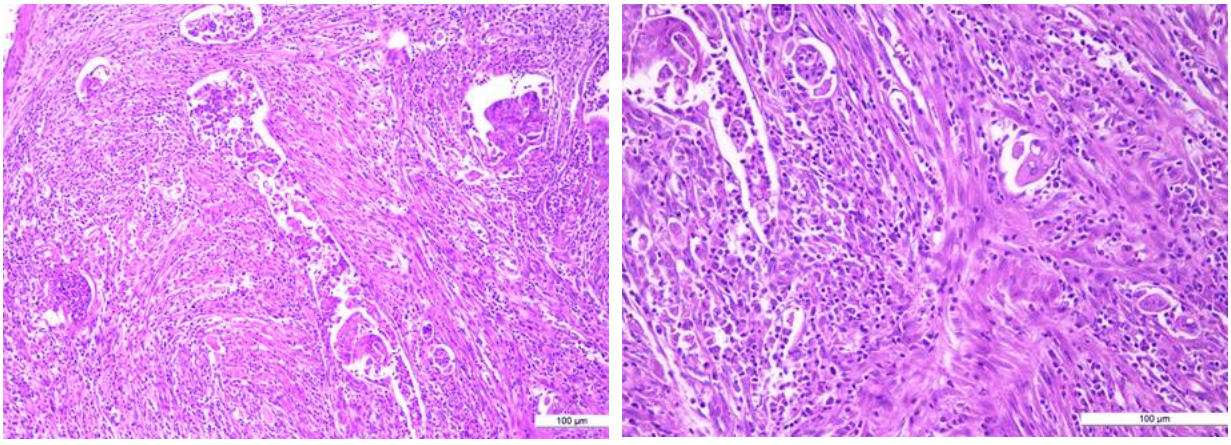
Figure 1.27. Diffusely infiltrating pattern of myoinvasion in endometrioid endometrial carcinoma. H&E: (a) x100; (b) x200.

Microcystic, elongated, and fragmented (MELF) glands

MELF pattern of invasion is reported with variable frequencies, ranging between 7% and 48% [Ali et al., 2007; Stewart, Little, 2009; Hertel et al., 2011; Pavlakis et al., 2011; Quick et al., 2012; Euscher et al., 2013; Dogan Altunpulluk et al., 2015; Park et al., 2017; Joehlin-Price et al., 2017; Kihara et al., 2017]. It was first defined by Murray et al., in a study focusing on epithelial and stromal alterations in myoinvasive endometrioid endometrial carcinoma [Murray et al., 2003].

Two types of stromal reaction were identified, respectively lymphocytic and fibromyxoid, the latter being associated with a distinctive type of invasive glands. The histological appearance of these glands, as cystic-dilated or slit-like, lined by flattened, endothelial-like epithelium or squamoid tumor cells, with eosinophilic cytoplasm, often with intraluminal tufts or fragmented, alongside with small groups or isolated tumor cells, led to their denomination as "microcystic, elongated and fragmented glands" (Fig. 1.28 – a, b).

Frequently, these glands show a dense neutrophilic infiltrate in their lumen and are situated deepest in the myometrium [Murray et al., 2003]. Their subtle appearance can create difficulties in assessing the depth of myoinvasion, but the associated fibromyxoid surrounding stroma and neutrophilic infiltrate, visible at low power can be helpful in their identification [Cole, Quick, 2013] (Fig. 1.28 – a, b). Given their histological aspect, these areas were initially thought to represent degenerative changes of tumor glands [Murray et al., 2003], but later studies suggested that they rather represent areas of intense tumor activity.



a b

Figure 1.28. MELF (Microcystic, elongated and fragmented) pattern of myoinvasion in endometrioid endometrial carcinoma. H&E: (a) x100; (b) x200.

The resemblance between MELF pattern of invasion and areas of EMT identified at advancing margins of other neoplasia, such as budding glands in colorectal cancer [Dogan Altunpulluk et al., 2015] led to the investigation of this phenomenon in the MELF-type glands. Several studies have shown a distinct immunophenotype of these glands, compared to those with conventional morphology. Intense expression of CK7 [Stewart, Little, 2009], CK19 [Stewart et al., 2011] and molecular markers of EMT, such as reduced expression of E-cadherin, aberrant expression of β-catenin, loss of hormone receptors, overexpression of cyclin D1, p16, galectin-3 and fascin and low Ki-67 reactivity were detected [Stewart, Little, 2009; Stewart et al., 2010; Stewart, Crook, 2010; Zaino, 2014]. Furthermore, a high expression of stem cell markers (CD44, CD133, Nanog1, Sall4), known to have the ability to induce EMT, was shown in MELF glands [Park et al., 2017]. MELF together with infiltrating pattern are associated with hormone receptors loss due to EMT induction and tumor progression [Hanekamp et al., 2002; Dai et al., 2002; Hanekamp et al., 2005; Stewart, Little, 2009; Wik et al., 2013]. Furthermore, the expression of E-cadherin is lost in MELF tumors, in association with an aberrant β-catenin expression, downregulation of ER and PR expressions, along with lymphovascular invasion and, consequently, poor prognosis [Murray et al., 2003].

The molecular changes identified in these areas reflect in clinicopathological parameters with prognostic significance in endometrial carcinoma. MELF pattern is most frequently found in low-grade endometrial endometrioid carcinoma, in association with a high depth of myometrial invasion, lymphovascular invasion, lymph node metastases, and a high FIGO grade [Dogan Altunpulluk et al., 2015].

The association between MELF pattern of invasion, lymphovascular invasion and lymph node metastases was proven by several studies [Hertel et al., 2011; Pavlakis et al., 2011; Euscher et al., 2013; Han et al., 2014; Dogan Altunpulluk et al., 2015; Park et al., 2017; Quick et al., 2012; Pavlakis et al., 2017; Sanci et al., 2018]. Recently, Joehlin-Price et al. have further confirmed this correlation by standardizing their study group, which included only FIGO I endometrial endometrioid carcinoma, eliminating other variables that could favor lymph node metastases.

They defined three morphological types of lymph node metastases, respectively sinusal, histiocytelike, solid and glandular and claimed that, in contrast to breast carcinoma, isolated tumor cells and micrometastases are not proven clinically relevant in endometrioid carcinoma [Joehlin-Price et al., 2017], even though they have been recently reported in literature [Han et al., 2014; Pelletier et al., 2018].

The high probability of lymph node metastases in endometrioid endometrial carcinoma with MELF pattern can lead to a better therapeutic management in low-grade tumors, where lymphadenectomy indication remains controversial. Given the favorable evolution in most cases of low-grade endometrial carcinoma, lymphadenectomy is generally avoided because of its possible complications (lower limb lymphedema, vascular or nerve injury, symptomatic lymphocysts, and chylous ascites) [Han et al., 2014; Zhu et al., 2014].

In this context, identification of MELF pattern could represent an indication for subsequent lymphadenectomy. Furthermore, sentinel lymph node mapping techniques are beginning to be recommended in endometrial cancer [Holloway et al., 2016]. Some researchers regard MELF pattern as a specialized variant of the infiltrative pattern of invasion, given their frequent concomitancy and the similar molecular changes
regarding EMT and stem cell phenotype [Park et al., 2017]. The pattern of invasive front may be significant for a poor prognosis, in MELF and infiltrating pattern, along with lymphovascular invasion, lymph node metastasis, and CSCs markers expression [Park et al., 2017], and may select a group of patients requiring an aggressive therapy [Park et al., 2017].

In this view, MELF type glands could appear because of stromal alterations and inflammation [Quick et al., 2012]. MELF pattern has also been associated with papillary architecture and mucinous differentiation [Kihara et al., 2017]. Even though it is highly

associated with lymphovascular spread and extravaginal recurrences [Moschiano et al., 2014; Roma et al., 2015], MELF pattern was correlated with an enhanced overall survival [Sanci et al., 2018]. In this perspective, Kihara et al. argue that MELF pattern could have any implications on prognosis, proving a high expression of cellular growth arrest and senescence markers (p16, p21), together with low Ki-67 expression in these areas. Also, in their study, no statistically significant association between MELF pattern and recurrence-free survival or disease-specific survival was found [Kihara et al., 2017]. These researchers question the causal relation between MELF pattern and lymph node metastases, which, in their opinion, can be determined by other associated factors such as tumor size, myometrial invasion, and lymphovascular invasion. In this view, MELF glands could only represent a morphological change in senescent glands, having no impact on prognosis [Kihara et al., 2017]. However, decreased cellular division has been demonstrated during tumor progression and local invasion in other neoplasia, especially during EMT [Stewart et al., 2010]. This observation could continue to support the theory of MELF being a morphological manifestation of EMF at the advancing margins. Further studies are needed to elucidate the clinicopathological implications of this pattern. Recently, new molecular markers associated to MELF pattern have been identified. High expression of S100A4 was correlated to myometrial and lymphatic invasion, demonstrating an aggressive phenotype, with strong and diffuse staining in MELF type glands [Tahara et al., 2016]. Also, increased microvessel density in the neoplastic stroma and high expression of vascular endothelial growth factor (VEGF) in tumor cells, correlated with the presence of MELF pattern, could be predictors of unfavorable outcome [Zinovkin et al., 2017]. In cases where the criteria for MELF diagnosis are incomplete, it is helpful to consider this pattern when at least two features are accomplished.

A practical application of the identification of this type of myoinvasion emerges from the large spectrum of histological features characteristic for MELF, which create heterogeneous tumoral areas. These areas raise the issue of the differential diagnosis with other types of invasion or with other types of tumors.

Moreover, the effect of autolysis should be eliminated when evaluating MELF pattern and an optimum preparation of specimens for grossing should be mandatory.

MELF-type features have also been observed in other cancers. In intraductal papillary mucinous neoplasm of the pancreas, they were frequently detected in association with high-grade dysplasia, deleted in pancreatic cancer 4 (DPC4) loss, and p53 overexpression, possibly suggesting stromal invasion [Park et al., 2018]. In ovarian endometrioid carcinoma, MELF pattern was seen with similar frequencies as in uterine endometrial carcinoma, but without impact on prognostic features. However, it was associated with clear cell features and mismatch repair protein loss, hence it could be an indicator for surgical staging and Lynch syndrome screening [Goldberg et al., 2018].

Due to the proven poor prognosis in these types of tumors, the histopathological report should contain a special mention about the possible identification of MELF myoinvasive pattern. Clinicians should be aware of MELF significance as a more aggressive tumor subtype, possible in association with other factors, in order to adjust the therapy to an appropriate approach, by different therapeutic means association.

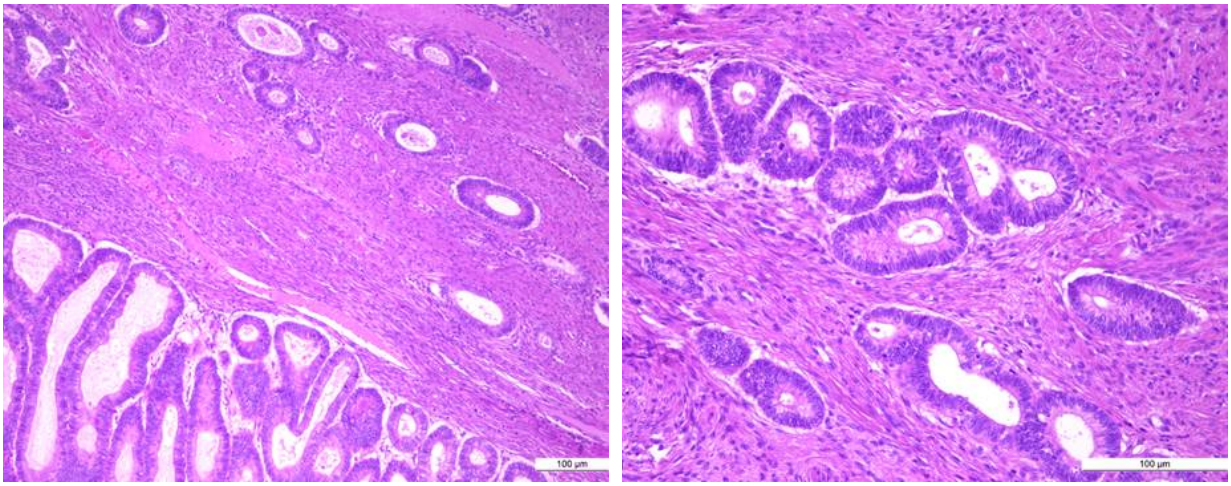
Single-cell invasion (SCI)

Previously considered as part of MELF spectrum [Stewart et al., 2009; Pavlakis et al., 2011; Quick et al., 2012; Cole, Quick, 2013], single-cell invasion (SCI) is now considered a distinct myometrial invasion pattern, being associated in 17% of endometrial endometrioid carcinomas with MELF [Euscher et al., 2013]. This finding led to the presumption that SCI represent a more aggressive type of MELF [Euscher et al., 2013]. The morphological features of SCI consist of isolated or cell groups with eosinophilic cytoplasm, without a precise

structure, usually situated in a myxoid or edematous stroma. Regardless of its association with MELF spectrum, SCI should raise the suspicion of lymph node metastases, its presence increasing the risk of advanced disease [Euscher et al., 2013; Mateva et al., 2021].

Adenoma malignum (minimal deviation endometrial adenocarcinoma)

Adenoma malignum or minimal deviation adenocarcinoma is typically described as an extremely well differentiated variant of gastric type adenocarcinoma of the uterine cervix. This type of cervical neoplasia is unrelated to human papilloma virus (HPV) infection. Its histogenesis resembles more to gastric carcinogenesis, possibly with lobular endocervical glandular hyperplasia, as its precursor lesion. Histologically, it is composed of glands with mucinous epithelium, with cells having abundant, clear pale eosinophilic cytoplasm, pleomorphic nuclei, and mitotic figures. The glands show an irregular outline, are dilated, fused or cribriform, disposed within a various amount of desmoplastic stroma [Kurman et al., 2014]. Adenoma malignum consists in regular, round, often widely spaced glands, with minimal nuclear atypia, lacking a desmoplastic or inflammatory stromal response [Quick et al., 2012; Renu et al., 2016]. It has rarely been described at the invasive front of endometrial carcinoma, as a distinct pattern of myoinvasion, with frequencies of 1% [Joehlin-Price et al., 2013] or 1.33% [Quick et al., 2012] (Fig. 1.29 – a, b).



a **b**

Figure 1.29. Adenoma malignum pattern of myoinvasion in endometrioid endometrial carcinoma. H&E: (a) x100; (b) x200.

In endometrial carcinoma, adenoma malignum pattern brings difficulties in assessing the tumor, grossly and microscopic. The diffuse, infiltrative spread of the glands, without an associated stromal reaction, can be misleading when appreciating the depth of myoinvasion [Kalyanasundaram et al., 2010], also causing an unobvious gross appearance, as a thickened, firm, grey-white myometrium [Renu et al., 2016] that can render the neoplasia undetectable at high-precision imagistic examinations [Nakao et al., 2014]. The complexity of the diagnosis increases, as this pattern of invasion can extend beyond the uterine body, with a frequent involvement of the cervix, or possibly, of the ovaries. Mainly, adenoma malignum areas must be distinguished from adenomyosis, synchronous adenocarcinoma of the cervix, deeply located endocervical glands, mesonephric remnants, cervical tubo-endometrioid metaplasia, cervical or ovarian endometriosis or cortical inclusion cysts of the ovary.

Adenomyosis can be excluded in the absence of endometrial stroma, the presence of mitotic activity, identification of carcinoma in the surface endometrium, with possible association of conventional invasion areas, and the absence of AM foci elsewhere in the myometrium. In

case of cervical involvement, synchronous adenocarcinoma of the cervix can be excluded in the presence of benign surface endocervical glands and also by distinctive IHC profile.

Deeply located endocervical glands can be ruled out given the location in the uterine body and the absence of mucinous overlying epithelium, respectively. The lack of a lobular architecture and the presence of mitotic activity help distinguishing adenoma malignum from mesonephric remnants. Tubo-endometrioid metaplasia and endometriosis can be excluded by the absence of endometrial stroma and ciliated cells, or hemosiderin-laden macrophages.

Ovarian cortical inclusion cysts can be ruled out in the absence of ciliated cells and the presence of minimal cellular atypia and mitotic activity [Kalyanasundaram et al., 2010]. The frequent association of uterine corpus adenoma malignum with endometrioid endometrial carcinoma [Kalyanasundaram et al., 2010], as well as the IHC profile supports its histogenesis from endometrial carcinoma, as a differentiated variant [Landry et al., 2003]. Uterine corpus adenoma malignum areas are positive for ER, PR, CK7, vimentin and negative for CK20, carcinoembryonic antigen (CEA) and p16. Immunohistochemistry is useful in cases of cervical involvement, for excluding a synchronous cervical adenocarcinoma, which is positive for CEA and p16 and negative for vimentin and ER [Landry et al., 2003; Kalyanasundaram et al., 2010].

Even though in the cervix, adenoma malignum is associated with a dismal prognosis [Kurman et al., 2014], a study showed that endometrial carcinomas with this pattern of invasion do not have a worse evolution compared to those with a conventional tumor front. Similar recurrence and survival rates were detected in both categories [Longacre TA, Hendrickson, 1999]. This observation is supported by Quick et al., which report a favorable evolution of one patient with adenoma malignum pattern of invasion in their study group [Quick et al., 2012]. Although there are studies which consider that endometrial carcinoma with this myometrial invasion pattern do not present a worse prognosis, overall, adenoma malignum suggest an increased aggressive behavior, compared with ECs with conventional tumor front [Longacre, Hendrickson, 1999; Quick et al., 2012; Mateva et al., 2021].

However, a small number of cases of mucinous, gastrictype adenocarcinoma of the uterine corpus, associated with chemoresistance and poor prognosis have been reported [Abiko et al., 2010; Hino et al., 2016]. They were often found in combination with endometrioid carcinoma and endometrial hyperplasia. These tumors are composed of cells with pale eosinophilic cytoplasm and well-defined borders, suggestive of gastric differentiation. Immunohistochemistry confirmed the histological appearance, showing positivity for markers of pyloric glands mucin (HIK1083 and/or MUC6), partial positivity for p53, and negativity for p16 [Hino et al., 2016]. These findings suggest that this neoplasia could arise on areas of gastric metaplasia [Abiko et al., 2010]. As opposed to the extremely well differentiated variant, respectively, adenoma malignum, these tumors showed desmoplastic stroma and presented grossly as well defined tumor masses within the myometrium [Abiko et al., 2010; Hino et al., 2016].

The similar morphological features of adenoma malignum in both cervix and endometrium may suggest possible common cancer stem cells for both locations, along with possible epigenetic factors acting in female genital tract. Adenoma malignum-like features were exceptionally described in other locations, such as in gallbladder adenocarcinoma [Tashiro et al., 2000] and urinary bladder adenocarcinoma [Kanomata, Muramaki, 2013], creating diagnostic difficulties, as they had to be distinguished from other benign conditions, such as Rokitansky–Aschoff sinus and adenomyomatosis of the gallbladder, or Müllerianosis of the urinary bladder, respectively.

Nodular fasciitis-like stroma and large cystic growth (phyllodes tumor-like)

Recently, Švajdler et al. have reported a new growth pattern at the invasive front of endometrioid endometrial carcinoma, in a 67-year-old woman, different from all other patterns of myoinvasion or morphological variations previously described in the literature. Its

particularities consist in stromal and glandular architecture. In these areas, the stroma was vaguely nodular, with fibromyxoid change, variable cellularity, without nuclear atypia, pleomorphism or mitotic activity and collagen deposition, similar to nodular fasciitis. Neoplastic glands were elongated, slit-like or large and cystic, lined by flattened cells, with variable degrees of squamous differentiation. Large stromal nodules, alongside interconnected glandular spaces give resemblance to the phyllodes tumor of the breast [Švajdler et al., 2016; Mateva et al., 2021].

This pattern is somewhat similar to MELF, but in the latter, there is only focal fibromyxoid change. As opposite to MELF pattern, in this case, the glands were interconnected, without fragmentations or single cells. Also, vascular invasion was not identified [Švajdler et al., 2016]. The appearance of the tumor in these areas produced diagnostic controversies, as it was considered, on a previous biopsy, a Müllerian adenofibroma, due to the benign-looking glands. This diagnostic was infirmed after examination of tissue samples following hysterectomy, in which there were found adjacent areas of well differentiated endometrioid endometrial carcinoma, with pushing border pattern of myoinvasion. Differential diagnosis of the unusual growth pattern was made with carcinosarcoma (malignant mixed Müllerian tumor), that was invalidated by IHC tests showing keratin negativity in the stromal component [Švajdler et al., 2016]. Given that it is the single reported case of such pattern of invasion in endometrial carcinoma, its prognostic value cannot be appreciated, especially since the patient died of surgical complications and therefore, a response to oncological therapy could not be assessed [Švajdler et al., 2016].

Further studies are necessary to validate this newly described pattern of myoinvasion and to provide clinicopathological correlations, mainly regarding the prognostic significance. A similar growth pattern, with exuberant nodular fasciitis-like stroma was described in a variant of papillary thyroid carcinoma, requiring differential diagnosis with fibrous thyroiditis (fibrous variant of Hashimoto's thyroiditis and Riedel's thyroiditis), solitary fibrous tumor, anaplastic carcinoma secondary to dedifferentiation of a conventional papillary thyroid carcinoma, and carcinosarcomas [Ginter, Scognamiglio, 2015].

1.4.6. FINAL REMARKS

The review of the main aggressive myoinvasive patterns in endometrioid endometrial carcinoma highlights the difference between MELF, diffusely infiltrative, single-cell invasion, and, possible, nodular fasciitis-like stroma and large cystic growth patterns as the most aggressive tumor subtypes, compared to adenoma malignum pattern, with a controversial prognosis, and with the broad front, AM-like patterns, which have a good prognosis.

Furthermore, these patterns may successfully complete the dual clinicopathological classification of the two types of endometrial tumors and may lead to new therapeutic approaches for each type. The knowledge and accurate evaluation of the multiple patterns of myoinvasion in endometrioid endometrial carcinoma has a great practical value, as demonstrated in our experience, firstly for the correct assessment of the depth of myoinvasion, one of the main criteria in staging.

Additionally, considering the results of numerous studies showing an association between patterns of myoinvasion and various features that influence the patient's evolution, we would like to highlight their possible prognostic value. The diversity of morphological aspects seen at the advancing margins of the tumor could be the result of a variable degree of molecular alterations, involved in carcinogenesis and tumor progression, explaining the higher aggressiveness of some patterns of myoinvasion.

In cases with mixed pattern of myoinvasion, a complex evaluation of the dominant pattern and the depth of myometrium invasion should be corroborated in order to obtain a good perspective about patients' prognosis.

Considering the possibility to identify specific molecular markers correlated to these patterns of myoinvasion, in correlation with EMT and cancer stem cells, future targeted therapies open promising perspectives in the treatment of endometrioid endometrial carcinomas. The identification of invasion patterns known to be associated with more aggressive tumors, even in the presence of an initial low-grade morphology and corresponding stage, could raise the idea of some similarities in the gene profile of these endometrial carcinomas type 1 with those of type 2, being known that high-grade endometrioid endometrial carcinomas share a specific gene expression profile with serous-type endometrial carcinomas [Cao et al., 2004]. In this regard, the study of the characteristic molecular expression of type 2 endometrial carcinomas occurrence in low-grade endometrioid carcinomas with aggressive myoinvasion pattern could provide new insights into endometrial carcinogenesis and could also represent valuable predictive factors.

1.5. ENDOMETRIAL STEM CELLS

1.5.1. INTRODUCTION

The uterus is a particular organ characterized by its morphological adaptation to the reproduction function. Both myometrium and endometrium are able to modify their histological structure to support the embryo development. Its implantation and nourishment is possible due to dramatic changes of endometrium and its protection and delivery is achieved by myometrium. The cyclical evolution of endometrium is governed by ovarian steroid hormones, under the modulation of neuroendocrine hypothalamo-hypophyseal system.

Considering these important functions, the endometrium is a unique and remarkable tissue characterized by a regeneration activity comparable to that of bone marrow, epidermis, and intestinal epithelium. In humans, it undergoes 400 - 500 menstrual cycles during a woman's reproductive lifetime and this high turnover has been speculative for scientists, regarding its mechanism, regulatory factors, and their significance for fertility and endometrial pathology [Bockeria et al., 2013; Mutlu et al., 2015]. Relatively recent scientific progresses due to genomics, proteomics, and transcriptomics have changed the knowledge in respect with endometrial regeneration and uterine-derived diseases. The latter seem to be based on unbalanced factors involved in proliferation regulation, being lately reflected in several WHO classifications changes, which have been traditionally based on clinicopathological features.

This distinct remodeling potential of the endometrium lead to the hypothesis of the existence of adult stem cells (ASCs) in the uterine mucosa, with corresponding cyclic activity [Prianishnikov, 1978; Teixeira et al., 2008]. Hence, alterations in this endogenous cellular population could be linked with various endometrial pathologic conditions, such as infertility [Deane et al., 2013], endometrial atrophy [Lebovitz, Orvieto, 2014], Asherman syndrome (AS) [Dreisler, Kjer, 2019], or endometriosis [Deane et al., 2013; de Miguel-Gómez et al., 2021].

Stem cells represent undifferentiated cells with a capacity for self-renewal and differentiation into multiple cell types under specific conditions [Dulak et al., 2015; Clevers, 2015], being generally classified conforming to differentiation potency and localization [de Miguel-Gómez, 2021].

In this regard, the totipotent cells are zygotes, which give rise to the whole embryo and extra-embryonic tissues, followed by pluripotent stem cells (PSCs), like induced PSCs and embryonic stem cell, which generate the all three germ layers, unipotent stem cells, with the most limited differentiation ccpacity, dividing themselves only in a single type of cell, and eventually, the multipotent stem cells, which produce specific cell lineages [Figueira et al., 2011; Zakrzewski et al., 2019; de Miguel-Gómez, 2021].

Adult or somatic or tissue stem cells [Maruyama, 2014] represent multipotent stem cells found in different organs, with the differentiation capacity toward limited mature cell lines in order to sustain homeostasis [Clevers, 2015]. The necessary conditions are provided by the specific anatomical location surrounding the ASCs. The specific microenvironment or the stem cell niche, provides necessary conditions through paracrine, autocrine, and systemic signaling, which permit the maintenance and differentiation of these stem cells into particular cell types involved in tissue regeneration and repair [Cervelló et al., 2015; Chacón-Martínez et al., 2018]. Although mostly of ASCs are located in the bone marrow, they were also found in other organs, having the same important roles in renewal, repair, and homeostasis [Gurusamy et al., 2018]. The peculiarities of these cells open up opportunities for research into the potential therapeutic approaches of different tissues such as skin [Gonzales et al., 2017], muscle [Mashinchian et al., 2018], endometrium [Cervelló et al., 2013], blood [Amouzegar et al., 2019], brain [Kelava, Lancaster, 2016], and intestine [Ayyaz et al., 2019; de Miguel-Gómez et al., 2021].

Conforming to their origin, endometrial stem cells (ESCs) can be classified as endogenous ESCs and exogenous ESCs [Kucia et al., 2005; de Miguel-Gómez et al., 2021].

The first description of endometrial ASCs was made by Gargett et al. in 2004, through recognition of colony-forming units (CFUs) or clonogenic cells in purified single-cell suspensions originating from hysterectomy specimens [Gargett et al., 2004].

Since then, there were discovered and described various types of endometrial stem/progenitor cells (ESCs). Currently, specific identification techniques define endometrial epithelial stem/progenitor cells, particular endometrium-derived mesenchymal stem cells (eMSCs), side population cells (SPs) [Gargett et al., 2016a; Bhartiya, 2016; Gargett et al., 2016b], and menstrual stem cells (MenSCs), for those derived from menstrual blood [Kong et al., 2021].

We highlight the recent progresses in classification of endometrial stem cells, addressing the current paradigm regarding endometrial regeneration, based on endometrial stem cells (ESCs) and endometrial regenerative cells (ERCs). Their unlimited potential of reconstruction of any type of tissue has been demonstrated and is currently in different trial stages in cell-based therapies opening promising perspectives in severe or lethal diseases. The paper is based on English language publications indexed in the main medical data bases, using as key words: “endometrium”, “stem cells”, “regeneration”, “mesenchymal-epithelial transition”.

1.5.2. OLD AND NEW IN ENDOMETRIAL REGENERATION THEORIES

Past theories of endometrial regeneration mechanism

Based on the endometrial zonation of primates [Kaiserman-Abramof, Padykula, 1989], there are several compartments, according to their glandular or epithelial content, as following: two basal layers (IV: base and III: middle of endometrial glands) and the two functional layers (II: upper endometrial glands and I: luminal epithelium) which have been later characterized by marked proliferation kinetics differences [Brenner et al., 2003] (Fig. 1.30).

In an attempt to explain the complex process of endometrial regeneration, two past mechanisms have been proposed during time.

The first mechanism has been initially elaborated several decades ago, based on the capacity of glandular epithelial cells to proliferate. It had been hypothesized that primate and human endometria are regenerating by epithelial cell proliferation of the upper ends of the gland stumps from basal layer [Ferenczy et al., 1979]. The endometrial regeneration has been considered a unique process in which basal layer acts as a germinal layer due to its particular vascularization and different hormonal influences when compared to the functional layer shed with each menstruation. The speculation that the “free-edge” effect initiates endometrial re-epithelialization has been launched considering that the lack of endometrial neighboring cells in the denuded area may elicit a growth signal [Heimark, Schwartz, 1985].

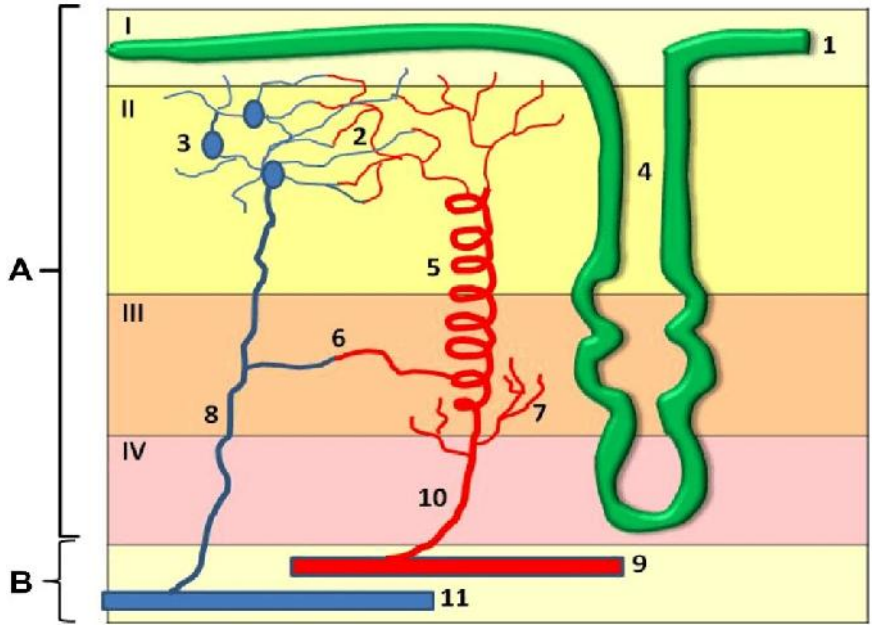


Figure 1.30. The endometrial zonation model. Based on the endometrial zonation of primates, there are four compartments: basal layer IV, containing the glandular bases, basal layer III, with the middle of the endometrial glands, functional layer II, containing the upper endo- metrial glands, and functional layer I corresponding to the luminal epithelium. A: Endometrium; B: Myo-metrium; I: Luminal epithelium; II: Upper endometrial glands; III: Middle of endometrial glands; IV: Basal endometrial glands; 1: Lining endometrial epithelium; 2: Capillary plexus; 3: Venous lake; 4: Endometrial gland; 5: Spiral artery; 6: Arteriovenous anastomosis; 7: Straight artery; 8: Vein; 9: Arcuate artery; 10: Radial branch/artery; 11: Arcuate vein.

Observing the capacity of remnant stromal cells to act under hormonal stimulus and regenerate the surface endometrium, the hypothesis of mesenchymal - epithelial transition has been proposed as a second mechanism. The mechanism of conversion from epithelial to mesenchymal phenotype is used in morphogenesis, in tissue repair [Thiery et al., 2009], and is also reinitiated during cancer invasion [Makker, Goel, 2016]. During this stepwise process, epithelial cells lose cell-cell adhesion molecules, the apical-basal polarity and achieve a more stromal-type histological phenotype [Thiery et al., 2009]. This is characterized by down-regulation of epithelial markers, such as E-cadherin, α and β-catenins, cytokeratins, and claudin, and acquisition of mesenchymal markers, such as N-cadherin, cadherin-11, along with S100A4, vimentin, fibronectin, α-smooth muscle actin (α-SMA), MMPs (MMP2, MMP3, and MMP9) expression, under the control of transcription factors like TWIST, SNAIL, SLUG, and ZEB1 [Cousins et al., 2014; Makker, Goel, 2016]. Conversely, the reverse process is represented by mesenchymal-epithelial transition (MET), as a necessary step in morphogenesis, to continue some differentiation pathways [Thiery et al., 2009], and seems to be reinitiated in tumor metastases. Therefore, malignant cells undergo EMT to invade and disseminate and then undergo a reverse process (MET) to form epithelial metastases in target organs [Makker, Goel, 2016].

The first evidences of the involvement of stromal cells in endometrial regeneration have been provided by electron microscopy [Ludwig, Spornitz, 1991; Garry et al., 2009] and later on by murine models [Patterson et al., 2013]. Thus, stromal cells are considered to be programmed to lose their mesenchymal traits and gain epithelial features, as demonstrated by co-expression of pancytokeratin with vimentin, after 24 hours of progesterone withdrawal [Patterson et al., 2013], expression of genes involved in MET, and identification of proliferative activity in both stromal and epithelial areas [Maybin, Critchley, 2015].

Furthermore, an important contribution of stromal cells from the functional layer in regeneration of extracellular matrix has been demonstrated by laser capture microdissection technique [Gaide Chevronnay, 2009].

In view of an analogy with a wound healing process, a regulated balance between MET and the reverse, EMT should be active in endometrium, in order to prevent extreme proliferation of apoptosis resistant myofibroblasts, with possible excessive production of type I collagen leading to fibrosis [Maybin, Critchley, 2015].

The endometrial regulation of the balance between these "mirror" processes is attributed to hypoxia, components of the extracellular matrix, cytokines, and growth factors [Gonzales, Medici, 2014], without an associated scarring process. Not surprisingly, stromal cells from endometriotic implants express higher levels of α-SMA when compared to stromal endometrial cells, leading to the hypothesis that alteration in the regulation of this process may result in endometriosis [Maybin, Critchley, 2015].

Recent theory of endometrial regeneration mechanism

The initial hypothesis of epithelial glandular stumps proliferation [Ferenczy et al., 1979] has been later questioned [Horne, Blithe, 2007] and further on invalidated by complex hysteroscopical – histological - electron microscopic studies that demonstrated the regeneration of surface endometrium by differentiation of stromal cells [Garry et al., 2009]. Supplementary, more and more evidences of stem cells location in endometrium, analogous to other organs counterparts, have been supporting the hypothesis of possible reconstruction of endometrium based on endometrial regenerative cells or stem cells.

The existence of human endometrial stem cells has been initially hypothesized by Prianishnikov, in 1978, who identified three types of endometrial proliferative cells, according to their correlation with steroids hormones, as follows: estradiol-sensitive, progesterone-sensitive, and estradiol- and progesterone sensitive cells [Prianishnikov, 1978]. Studies of endometrial derived colony-forming units have been later added to support this idea [Gargett et al., 2009]. Moreover, murine models have been very useful in identifying stem cells, by 5-bromo-2'-deoxyuridine (BrdU) labeling and proliferating cell nuclear antigen (PCNA) immunofluorescence [Chan, Gargett, 2006].

The identification of predecidual cells sharing bone marrow derived features, in 1982 [Kearns, Lala, 1982], together with the expression of telomerase gene [Kyo et al., 1997], along with c-kit and OCT-4 (markers of stem/puripotential cells) [Cervelló et al., 2007] lead to the hypothesis of stem-like endometrial cells. Later on, clonogenicity studies have identified cells capable of stromal and epithelial generation [Chan et al., 2004; Schwab et al., 2005].

Starting from the latest findings, a recent model of both ectopic and eutopic implantation has been proposed [Maruyama et al., 2010], as an alternative mechanism of functional layer regeneration, based on basalis persistence during menstruation. This model involves stem/progenitor cells, possible bone marrow-derived, located in the vascular or perivascular areas, in both basal and functional layers and contained in the sloughed endometrium. They may either implant in ectopic sites, by retrograde menstruation, either remain inside the uterine cavity after menstruation and later on these stem cells reimplant in regenerating endometrium. The latter event is also supported by previous observations of heterogeneous areas regarding their development stage, noticed in normal endometrium [Garry et al., 2009].

The endothelial location of stem cells is in agreement with the speculations about their bone marrow origin and their ability to participate in neovascularization and neoendothelialization [Masuda et al., 2007] and, furthermore, to epithelial and stromal endometrial cells regeneration [Taylor, 2004].

Moreover, circulating stem cells, mainly introduced after surgical trauma or due to increased vascular turbulence, may result in lymphovascular dissemination, followed by ectopic implantation [Blann et al., 2005] (Fig. 1.31).

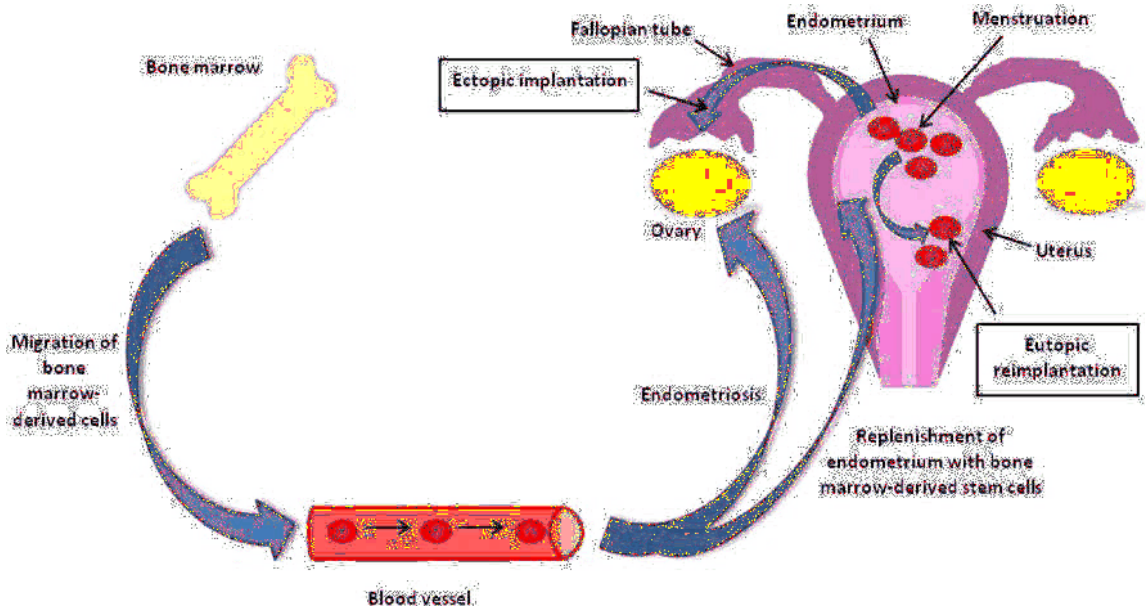


Figure 1.31. Current model of endometrial regeneration. The recent model of both ectopic and eutopic implantation is based on stem/progenitor cells, possible bone marrow-derived, located in both basal and functional layers and also contained in the sloughed endometrium; endometrial stem cells may either implant in ectopic sites, by retrograde menstruation, either remain inside the uterine cavity and later on they reimplant and regenerate the endometrium; circulating stem cells may also result in lymphovascular dissemination, followed by ectopic implantation.

1.5.3. ADULT STEM CELL TYPES IN NORMAL ENDOMETRIUM

According to their origin, endometrial stem cells (ESCs) can be included in endogenous ESCs, represented by highly proliferative clonogenic endometrial cells population (epithelial and stromal), and exogenous ESCs, originating mainly in the bone marrow, with hematopoietic, progenitor, and non-hematopoietic stem cells [Kucia et al., 2005], considered an extrinsic ASCs source of endometrial stem cells [de Miguel-Gómez et al., 2021].

The review of the literature reveals a large variability in the definition and classification of the stem cells found in the endometrium, with different perspectives on their exogenous or endogenous origin.

Within this framework, the menstrual blood-derived stem cells (MenSCs) represents a matter on debate with controversial opinion regarding their source, due to the inconsistencies in the phenotype of cell lineages in the menstrual blood – most of them presenting the same markers as the resident endometrial stem cells, but few having specific markers for bone marrow mezenchimal stem cells.

Therefore, as a results of our personal vision on this topic, Table 1.22 summarizes a comprehensive image of all types of ESCs, that allow an insight into the phenotype similarities which indicate an overlapping degree in behaviour, and into the differences which define distinct properties and functions.

Table 1.22. Categories of endometrial stem cells, origin, and their specific markers

ORIGIN	EXOGENOUS STEM CELLS				ENDOGENOUS STEM CELLS				
Stem cells types	BMDSCs	UC-MSCs	HAMSCs	AD-MSCs	eEPCs	eMSCs	SPCs	MenSCs	ERCs
Source	Bone marrow	Umbilical cord	Amnios	Adipose tissue	Endometrium		Menstrual blood		
Specific cell markers	CD73	CD73	CD44	CD10	N-Cadherin	CD10	CD9	CD73	OCT4
	CD90	CD90	CD59	CD13	SSEA-1	CD13	CD31	CD13	CD9
	CD105	CD105	CD90	CD29	Axin2	CD29	CD34	CD9	CD29
	CD133		CD105	CD34	SOX9	CD44	CD73	CD29	CD41a
	DNp63			CD44	LGR5	CD73	CD90	CD90	CD44
				CD49e	TROP2	CD90	CD45	CD41a	CD59
				CD59		CD105	CD105	CD44	CD73
				CD73		CD140b	CD133	CD59	CD90
				CD90		CD146	STRO-1	CD105	CD105
				CD105		CD117			MSI1
						STRO-1			Notch1
						NTPDase2			
						SUSD2			

BMDSCs – bone marrow-derived stem cells; UC-MSCs – umbilical cord mesenchymal stem cells; HAMSCs – human amnion-derived stem cells; AD-MSCs – adipose tissue-derived mesenchymal stem cells; eEPC – endometrial epithelial progenitor cells; eMSCs – endometrial mesenchymal stem cells; SPCs – side population cells; MenSCs – menstrual blood-derived stem cells; ERCs – endometrial regenerative cells; DNp63 - truncated N-terminus isoform of p63 gene; SSEA-1 - stage-specific mouse embryonic antigen; Axin2 - axis inhibition protein 2; SOX9 - SRY-Box Transcription Factor 9; LGR5 - Leucine Rich Repeat Containing G Protein-Coupled Receptor 5; TROP2 - Trophoblast cell surface antigen 2; STRO-1 – mesenchyme 1; NTPDase2 - Nucleoside Triphosphate Diphosphohydrolase-2; SUSD2 - sushi domain containing-2; MSI1 - Musashi RNA Binding Protein 1; Notch1 - Neurogenic locus notch homolog protein 1.

Adult, somatic, or tissue-specific stem cells are dispersed throughout the whole body, in bone marrow [Friedenstein et al., 1968], cord blood [Oh et al., 2008], Wharton's jelly [Chao et al., 2008], dental pulp [Jo et al., 2007], peripheral blood [He et al., 2007], and Fallopian tube [Jazedje et al., 2009]. Longtime after the embryonic development, these cells maintain their undifferentiated state, their capacity of selfrenewal, by their capacity to generate identical daughter cells. These are resting in quiescent functional status, being able of multi-lineage differentiation, by asymmetrical divisions, and transformation into committed cells that may reconstitute the tissue where they reside. Due to their role in replenishment and regeneration of damaged or dead tissues, they possess the capacity of morphological and functional tissue maintenance. This unique characteristic led to the idea that they have the ability to regenerate the entire organ where they are located [van der Kooy et al., 2000].

A viable solution in the treatment of numerous degenerative diseases is represented by stem cells therapies and thus clinical trials are currently trying to assess their compliance for human application. Due to ethical controversies and to the risk of tumorigenesis, the practical exploitation of stem cells has been delayed. By elimination of the main drawbacks, adult mesenchymal stem cells (MSCs), firstly identified in bone marrow, and later on in periosteum, skeletal muscle, pancreas, placenta [Crisan et al., 2008], adipose tissue [Gimble et al., 2007], and dental pulp [Gronthos et al., 2000] emerged as a viable solution in both heterologous and autologous cell transplant [Mutlu et al., 2015]. MSCs are defined by the Committee of the International Society for Cellular Therapy as plastic adherent multipotent cells, able to differentiate in vitro into adipocytes, chondroblasts, and osteoblasts ("orthodox pathway"), with

positive expression of CD73, CD90, and CD105, and negative expression of human leukocyte antigen–antigen D related (HLA-DR), CD11b, CD14, CD19, CD34, CD45, and CD79a [Dominici et al., 2006]. Their potential of differentiation into osteocytes and chondrocytes had been already exploited in bone and cartilage repair [Caplan, 2017].

Beside the "orthodox" differentiation, a "non-orthodox pathway" has been also demonstrated, towards muscle [Liu et al., 2007], neurons [Trzaska et al., 2009], pancreatic islets [Xie et al., 2009], and hepatocytes [Chivu et al., 2009].

● ***Exogenous endometrial stem cells***

Interestingly, one of the main exogenous sources of endometrial stem cells is the bone marrow, contributing to the cellular turnover and being able to react to inflammatory stimuli [Taylor, 2004], as reflected into endometrial glandular chimerism.

Bone marrow-derived stem cells

Bone marrow-derived stem cells (BMDSCs) provide some of the mesenchymal stem cells (MSCs), being located in different tissues and having the ability to supply cells to non-hematopoietic tissues [de Miguel-Gómez et al., 2021].

It was demonstrated, in both mouse and human models, that the BMDSCs can populate the stem cell niche of the endometrium, differentiating into specific stem cells [Taylor, 2004], such as endothelial, epithelial, and stromal cells [Taylor et al., 2004; Ersoy et al., 2017; Tal et al., 2019]. In this regard, one study confirmed the existence of MDSC, as a source of exogenous endometrial stem cells, in patients with bone marrow transplants [Cervelló et al., 2012].

A noteworthy aspect is the difficulty in evaluating characteristic markers of these cells, due to the complexity of the endometrial microenvironment [Campo et al., 2020]. Thereby, another study showed that, in vitro, the clonogenic endometrial cells share the same properties with mesenchymal stem cells, which express several surface markers (CD44, CD29, CD90, CD73, and/or CD105) and are negative for others (CD34, CD45, CD14, and/or HLA-DR) [Cervelló et al., 2010; Ullah et al., 2015; de Miguel-Gómez et al., 2021].

Accordingly, is still unsettled if these BMDSCs are envolved in the endometrium remodeling or they represent just stem cells with endometrial phenotype after their migration [Gargett et al., 2016a]. However, these exogenous stem cells appear to provide great opportunities for infertility therapy due to endometrial imbalance [de Miguel-Gómez et al., 2021].

Various animal studies concentrated on other markers related to BMDSCs, like CD133 and DNp63, which are biomarkers of Asherman syndrome, demonstrated the therapeutic effects of these cells in Asherman syndrome [Alawadhi et al., 2014; Zhao et al., 2017; Gao et al., 2021]. Hence, it was revealed that the injected CD133+ BMDSCs in an immunodeficient mouse model with Asherman syndrome were located especially around stem cell niche, close to damaged endometrial blood vessels, promoting cells proliferation via IGF-1 (insulin-like growth factor 1) and thrombospondin 1, which, in turn, activate surrounding cells mitosis and thus, the endometrial regeneration [Alawadhi et al., 2014; Cervelló et al., 2015; Gao et al., 2021]. DNp63 was proved to induce endometrial quiescence. Moreover, DNp63+ cells presented a characteristic Asherman syndrome stemness modification. It was assumed that BMDSCs can reverse endometrial inactivity and stemness changes DNp63 induced in patients with Asherman syndrome [Zhao et al., 2017; Gao et al., 2021].

The study of bone marrow-derived MSCs (BM-MSCs) has demonstrated their role in the inhibition of immune system, by suppressing T-cells proliferation and diverting their differentiation into tolerogenic T-regulatory cells instead of proinflammatory Th-cells [Németh et al., 2009]. Supplementary, BM-MSCs suppress natural killer (NK) cells, switch macrophages phenotype from type 1 to type 2 (proinflammatory to anti-immunomodulatory) [Németh et al., 2009], induce a dendritic cells tolerogenic phenotype [Deng et al., 2014], secrete chemokines for

MSCs recruitment (C–C motif chemokine ligand 2 (CCL2), CXCL8, and CXCL12), and a variety of cytokines and growth factors with antiapoptotic [TGF-β, bFGF, IGF-1, and hepatocyte growth factor (HGF)], angiogenic [VEGF, bFGF, phosphatidylinositol-glycan biosynthesis class F protein (PIGF), and monocyte chemoattractant protein-1 (MCP-1)], and supportive functions (macrophage colony-stimulating factor (M-CSF), IL6, and SDF-1) [Mutlu et al., 2015]. Their ability of immunomodulation had been already demonstrated both in vitro and in vivo, creating the premises for their use in both autologous and heterologous applications [Wang et al., 2014].

Other exogenous stem cells

Umbilical cord-derived mesenchymal stem cells

Also named Wharton's Jelly-derived mesenchymal stem cells (WJMSCs), human umbilical cord-derived MSCs (UC-MSCs) are found in the Wharton's jelly and perivascular areas of the umbilical cord, being extensively tested in regenerative medicine. Unlike BMSCs, UC-MSCs have a higher proliferative capacity and an elevated cartilagenous differentiation rate [Hua et al., 2013]. Due to their effortless availability, low immunogenicity, easy quality management during aquisition, and inoffensive collection technique, UC-MSCs are considered better candidates for damage endometrial repair [Wang et al., 2010]. Because of their proved qualities to relatively damage the endometrial stromal cells both in vivo and in vitro [Yang et al., 2011; Chen et al., 2016], UC-MSCs are suitable for stem cell-based therapy in Asherman syndrome [Gao et al., 2021]. In this regard, another study, which used Asherman syndrome rats, showed that transplantated UC-MSCs can restore endometrial damage and thus improve fertility through inhibition of inflammation and fibrosis and enhancement of endometrial proliferation [Zhang et al., 2018; Gao et al., 2021]. As Wharton's jelly represents one of the best sources of autologous mesenchymal stromal cells for targeted therapy, it can be presumed that WJ-MSCs have to meet the same three criteria as MSCs: (i) fibroblast-like morphology, with adherence to plastic surface; (ii) differentiation capacity towards chondrogenic, osteogenic, and adipogenic lineages; (iii) positive in more than 95% for CD105, CD90, and CD73 [Dominici et al., 2006; Ranjbaran et al., 2018; Liau et al., 2019].

Human amnion-derived mesenchymal stem cells

Human amnion-derived MSCs (HAMSCs) represent a new source of mesenchymal stem cells originated from human discarded tissue, being simple to obtain and without ethical aspects [Gao et al., 2021]. Moreover, these cells characterize by positivity for markers of stem cells, like CD105, CD90, CD44, CD49d, and CD59, as well as differentiation potentials [Miki et al., 2005; Lindenmair et al., 2012]. Hence, HAMSCs, together with BMDSCs, could have a crucial role in allograft and xenograft transplantation, in intrauterine adhesion, Asherman syndrome, promoting endometrial regeneration by decreasing proinflammatory cytokines (IL-1b, IL-8, TNF-a) and overexpressing anti-inflammatory cytokines (IL-6, IL-10), FGF-b, VEGF, HGF [Gao et al., 2021].

There are also described the human amniotic-derived epithelial stem cells, with differential potential and demonstrated capacity for fertility restoration in mice with intrauterine adhesion, stimulating the autophagy and improving damage repair [Li et al., 2019; Gao etal., 2021].

Adipose tissue-derived mesenchymal stem cells

Adipose tissue-derived MSCs (AD-MSCs) are little studied regarding their envolvement in endometrial repair [Lv et al., 2021]. These cells are easy obtained, create in vivo large reserves, present high proliferation capacity, and proved to have large applications in the therapy of osteoarthritis, nerves injury repair, and diabetes [Liu et al., 2016; Lee et al., 2019; Bydon et al., 2020]. It was demonstrated that, in rats with intrauterine adhesion, AD-MSCs together with their exosomes stimulate endometrial regeneration, increase endometrial

receptivity, and adjust fertility [Shao et al., 2019; Zhao et al., 2020]. Other studies assumed that endometrial carcinoma-pretreated adipose stem cells stimulate carcinogenesis and migration through the STAT3 signaling pathway activation [Chu et al., 2018], suggesting that these cells are influenced by the tumor microenvironment [Lv et al., 2021]. The most frequently positive markers for AD-MSCs are CD10, CD44, CD29, CD90, CD13, CD34, CD105, CD73, CD49e, CD59, and CD166, while the most frequently negative markers are CD14, CD11b, CD19, CD31, CD34, CD45, CD56 and CD146 [Mildmay-White, Khan, 2017].

● ***Endogenous endometrial stem cells***

The endogenous category of endometrial stem/progenitor cells come from the specific tissue reserve cells, being significant for endometrial function recovery [Gao et al., 2021].

Through clonogenicity at very low densities there were indentified and characterized in vitro many of the human ASCs, including clonogenic epithelial and stromal stem cells [van Os et al., 2004; Chan et al., 2004; Gargett et al., 2007].

It is widely accepted that endometrial clonogenic cells possess multilineage mesenchymal capacity, being able to differentiate towards smooth muscle cells, adipocytes, chondrocytes and osteoblasts [Schwab et al., 2007]. In this regard, it can be assumed that these cells act as stem cells, with multipotency and self-renewal properties, demonstrating endometrial regenerative capacity [Maruyama, 2014].

According to the cell types and different identification methods, the endogenous endometrial stem/progenitor cells include endometrial N-cadherin+ epithelial stem/progenitor cells, SUSD2+ CD140b+ CD146+ endometrial mesenchymal stem cells (eMSCs), side population cells (SPs) [Gargett et al., 2016a; Bhartiya, 2016; Gargett et al., 2016b],with predominantly endothelial cells, and menstrual stem cells (MenSCs), which are menstrual blood-derived [Cousins et al., 2018; Gao et al., 2021; Kong et al., 2021]

The ERCs control the endometrial regeneration, the epithelial progenitor cells reside in the basalis layer of the endometrium, at the base of the endometrial glands, and the endometrial MSCs are located in the perivascular niche, mediating stromal regeneration and angiogenesis [Cousins et al., 2018]. There are several criteria for endometrial stem cells identification, as follows: invivo capacity for endometrial regeneration using xenograft animal models, long-term culture property, their clonogenicity, multi-lineage differentiation capacity, stem cell markers expression [Masuda et al., 2010; Cervello et al., 2011; Masuda et al., 2012; Miyazaki et al., 2012; Cervello et al., 2017; Gao et al., 2021].

Endometrial epithelial stem/progenitor cells

Human endometrial epithelial stem/progenitor cells (EPCs) are located in the basal layer of the endometrium, being not normally shedded [Chan et al., 2004; Gargett, Masuda, 2010]. They are defined as large epithelial colony-forming units (CFUs), with elevated proliferative capacity, and may be differentiated in 3D (three-dimensional) cell culture, which contain various niche factors, into gland-like structures [Gargett et al., 2009; Gao et al., 2021].

Endometrial EPCs were initally isolated in 2009 [Gargett et al., 2009], individual colonies meeting the criteria, in the differentiation activation medium, of ASCs: self-renewal properties, differentiation potential, and high single epithelial proliferative capacity [Kong et al., 2021].

It is widely accepted that epithelial cells CD15 or SSEA-1 (stage-specific embryonic antigen 1) positive, as well as nuclear SOX9 positive can generate in vitro gland-like structures [Valentijn et al., 2013; Turco et al., 2017]. SSEA-1 has the highest expression in the glandular endometrial epithelium from both postmenopausal and menstruating women [Gao et al., 2021]. In 3D cell culture, SSEA-1+ endometrial epithelial cells present features of the basal layer epithelium and elevated telomerase activity, with diminished proliferation rates and longer telomeres [Valentijn et al., 2013; de la Gao et al., 2021; Kong et al., 2021]. Hence, the SSEA-1

is considered a marker for basal glandular endometrial epithelial cells, and can be used in differentiation of the basal layer epithelium from functional layer one [Valentijn et al., 2013; Nguyen et al., 2012; Kong et al., 2021].

It is postulated that in human endometrium exist various epithelial progenitor cells phenotypes. Thus, there are described SSEA-1++SOX9++LGR5+ EPCs, located in the basalis, which are involved in functional layer regeneration after cyclic menstruation or after birth, and LGR5++ SOX9+SSEA-1+ epithelial stem cells, which are responsible for embryo implantation promotion and luminal epithelial cells replacement [Tempest et al., 2018; Gao et al., 2021].

Another recently discovered EPC specific marker is N-cadherin, these cells with positive expression of N-cadherin being located at the myometrial-endometrial interface, in the basal glandular epithelium, presenting in vitro the capacity to differentiate into gland-like structures with cytokeratin+ epithelial phenotype [Nguyen et al., 2017]. This study also compared SSEA-1+ EPCs with N-cadherin+ EPCs, revealing significant epithelial differentiation of the endometrial glands [Nguyen et al., 2017; Gao et al., 2021].

Another specific studied marker is Axin2, an important WNT signaling pathway negative regulator, which is expressed in various stem cells [Nusse, Clevers, 2017], and was also found in bipotent epithelial progenitors' cells from endometrial glands [Syed et al., 2020]. While cytoplasmic Axin2 is expressed in the functional endometrial layer of the premenopausal women in the epithelium of the proliferative and secretory endometrial glands [Kong et al., 2021], the nuclear Axin2 is expressed in the basal layer of the endometrium from premenopausal and menopausal women, in proliferative and secretory epithelia [Nguyen et al., 2012]. Moreover, it was shown that, in vivo, Axin2-positive endometrial epithelial glandular cells express some stem cell markers, like SOX9, LGR5, and TROP2, which promote epithelial growth, and in vitro, the same Axin2+ cells can create functional endometrial organoids [Syed et al., 2020]. These findings evidentiate the participation of the mesenchymal-to-epithelial transition (MET) in the endometrial regeneration and maintenance [Ghosh et al., 2020; Cousins et al., 2021; Kong et al., 2021].

Endometrial mesenchymal stem cells

The endometrial MSCs (eMSCs) were defined in 2007, by CD146 and PDGF-Rb (platelet-derived growth factor receptor b) coexpression, known as CD140b [Schwab et al., 2007]. Because of their perivascular location of the small vessels from functional and basal layers, eMSCs are considered pericytes capable to stimulate endometrial homeostasis, regeneration, and maturation [Caplan et al., 2008; Spitzer et al., 2012].

In humans, the endometrial CD146+CD140+ cells population present gene profiles related to menstrual blood-derived MSCs [38] and other MSCs types [Spitzer et al., 2012; Gao et al., 2021].

The perivascular CD146+CD140b+ cells have the capacity to differentiate into various lineages, like adipogenic, myogenic, chondrogenic, and osteogenic, particular smooth muscle cells and fibroblasts [Schwab et al., 2007; Masuda et al., 2012; Spitzer et al., 2012]. This cells population expresses MSCs markers (CD29, CD44, CD73, CD90, and CD105), but do not express hematopoietic or endothelial markers (CD45, CD31, and CD34) [Gargett et al., 2009]. Although the proportion and clonal ability of CD140b+ CD146+ MSCs are unchanged during menstrual cycle, these correspondent cells located in the menstrual endometrium present more self-renewal rounds than during secretory phase, suggesting that this cell phenotype can be triggered during menstruation for cyclic endometrium regeneration. Moreover, CD140b+CD146+ cells present a specific gene profile, with a high expression of genes implicated in different processes, as steroid hormone/hypoxia responses, angiogenesis, cell communication, immunomodulation, inflammation, and proteolysis/inhibition, as well as of signaling molecules (IGF, Notch, Hedgehog, TGF-β, and G protein-coupled receptor) [Spitzer et al., 2012; Kong et

al., 2021]. Another specific feature of human endometrium CD146+ cells is that they can form CFUs [Schwab et al., 2008], being able to differentiate towards osteoblasts, adipocytes, glial-like cells, and neural progenitors' lineages [Fayazi et al., 2015; Li et al., 2019; Kong et al., 2021].

Other specific cell surface marker of eMSCs is SUSD2 (sushi domain containing-2), also named W5C5, can help in clonogenic endometrial MSCs selection [Masuda et al., 2012; Sivasubramaniyan et al., 2013; Gao et al., 2021; Kong et al., 2021]. These cells are mainly perivascular located in both endometrial basalis and functionalis [Kong et al., 2021].

Endometrial SUSD2+MSCs express a stromal basalis marker, known as nucleoside triphosphate diphosphohydrolase 2 (NTPDase2), characteristic for pericytes and stem cells, which appears to be important for MSCs isolation in regenerative medicine, as their localization and expression level remain constant during menstrual cycle [Zimmermann et al., 2012; Yegutkin, 2014; Trapero et al., 2019; Gao et al., 2021; Kong et al., 2021].

Moreover, in vitro, SUSD2+ cells have the property to differentiate into diverse cell lineages, such as endothelial cells, adipocytes, chondrocytes, osteocytes, myocytes, while, in vivo, they can generate endometrial stromal-like tissues [Kong et al., 2021]. It was also observed that isolated SUSD2+ cells present MSC markers positivity (STRO-1, CD73, CD90, CD29, CD44, CD117, CD105, CD140b, and CD146) [Kong et al., 2021].

Side population cells

Stem subpopulation or "side population" (SP) cells isolated in mammals are associated with adenosine triphosphate (ATP-binding cassette transporter protein (ABCG2/Bcrp1) and show multilineage development capacities [Kato, 2012].

SP cells are round, small, have self-renewal abilities, a long lifespan and proliferation activity, acting as progenitor cells [Kato, 2012]. SP are negative for CD9 (endometrial glandular marker) and CD13 (endometrial stromal marker), are able to extend podia, and may be maintained in cultures up to nine months [Kato, 2012].

The enhanced tumorigenicity of SP cells, together with the bipotent epithelial and stromal population generation, along with enhanced migration ability might be linked to EMT [Kato, 2012]. An important recent finding was that of constant identification of endometrial MSCs CD140b+/PDGFRβ and CD146+ not only in basal, but also, in a lesser extent, in functional endometrium, showing the maximum capacity of self-renewal in menstrual phase but also continued in proliferative phase [Xu et al., 2015]. Therefore, these cells are those responsible for endometrial regeneration, representing approximate 1.5% of endometrial stromal cells (quiescent and activated stem cells) [Xu et al., 2015].

The identification of stem cells in functionalis zone supports the correlation with endometriosis, as basal layer is not involved in menstruation [Xu et al., 2015] and furthermore endometrial MSCs inability to decidualize, due to aprogesterone resistance, may be attributed to a stem cell disease [Xu et al., 2015].

Moreover, it has been hypothesized that stem/progenitor cells from basal zone may form large colony-forming units (CFUs), while transit amplifying cells from functionalis zone, more differentiated, form small CFUs [Zhu et al., 2014]. At the endometrial–myometrial junction and in perivascular locations a CD146+ PDGFRβ MSC population has been identified [Schwab et al., 2007; Gargett et al., 2008], exhibiting multilineage abilities. Thus, a common origin with bone marrow-derived human mesenchymal has been suggested [Gargett et al., 2009].

It is widely accepted that SP cells express various cell markers, including undifferentiated cell markers (OCT-4 and c-KIT), endothelial cell markers (CD34 and CD31), epithelial cell marker (EMA), as well as MSCs markers (CD90, CD105, and CD146) [Tsuji et al., 2008]. In hypoxic milieu, SP cells present a medium telomerase length and the potential for clining, proliferation, and differentiation into osteoblasts and adipocytes [Cervelló et al., 2010; Cervelló et al., 2011]. In the same way as SUSD2+ eMSCs, side population cells do not have

ERα or PR expression, but present ERβ expression, as these represent mainly endothelial cells [Masuda et al., 2010]. It was demonstrated that in vitro, endometrial SP cells may differentiate into epithelial, stromal, and and endothelial cells [Masuda et al., 2010; Lv et al., 2021].

It was hypothesized that endometrial endothelial stem cells are part of the stem cell niche. These endothelial cells have CD31/CD34 (classical endothelial markers) positivity, being found between endometrial SP cells from the basal layer as well as from the endothelium [Tsuji et al., 2008]. Moreover, the endothelial markers are higher expressed in endometrial SP cell population than in non-SP cells [Masuda et al., 2010; de Miguel-Gómez et al., 2021].

Menstrual blood-derived stem cells

Menstrual blood-derived stem cells (MenSCs), initially discovered in 2007 from menstrual blood [Meng et al., 2007], include mainly endometrial stromal fibroblasts and MSCs. MenSCs present a heterogenous markers expression, being positive for CD9, CD13, CD29, CD41a, CD44, CD59, CD73, CD90, CD166, OCT-4, as well as for CD105, and negative for HLA-DR, CD117, CD130, CD19, CD34, CD133, and CD45 [Meng et al., 2007; Zemelko et al., 2011; Khoury et al., 2014; Lv et al., 2018; Kong et al., 2021; Lv et al., 2021]. MenSCs present the property of differentiation towards numerous lineages, such as cardiomyocytic [Hida et al., 2008], adipocytic [Khanmohammadi et al., 2014], osteogenic [Darzi et al., 2012], neurocytic [Liu et al., 2018], as well as endothelial, respiratory epithelial, germ-like [Lai et al., 2016; Eyni et al., 2017], myocytic, hepatic [Mou et al., 2013], and pancreatic cells [Meng et al., 2007; Patel et al., 2008; Kong et al., 2021].

Menstrual blood-derived stem cells (MenSCs) represent a new menstrual fluid source, being simple and noninvasive to obtain, comparative with other ASCs [Meng et al., 2007; Lv et al., 2021]. They characterize by an elevated proliferative potential, multilineage differentiation capacity, low tumorigenicity, decreased immunogenicity, as well as karyotype maintenance after 68 passages [Lv et al., 2018]. Accordingly, MenSCs can represent perfect regenerative cells for various female reproductive system conditions (intrauterine adhesions, premature ovarian failure, pelvic prolapse), or other diseases (stroke, myocardial infarction, acute lung injury, liver injury, and Duchenne dystrophy) [Lv et al., 2018; Rossignoli et al., 2013]. Moreover, it was shown that, to a certain degree, they can exhibit antitumor effects in neuroblastoma, cervical cancer, and lung cancer [Chen et al., 2019; Moreno et al., 2017; Moreno et al., 2019; Lv et al., 2021].

MenSCs do not represent a variant of eMSCs, as these two categories are relative but different but cell types. For the definition and identification of MenSCs, three conditions should be respected: (i) menstrual blood isolation; (ii) specific markers expression as presented above; (iii) MenSCs have multilineage differentiation capacity, and may be cultured in plastic-adherent containers [Chen et al., 2019]. Hence, for future studies, must be taken into account factors that influence the MenSCs viability, such as the donor age, various features of stromal cells, and contraceptive use, and heterogeneous characteristics of stromal cells [Liu et al., 2018; Lv et al., 2018; Lv et al., 2021].

Endometrial regenerative cells

The endometrial regenerative cells (ERCs) represent multipotent mesenchymal stem cells from menstrual blood, which have an elevated proliferative potential [Gargett et al., 2014]. As ERCs are situated in the endometrial perivascular areas of the basalis and functionalis and are cyclical shed, they can provide a reliable noninvasive source in regenerative medicine research [Khoury et al., 2014; Gao et al., 2021].

Since 1978, the existence of stem cells in the endometrium has been speculated, firstly as estradiol-sensitive, progesterone-sensitive, and estradiol-progesterone-sensitive cells [Prianishnikov, 1978], then correlated to a bone marrow origin [Kearns, Lala, 1982], and later supported by telomerase expression in proliferative endometrium [Kyo et al., 1997].

Within this context, the endometrium has been revealed as a source of stem cells useful in therapy, according to two independent research teams [Meng et al., 2007; Patel et al., 2008]. The first group used cells derived from menstrual blood followed by cloning in order to obtain a pluripotent population, named ERCs [Meng et al., 2007]. The second group used c-kit selection of mononuclears from the menstrual blood that have also showed a marked proliferative ability [Patel et al., 2008].

The identification of endometrial pluripotent stem cells from menstrual blood, generated a population showing telomerase+, octamer-binding transcription factor 4 (OCT4)+, CD9+, CD29+, CD41a+, CD44+, CD59+, CD73+, CD90+, CD105+, MSI1+, NOTCH1+ [Schwab et al., 2007; Schwab et al., 2008; Schüring et al., 2011], along with CD34+ and CD117+, in the basal layer [Cho etal., 2004], and CD133+ in the epithelial component [Schwab et al., 2008], while other markers showed lack of expression (NANOG-1-, STRO1-, CD14-, and CD45-) [Meng et al., 2007]. These cells exhibited the ability to differentiate in vitro into 11 different lineages: endothelial, respiratory epithelium, adipocytic [Ai et al., 2012], chondrogenic [Schwab et al., 2007; Su et al., 2014], osteogenic [Schwab et al., 2007; Fayazi et al., 2016], myocytic [Shoae-Hassani et al., 2013], neural [Noureddini et al., 2012], hepatic, pancreatic lineages, oligodendrocytic [Fayazi et al., 2016], and odontoblastic [Tabatabaei et al., 2013] and have been named "endometrial regenerative cells" (ERCs) [Meng et al., 2007] (Fig. 1.32).

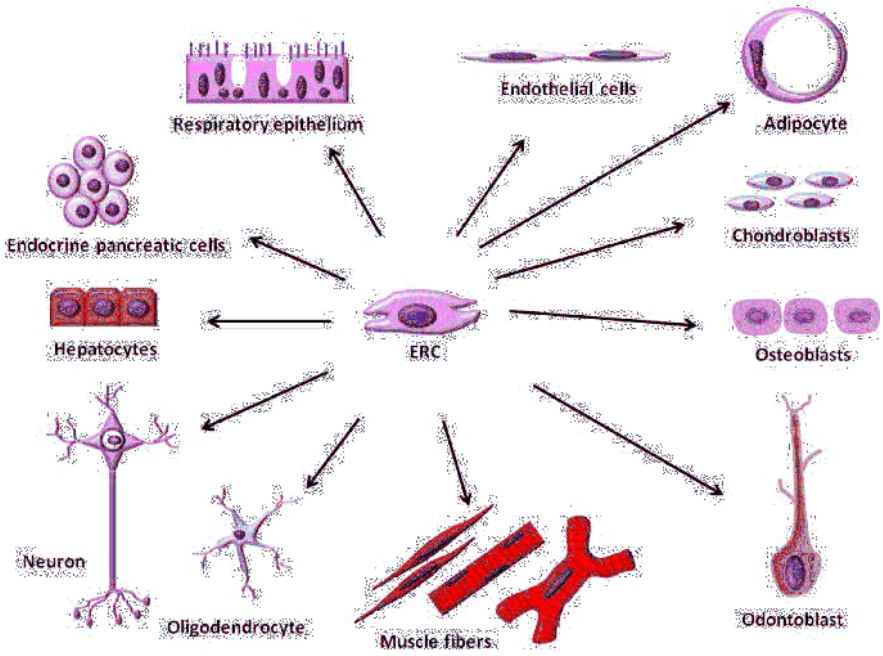


Figure 1.32. Multipotentiality of endometrial regenerative cells (ERCs). Endometrial regenerative cells (ERCs) have demonstrated in vitro ability to differentiate into eleven different types of cells.

Using menstrual blood mononuclear cells c-kit selection, similar cells have been identified [Patel et al., 2008]. During ERCs reprogramming, endogenous NANOG becomes expressed [Park et al., 2011]. ERCs have a high proliferative activity, with ≥30 doublings [Gargett, 2007; Gargett et al., 2009] and display important role in angiogenesis, as demonstrated in a hind limb ischemia model [Murphy et al., 2008]. Putative endometrial stem cells located in the basalis have been firstly suggested decades ago [Prianishnikov, 1978] and much later the demonstration of monoclonality added a convincing support to this supposition [Tanaka et al., 2003]. In humans, endometrial stem cells have been identified by their ability to form colonies in cultures [Chan et al., 2004]. While stromal cells exhibited an increased in vivo capacity, with a peak clonogenicity in proliferative phase, their epithelial counterparts, later on identified, demonstrated their highest activity in secretory phase [Schwab et al., 2005]. Their

characterization has been further performed [Taylor, 2004; Gargett, 2007], with both stromal and epithelial cells exhibiting clonogenicity. The endometrial stem cells have been described as fibroblast-like cells, with adherence to plastic ability and multipotentiality in vitro [Schwab et al., 2007]. In terms of the required growth factors, in serum-free medium, two types of clones may be obtained [Chan et al., 2004; Schwab et al., 2005], considered to belong to different endometrial niches (epithelial vs. stromal), as illustrated in Fig. 1.33.

Endometrial pluripotent cells may be associated with endometrial angiogenesis [Bockeria et al., 2013], considering their coexpression of MMPs and angiogenic factors [Meng et al., 2007]. It is already recognized the supporting role of estradiol in VEGF production and, consequently, in endometrial vasculogenesis [Niklaus et al., 2002; Meng et al., 2007; Patel et al., 2008]. Beside NK cells [Zhang et al., 2011], neutrophils [Gargett, Rogers, 2001], and circulating endothelial progenitor cells [Lemieux et al., 2009], endometrial pluripotent cells appear to play a pivotal role in the multifactorial process of angiogenesis [Bockeria et al., 2013].

More and more evidences are confirming the hypothesis that adult stem cells are also present in human and mouse female reproductive tracts [Gargett, 2007; Maruyama et al., 2010]. Although a plethora of experimental and in vitro models have been tested, due to major differences between rodents and humans and poor reflection of the steps involved in the endometrial regeneration, the progresses in understanding the phenomena has been delayed.

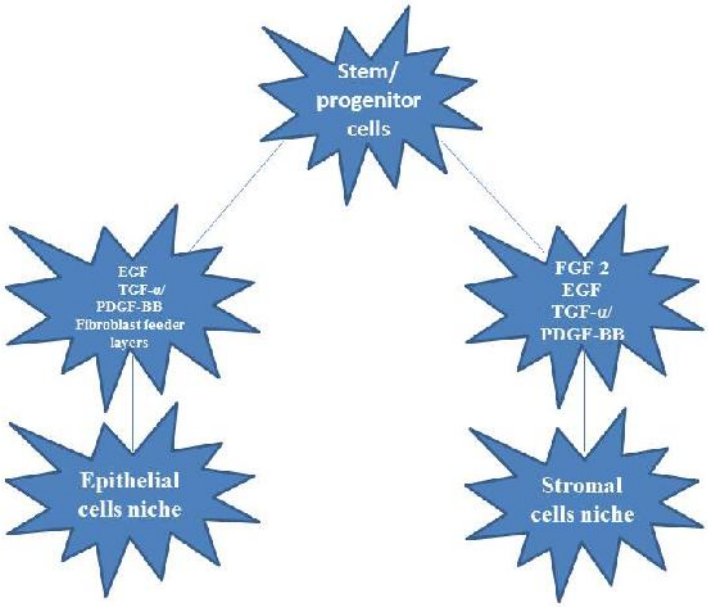


Figure 1.33. Clonogenic development of epithelial or stromal endometrial cells. Different types of medium are necessary for the clonogenic development of either epithelial or stromal endometrial cells (EGF, TGF-α/ PDGF-BB, on fibroblast feeder layers vs. FGF-2, EGF, TGF-α/PDGF-BB). EGF: Epidermal growth factor; FGF-2: Basic fibroblast growth factor (bFGF); TGF-α/ PDGF-BB: Transforming growth factor-alpha/Platelet- derived growth factor two B subunits.

Several teams of research have identified endometrial stem cells not only in the basal layer but also in the functional layer, being associated with the endothelium [Tsuji et al., 2008; Masuda et al., 2010]. In vivo animal models developed to study endometriosis (ectopic functional endometrial-like tissue) provided useful information regarding the endometrial regeneration. The experiments using transplanted human endometrial cells under the renal

capsule of immunodeficient NOG mice demonstrated the existence of human endothelial cells/progenitors able to form a chimeric vascular system [Masuda et al., 2007].

Moreover, using the study of epigenetic changes, by analyzing the methylation patterns, a diversity of stem cells has been also identified in aging atrophic endometrium demonstrating their persistence throughout entire life, in correlation with clinical data of the regenerative capacities elicited by hormonal replacement therapies [Schwab et al., 2005]. ERCs possess a larger spectrum of potentiality than expected and thus its potential use in regenerative medicine seems very extensive.

Furthermore, new molecular techniques [enzyme-linked immunosorbent assay (ELISA), reverse transcription–polymerase chain reaction (RT-PCR), high performance liquid chromatography (HPLC) quantification, flow cytometry, ribonucleic acid (RNA) and protein microarray, fluorescence-activated cell sorting (FACS), and whole transcriptome shotgun sequencing (WTSS)] may identify the trophic substances involved in the regulation of this process and thus, may reveal their potential utility in stimulation of stem cells activity [Janzen et al., 2013; Mutlu et al., 2015].

However, the specific conditions required for the niche to exhibit its stem cell activity are difficult to reproduce in different in vitro and in vivo experiments, mainly because the host cell population also contains precursors/progenitors [Gargett, 2007; Maruyama et al., 2010].

1.5.4. ENDOMETRIAL CANCER STEM CELLS

Recent research has identified a particular type of stem cells which have been considered as responsible of invasion, metastasis, and the development of resistance to conventional therapy, called cancer stem cells (CSCs) [Park et al., 2017], having its counterpart in endometrium.

Moreover, tumor milieu has reciprocal interactions with malignant cells. Therefore, stromal matrix is inducing CSCs proliferation, while a specific epithelial cells phenotype induces EMT, followed by invasion, metastasis, along with hormonal, chemo-, and radiotherapy resistance acquisition in different tumors, including endometrial carcinoma [88]. Several markers have emerged as useful for identification of CSCs, such as human prominin-1 (CD133), CD44, Nanog1, Sall4 [Park et al., 2017], along with CXCR4, c-Myc, Sox-2, Oct4A, ATP-binding cassette subfamily G member 2 (ABCG2), BMI-1, CK18, nestin, β-actin [Sun et al., 2017], and telomerase [Hapangama et al., 2017]. CD133 represents a member of the prominin family, a membrane glycoprotein, which is associated with poor prognosis in endometrial endometrioid carcinoma [Park et al., 2017].

A known adhesion molecule, CD44, is another CSC marker, and its expression seems to be correlated with a higher aggressiveness of endometrioid endometrial carcinoma [Park et al., 2017]. As a consequence, both CD133 and CD44 may be associated with carcinoma progression and poor prognosis [Park et al., 2017]. Nanog, Oct4, and Sox-1 may activate their own genes, resulting in self-renewal abilities [Sun et al., 2017]. Sall4 [Tatetsu et al., 2016] is a member of the spalt-like (SALL) gene family, which is responsible for the persistence of selfrenewal and pluripotent capacities of embryonic stem cells (ESCs). While SALL4 is registering a progressive loss in expression after birth, being absent in most adult tissues, it becomes re-expressed during carcinogenesis [Li et al., 2015; Liu et al., 2015]. Therefore, it has been identified in different cancers, including endometrial cancer, being associated to their ability to metastasize and to develop drug resistance [Li et al., 2015; Liu et al., 2015].

CXCR4 represents a stromal cell-derived factor-1 receptor and its stimulation results in several tumor characteristics, which increase its aggressive behavior [Sun et al., 2017]. ABCG2 is a marker of a fraction of SP cells, containing ATP-binding cassette transporter G2 that result in the capacity to pump out intracellular deoxyribonucleic acid (DNA)-binding dye Hoechst

33342 [Maruyama et al., 2010]. BMI-1 belongs to polycomb genes and is associated with the self-renewal capacity [Bokhari et al., 2016].

CK18 has been identified as an independent factor associated with poor prognosis in some cancers [Lu et al., 2015]. Nestin is an intermediate filament protein identified as a stem cell marker in endometrial cancer [Feng et al., 2013]. However, if the markers of EMT are permanently expressed, a correlation with the development of carcinosarcomas has been demonstrated [Park et al., 2017].

Thus, the downregulation of hormone receptors may be significant for invasion and metastasis and, added to the expression of CSCs markers and loss of E-cadherin expression led to the hypothesis that CSCs possess the capability of EMT [Park et al., 2017].

Besides their multilineage developmental capacity and increased carcinogenesis potential [Kato, 2012], CSCs express a wide range of phenotypical epithelial, stromal, leukocytes, and vascular markers [Masuda, 2010], as well as telomerase activity, which is considered the immortality gene [Kyo et al., 1997]. Although endometrial carcinoma is associated with high telomerase activity [Hapangama et al., 2017], the mechanisms of causing exuberant cell proliferation during endometrial carcinogenesis are not fully known. Telomerase activity is also increased in normal endometrial cells due to specific hormonal influences. On the other hand, other concurrence factors occur because of interactions of endometrial cells with cell populations not specific to the host environment that can influence telomerase and endometrial cell telomerase activity [Hapangama et al., 2017]. In this sense, understanding the mechanisms of regulating telomerase activity may lead to new treatment perspectives in endometrial pathology, involving stem cells [Hapangama et al., 2017].

However, the most probable mechanism considered to explain this phenomenon would be related to the relationship between the decreased postmenopausal estrogen level and telomerase activity deficiency, which cannot sustain the endometrial telomeres length and integrity, this leading to genetic instability and susceptibility of malignant transformation of improper proliferated epithelial cells [Hapangama et al., 2017]. By emphasizing the implication of telomerase activity in carcinogenesis and cell senescence processes, other studies highlighted the fact that inhibition of telomerase activity would be the target for a complementary therapy to existing chemotherapy [Hsu et al., 2005]. Since telomerase activity has also been observed in other cell lines (germ cells, blood mononuclear cells, and fibroblasts), the potential effects of inhibition should be carefully evaluated, with consequences varying according to the pathway of the inhibition mechanism [Hsu et al., 2005].

1.5.5. ENDOMETRIAL STEM CELLS CURRENT THERAPEUTICAL APPLICATIONS

ESCs potential has been tested in experimental studies, using in vitro and animal models and later on, as their safety has been demonstrated and clinical studies have been already performed or are being planned for the next future.

It can be stated that MenSC represent the most accessible stem cells sources with great opportunities in tissue engineering and the regenerative medicine, because they can be regularly and noninvasively harvested. Moreover, these cells can be perfect candidates in the stem cell-based therapy for immune diseases and inflammation, being envolved in cellular and humoral immunity regulation [Kong et al., 2021].

Besides the large briefly mentioned demonstrated and potentially applicability in female genital system conditions, other studied therapies of stem cells are: diabetes mellitus [Fiorina et al., 2009], tooth regeneration [Tziafas, Kodonas, 2010; Nanci, 2013], angiogenesis [Bockeria et al., 2013; Meng et al., 2007], megakaryocyte and platelets production [Wang et al., 2012], acute liver failure [Kademi et al., 2014; Fathi-Kazerooni et al., 2017], musculo-skeletal diseases [Law et al., 1991; Gang et al., 2009; Ichim et al., 2010; Macdonald et al., 2011], bladder tissue

reconstruction and pelvic prolapse repair [Sharma et al., 2011; Shoae-Hassani et al., 2013; Kazemnejad et al., 2013], future challenges for neural regeneration [Borlongan et al., 1998; Rodrigues et al., 2012].

The role of human ERCs in angiogenesis and its high level of immune privilege have been demonstrated in a murine competent hindlimb ischemia model [Murphy et al., 2008]. The potential risk of uncontrolled ERC proliferation in recipients and genesis of endometriosis-like or fibroblasttype tumors has been infirmed, as no endometriosis has been developed after therapy with ERCs in mice experiments [Han et al., 2009]. Considering the angiogenic effect of ERC, the concern that ERC could activate dormant tumors has been addressed by another experiment, which demonstrated an unexpected inhibitory effect on tumor growth [Han et al., 2009]. ERCs in cell-based therapy of acquired endometrial diseases Asherman syndrome is characterized by intrauterine fibrotic synechiae with the destruction of the endometrial basal layer, following miscarriage or curettage, being attributed to the destruction of endometrial stem cells [Mutlu et al., 2015]. Relatively recent, stem cell administration has been suggested as a possible therapy of this disease. Therefore, experiments on murine models of Asherman syndrome have been tested with successful results, using different types of stem cells measured by a higher pregnancy rate of treated animals attributed either to bone marrow-derived stem cells or either to trophic substances [Alawadhi et al., 2014].

The application of an analogous procedure has been also tested in humans. The sources used during time in endometrial regeneration of this syndrome as providers of stem cells have been variable. The first use of autologous bone marrow-derived mesenchymal stromal cells in a patient with Asherman syndrome resulted in successful consecutive in vitro fertilization (IVF) [Nagori et al., 2011]. Other sources have been also used, such as human amniotic mesenchymal stromal cells and autologous menstrual mesenchymal stromal cells [Tan et al., 2016; Gargett et al., 2016a]. The use of menstrual MSCs resulted in increased endometrial thickness and the possibility of pregnancy (in two of seven cases) but limitations related to the sterility of the material and the purification methods remained to be further employed [Tan et al., 2016; Gargett et al., 2016a].

Future research of the molecular pathogenic mechanisms of ESCs in endometriosis may contribute to molecular-based therapy for the related reproductive conditions and cancerous diseases [Kong et al., 2021].

1.5.6. FINAL REMARKS

Recent progresses in endometrial stem cells research made in the last decade may generate new hypotheses regarding eutopic regeneration and ectopic implantation. The facility in obtaining stem cells from endometrium and their high proliferative ability make them ideal candidates for cell-based therapies. The identification of new markers of endometrial stem cells is necessary in order to facilitate their isolation and promising applications.

Currently, beside uterine-acquired diseases and infertility, endometrial stem cells have been tested in a large spectrum of clinical applications. Although their application is yet limited, more clinical trials are necessary to surpass these limits, to improve, and to extend the spectrum of endometrial stem cells exploitation.

The perspectives of development of an endometrial stem cells bank with a large spectrum of HLA-typed cell lines may prevent immunorejection, as the main risk of their application. In order to ameliorate drawbacks of stem cells and to enhance their synergistic activity, a useful approach is their combination resulting in an enhanced trophic, antiinflammatory, and angiogenic effect. The great potential of endometrial cells for cell therapies arise from their completely xeno-free derivation, allogenic use, possibility of large-scale therapeutic doses production, safety, reproducibility, and chance to overcome the drawbacks associated with autologous therapies.

In order to overcome hostile environment of an injured tissue, the association of endometrial stem cells with other stem cells, possibly with added medium, in particular cases opens the perspective of specific combination available as standardized therapeutic means in the next future.

1.6. GRANULOSA CELLS OVARIAN TUMOURS

1.6.1. INTRODUCTION

Among ovarian sex cord-stromal neoplasms, granulosa cell tumors (GCTs) have the highest frequency of clinically malignant tumors within this category [Young, 2011]. There are two distinct subtypes, with different clinical and histopathological parameters, juvenile and adult GCT, the former diagnosed around puberty (44% of patients being 10 years old or younger), and the latter being commonly diagnosed (95% from all the GCTs), with a higher prevalence in premenopausal and young postmenopausal women (with a median age of 50–54 years old) [Jamieson, Fuller, 2012; Kitamura et al., 2017]. Rarely, juvenile GCT can be encountered in the median interval of age characteristic for adult type, and similarly, adult GCT can be found in children [Young, Scully, 1992].

The major risk factors of granulosa cell tumors are represented by family cancer history, nulliparity, oral contraceptives, and obesity [Li et al., 2018].

Despite they share some biomolecular features, recent studies revealed that FOXL2 mutation status represents the key element responsible for the different molecular pathology feature of juvenile and adult GCT [Jamieson, Fuller, 2012].

Current research showed that various signaling pathways are involved in GCT, affecting cell proliferation and apoptosis, such as: fork head box protein L2 (FOXL2), PI3K/AKT, TGF-β, Notch [Anttonen et al., 2014; Farkkila et al., 2014; Chang et al., 2014; Hua et al., 2016; Leung et al., 2017; Li et al., 2018].

It is widely accepted that in the GCT tumorigenesis, the numerous cell signaling pathways interconnected in a complex network [Li et al., 2018]. In this regard, the major mutations encountered in GCT development characterize FOXL2 gene. Moreover, studies assumed that FOXL2 signaling pathway interacts with TGF-β pathway. Thus, mutations in FOXL2 gene inactivate SMAD3 (drosophila mothers against decapentaplegic protein), collaborating with activing A, BMPs (bone morphogenetic proteins), and follistatin [McTavish et al., 2013]. Furthermore, FOXO1/3 (forkhead box O1/3) has also an inhibitory action upon SMAD3 [Wang et al., 2014]. Another study demonstrated the intercommunication between PI3K/AKT and Notch signaling pathways in granulosa cell tumors development [Irusta et al., 2013; Li et al., 2018].

Considering the higher incidence of the adult type, the present study is focused on this type of GCT. These tumors are originating in hormonally active granulosa cells, responsible for estradiol production, but the clear etiology of GCT remains obscure, as no specific risk factors have been yet identified [Kanthan et al., 2012]. Due to their common indolent course, with late recurrences and low metastatic rate, the long-term follow-up of patients is currently advised [McCluggage, 2000; Reed et al., 2010].

The proportion of adult granulosa cell tumors (AGCTs) which secrete estrogens is not precisely established, because of difficulty in acquiring an endometrial specimen for the evaluation of the estrogenic stimulation effect [Young, 2011].

Moreover, AGCTs histopathological diagnosis is often difficult due to the variable morphological phenotypes exhibited by this type of tumors [Yousefi et al., 2009]. Thus, adequate immunohistochemical panels may prove useful in the assessment of the diagnosis and may also efficiently orientate the management and prognosis of these malignancies.

The literature contains reports of independent or panels of markers used by different research teams for the characterization of these ovarian tumors. It is accepted that inhibins, estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are mostly useful in postmenopausal and post-ablation period follow-up [Reed et al., 2010].

During the reproductive life, the major source of estradiol is represented by granulosa cells, due to their production of aromatase, which irreversibly converts androstendione. In GCTs, the tumor cells generally secrete high levels of estradiol, because of the unregulated aromatase expression [Bulun, Simpson, 2008], thus making it a reliable marker for this type of tumors [Jamieson, Fuller, 2012].

Inhibin represents a heterodimeric 32-kDa glycoprotein produced by normal ovarian granulosa cells and granulosa cell tumors, with two subunits, alpha and beta, the latter divided in A and B type, which form inhibin A and inhibin B, considered to have the same biological activity [McCluggage, 2000; Shah et al., 2003]. Numerous studies revealed that inhibin A is very useful in the assessment of granulosa cell tumors [Hildebrandt et al., 1997; McCluggage et al., 1997; Matias-Guiu et al., 1998; Pelkey et al., 1998; Shah et al., 2003]. Although inhibin, together with steroid hormones, characterizes the hormonal activity of the most AGCTs, it may not be expressed in some of these tumors [Yousefi et al., 2009].

Calretinin, a 29-kDa calcium binding protein expressed in ovarian thecal and isolated stromal cells, was found positive in some AGCTs, being also compatible with sarcomatoid areas [Shah et al., 2003; Roth, 2006; Kanthan et al., 2012; Sonoyama et al., 2015; Duraisamy et al., 2017]. Other studies revealed that both adult and juvenile GCT staining positive with inhibin and calretinin, making them reliable markers for GCT diagnostic and follow-up [Yousefi et al., 2009; Reed et al., 2010; Kottarathil et al., 2013].

The proliferative marker Ki67 may be correlated to prognosis. The literature data concerning Ki67 value are controversial, as Ki67 index demonstrated a good correlation with GCT clinical behavior and stage in one study [Rajagopal, Ramesh, 2016], while another research team demonstrated no consistent correlation between Ki67 expression in primary GCT and recurrent neoplasms, with no association between Ki67 and the metastasis-free interval [Stewart et al.,2009]. Although numerous studies evaluated the Ki67 proliferative index in GCT, there are no straightforward conclusions regarding Ki67 index value in tumor behavior prediction [Gebhart et al., 2000; Le et al., 2004].

Aim

In this context, the aim of our study was to assess the immunoexpression of estrogen receptor (ER alpha), Ki67, calretinin, and inhibin A in AGCTs, in order to evaluate their significance in diagnosis and prognosis of this type of tumors.

1.6.2. MATERIALS AND METHODS

Patients

The retrospective study group comprised 21 pre- and postmenopausal women (range between 47–77 years), previously diagnosed with AGCTs, between 2001 and 2013, in the Department of Pathology, “Elena Doamna” Clinical Hospital of Obstetrics and Gynecology, Iaşi, Romania. The surgical treatment for all cases was total abdominal hysterectomy with bilateral salpingo-oophorectomy, associated with partial omentectomy in six cases. The conventional histopathological examination was performed on paraffin-embedded sections, stained with Hematoxylin-Eosin (HE), following the identification of the characteristic morphological elements of adult granulosa cell tumors: growth pattern, nuclear and cytoplasmic features, mitotic rate, differentiation, and nature of the stromal component. According to the

pTNM staging, 19 cases with pT1A, one case with pT1B, and one case with pT1C have been categorized.

Immunohistochemical exam

IHC stainings for ER alpha, calretinin, inhibin A, and Ki67 were performed in all 21 cases of AGCTs. These were carried out on paraffin sections of 4 μm thickness, displayed on silanized slides (coated with poly-L-lysine). Deparaffinization and hydration were performed, in two successive baths with xylene, 15 minutes each, in five successive baths with decreased alcohol concentrations, 5 minutes each (100%, 90%, 80%, 70%, and 50%, respectively), followed by distilled water. Antigen unmasking was performed by immersion of the slides into citrate buffer retrieval (pH 6), in water bath, at 98^0C, for 30 minutes, followed by slow cooling at room temperature. After endogenous peroxidase blocking, the sections were incubated with primary antibodies at an optimal dilution (Table 1.23), for 60 minutes, at room temperature. The reaction was amplified using the Novolink™ (Novocastra) detection system kit.

The following steps were performed: incubation of the sections with secondary antibody, 30 minutes at room temperature and incubation with the enzyme, 30 minutes at room temperature, with three successive washes with phosphate-buffered saline (PBS), after each incubation step (primary antibody, secondary antibody, and enzyme). The slides were developed in 3,3'-diamino benzidine (DAB) medium, 5 minutes at room temperature. After counterstaining with Hematoxylin, the immunohistochemical reaction was interpreted using a standard light microscope. Negative controls, where the primary antibody was omitted and replaced with distilled water, were included. Positive controls for each antibody were also used: testis with Leydig and Sertoli cells for anti-calretinin and anti-inhibin A, normal breast tissue for anti-ER, and breast carcinoma with known immunoreactivity for Ki67.

Table 1.23. The antibodies clones and dilutions used for the immunohistochemical technique

ANTIBODY	CLONE	DILUTION
ER	NCL-L-ER-6F11	**1/100**
Ki67	NCL-L-Ki67-MM1	**1/200**
Inhibin A	NCL-L-Inhibin A	**1/100**
Calretinin	**NCL-L-Calret-566**	**1/100**

ER – estrogen receptor

Immunohistochemical assessment

Positive IHC reaction for calretinin and inhibin A, exhibited as granular cytoplasmic staining, was assessed by a semi-quantitative score with the following values: 0 – no positive cells, 1 – <10% positive cells, 2 – 11% to 50% positive cells, and 3 – 51% to 100% positive cells [Dong et al., 2011]. Five to 10 positive high-power fields (HPFs) were examined for each section.

Ki67 scores were recorded as the percentage of positively stained nuclei across the tumor, without consideration of staining intensity, calculated within three HPFs, arbitrarily selected [Kitson et al., 2017]. The median score of 40% immunopositive cells has been considered the cutoff, to separate the lower expression (Ki67 score less than 40%) from higher expression (Ki67 score more than 40%). The extent of immunoexpression for ER alpha was scored based on the percentage of positive cells, as follows: 0 – ≤5% positive cells, 1 – 6–25% positive cells, 2 – 26–50% positive cells, 3 – 51–75% positive cells, and 4 – 76–100% positive cells [Farinola et al., 2007].

Statistical analysis

Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) v. 20 program (SPSS Inc., IBM Corporation, Chicago, IL, USA).

1.6.3. RESULTS

Clinicopathological features of the study group

The cases included in this study were individually diagnosed and reviewed by two pathologists. The histopathological diagnosis was of AGCT for all 21 cases and, according to World Health Organization (WHO) classification of tumors of the female reproductive organs [Cheung et al., 2020], 17 cases had pT1a stage, one case pT2a, one case pT1c2, with microscopic tumor invasion of the capsules of both ovaries, and two with pT2b, because of the invasive peritoneal implants (Table 1.24).

The growth pattern of the tumor granulosa cells was predominantly diffuse, combined with cords and trabeculae. In decreasing order of frequency, tumors exhibited microfollicular type characterized by small cavities filled with an eosinophilic fluid and bordered by well-differentiated granulosa cells with pale, scanty cytoplasm and angular, often grooved nuclei, and macrofollicular type, with cystic cavities lined by neoplastic granulosa cells, focally surrounded by theca cells. The mitotic rate was generally low, less than two mitotic figures per 10 HPFs in 16 (76.19%) cases, and more than two mitotic figures per 10 HPFs in five (23.8%) cases, which mainly corresponded to the higher stage tumors. The tumoral stroma was nearly absent in the diffuse areas or represented by evident fibroblasts and theca cells around cavities and trabeculae (Table 1.24).

Two cases had been diagnosed with synchronous endometrioid endometrial adenocarcinoma (pT1bNxG1 and pT1aNxG1), one case with cervical adenocarcinoma in situ, and three tumors were bilateral, one of which had synchronous endometrioid endometrial carcinoma (Table 1.24).

Table 1.24. Histopathology, tumor stage and the clinical diagnosis of the studied cases

HISTOPATHOLOGICAL DIAGNOSIS	PTNM/ GRADE	ASSOCIATED MICROSCOPIC FINDINGS	CLINICAL DIAGNOSIS
AGCT	pT1aNxG1	Diffuse and microfollicular pattern LVI	Ovarian cyst
Bilateral AGCT	pT2bNxG1	Diffuse and microfollicular pattern LVI Leiomyoma Invasive peritoneal tumor implants	Uterine fibroma Ovarian tumor
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma Endometrial hyperplasia with no atypia	Hemorrhagic uterine fibroma
Bilateral AGCT	pT2aNxG1	Diffuse and microfollicular pattern Fallopian tube invasion, uterine invasion, peri- and intratumoral lymphocytic infiltrate	Pelvic tumor

HISTOPATHOLOGICAL DIAGNOSIS	PTNM/ GRADE	ASSOCIATED MICROSCOPIC FINDINGS	CLINICAL DIAGNOSIS
AGCT	pT1aNxG1	Predominantly cystic tumor, compact areas with macro- and microfollicular patterns Leiomyoma	Pelvic tumor
AGCT	pT2bNxG2	Predominantly trabecular pattern, with focal micro- and macrofollicular areas, mixoid and luteinized areas Adenocarcinoma in situ of uterine cervix Endometrial hyperplasia with no atypia Invasive peritoneal implants	Ovarian tumor
AGCT	pT1aNxG1	Predominantly diffuse pattern, with macro- and microfollicular areas Leiomyoma Endometrioid carcinoma Intraglandular cervical neoplasia	Ovarian cyst Uterine neoplasia
Bilateral AGCT	pT1c2NxG1	Solid pattern, focal microfollicular Microscopic tumor invasion of both ovarian capsules Villoglandular endometrioid endometrial carcinoma	Metrorrhagia
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular patterns Leiomyoma	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly trabecular and solid patterns, and focally cystic Leiomyoma Endometrial hyperplasia with no atypia	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma	Ovarian tumor
AGCT	pT1aNxG1	Trabecular and solid pattern LVI Leiomyoma Endometrial hyperplasia with no atypia	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly trabecular and solid pattern, and focally cystic Leiomyoma	Ovarian tumor
AGCT	pT1aNxG1	Diffuse and microfollicular pattern Leiomyoma Endometrial hyperplasia with no atypia	Hemorrhagic uterine fibroma

HISTOPATHOLOGICAL DIAGNOSIS	PTNM/ GRADE	ASSOCIATED MICROSCOPIC FINDINGS	CLINICAL DIAGNOSIS
AGCT	pT1aNxG1	Diffuse and microfollicular pattern Leiomyoma Endometrial hyperplasia with no atypia	Ovarian tumor
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma	Ovarian cyst
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular pattern Leiomyoma	Metrorrhagia
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular pattern Leiomyoma	Pelvic tumor
AGCT	pT1aNxG1	Diffuse and microfollicular pattern	Ovarian cyst
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular pattern Leiomyoma LVI Endometrial hyperplasia with no atypia	Ovarian tumor
AGCT	pT1aNxG1	Diffuse and microfollicular pattern Leiomyoma Endometrial hyperplasia with no atypia	Hemorrhagic uterine fibroma Pelvic tumor

AGCT: Adult granulosa cell tumor; LVI: Lymphovascular invasion; EIN: Endometrial intraepithelial neoplasia.

ER alpha expression

Regarding steroid receptors, ER alpha was positive in nine (44.44%) tumors. Among these positive cases, ER exhibited a moderate extent, irrespective of staining intensity, which was also moderate (Table 1.25, Fig. 1.34).

Calretinin and inhibin A expression

Calretinin showed positive immunoreactivity in 16 (77.77%) granulosa cell tumors, for which the cytoplasmic staining was mostly strong and consistently diffuse (Table 1.25, Fig. 1.35). Inhibin A was positive in 14 (66.66%) cases, the cytoplasmic staining being predominantly moderate to severe and diffuse (Table 1.25, Fig. 1.36 – 1.37).

Table 1.25. The immunostaining pattern for ER alpha, calretinin and inhibin A

Score	ERalpha	Calretinin	Inhibin A
	No. of cases (%)		
0	12 (57.14)	5 (23.8)	7 (33.33)
1	-	2 (9.52)	3 (14.28)
2	3 (14.28)	4 (19.04)	6 (28.57)
3	5 (23.8)	10 (47.61)	5 (23.8)
4	1 (4.76)	-	-

ERalpha: estrogen receptor alpha

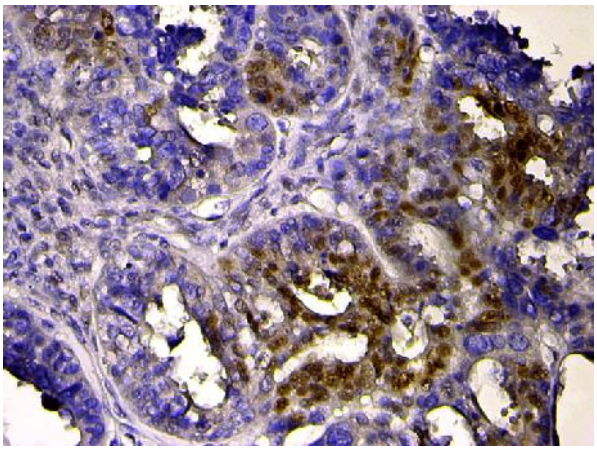


Figure 1.34. Moderate ER expression, nuclear staining in tumor cells (anti-ER alpha, x400).

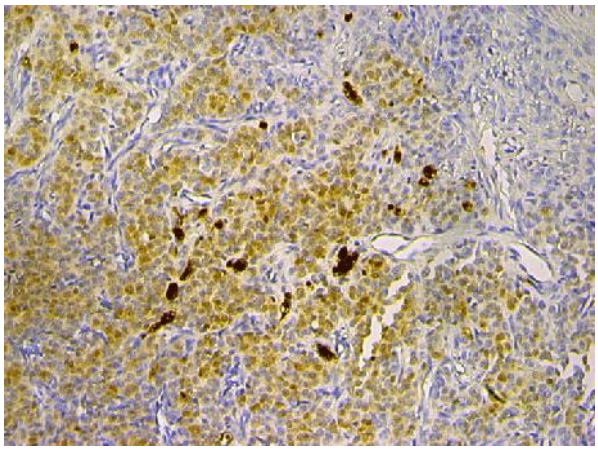


Figure 1.35. Strong and diffuse calretinin expression, cytoplasmic staining in tumor cells (anti- calretinin, x200).

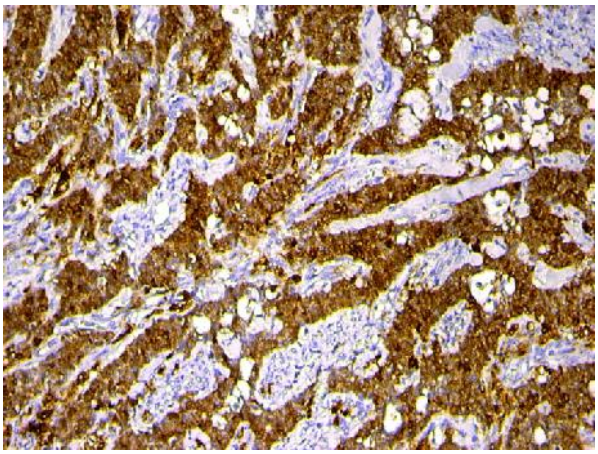


Figure 1.36. Strong and diffuse inhibin A expression, cytoplasmic staining in tumor cells (anti- inhibin A, x200).

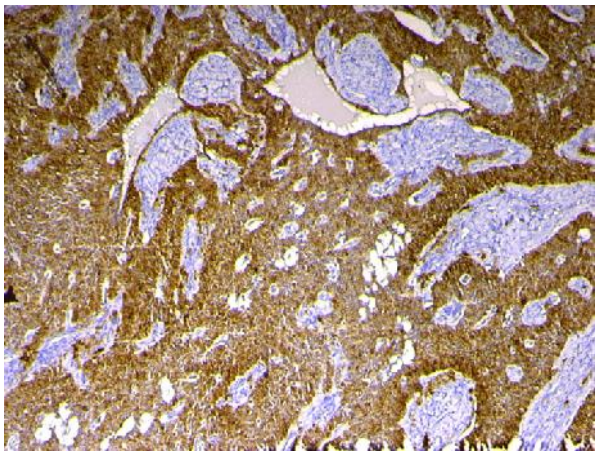


Figure 1.37. Moderate to strong and diffuse inhibin A expression, cytoplasmic staining in tumor cells (anti-inhibin A, x100).

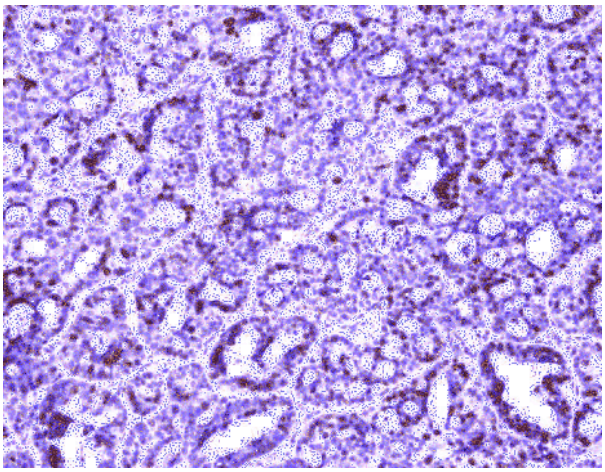


Figure 1.38. Weak and focal Ki67 expression, nuclear staining in tumor cells (anti-Ki67, x200).

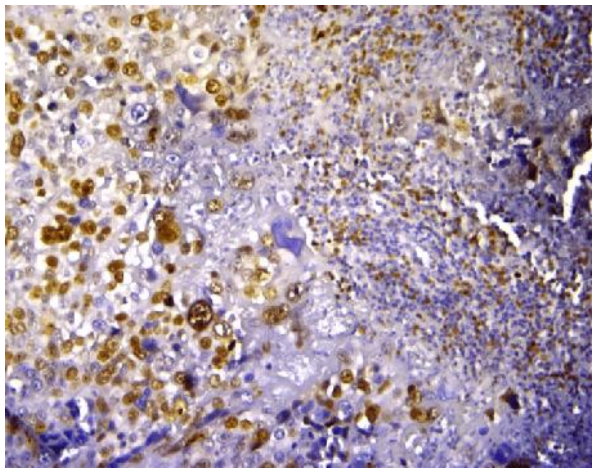


Figure 1.39. Strong and diffuse Ki67 expression, nuclear staining in tumor cells (anti-Ki67, x400).

Ki67 expression

Ki67 was expressed in 12 (57.14%) cases. Ki67 positive cells showed a heterogeneous immunoreactivity, ranging from weak and focal (Fig. 1.38) to strong and diffuse (Fig. 1.39).

Ki67 index was high in five (23.8%) cases, being correlated with the higher stages of the tumors (pT1c2, pT2a, and pT2b), and one with a case with lymphovascular invasion, nuclear atypia, and more than two mitotic figures per 10 HPFs. In seven (33.33%) cases, Ki67 index was low and in nine cases, Ki67 was negative, with no nuclear expression.

1.6.4. DISCUSSIONS

Among the two GCTs histological subtypes, adult form has a frequency of 95%, being characteristic in peri- and postmenopausal women [Bacalbasa et al., 2015]. Usually, the most GCTs (80–90%) are diagnosed in stage I [Aymen et al., 2016]. These clinicopathological features are in agreement with our findings, where 18 (85.71%) cases were diagnosed in stage I (17 cases with pT1a and one case with pT1c).

GCTs represent a rare category of malignant ovarian tumors, being the most common estrogen secreting ovarian tumor, responsible for patients' hyperestrogenism [Koukourakis et al., 2008; Iavazzo et al., 2015; Aymen et al., 2016]. Therefore, the hormonal spectrum of manifestations and the frequent association of these tumors with leiomyoma, endometrial hyperplasia, and endometrial adenocarcinoma are directly correlated to permanent and unbalanced ovarian estrogen secretion [Pekin et al., 2007; Ukah et al., 2011; Sekkate et al., 2013]. Usually, the associated endometrial pathology to AGCT is manifested in women aged 40 years or older [Ottolina et al., 2015; Abuali et al., 2022].

Our study confirms these literature data, with the hormonal spectrum of diagnoses confirmed by multiple histopathological diagnoses association, as following: 16 (76.19%) patients with AGCTs presenting also leiomyoma, eight (23.81%) patients had also endometrial hyperplasia, two (9.52%) cases had been diagnosed with synchronous endometrioid endometrial adenocarcinoma, and one case (4.76%) with cervical adenocarcinoma in situ. Although estradiol is the hormone responsible for the clinical manifestations of this type of tumor and can be used as a reliable tumor marker for GCTs, its fluctuating levels in patients cannot predict the tumor activity [Koukourakis et al., 2008]. A possible mechanism responsible for estradiol variability is that theca cells are sometimes absent in GCTs stroma and consequently there is a shortage of androstendione production, as the source of estradiol due to enzyme cytochrome P450 aromatase action in granulosa cells [Koukourakis et al., 2008]. While these aspects are conclusive for some cases, other studies found no correlation between hormonal levels and the tumor progression or disease recurrence [Lappöhn et al., 1989; Rey et al., 1996]. In this regard, estradiol is considered useful for postoperative follow-up of selected patients, but not constant enough to become a confident GCT marker [Jamieson, Fuller, 2012].

Although GCT are characterize by hormone activity, patients do not always show specific symptoms [Gica et al., 2021]. The tumors considered for the differential diagnosis include ovarian thecoma, carcinoid tumors, adenocarcinoma, and hypercalcemic small cell carcinoma [Wang et al., 2020; Bitterman et al., 2022]. The morphology of AGCT is more characteristic than of juvenile variety, exhibiting in almost half of the cases uniform coffee bean grooved nuclei and presenting positive immunoexpression for inhibin [Schumer, Cannistra, 2003; Parikshaa et al., 2021]. There are described various histological patterns in GCT, as follows: microfollicular, macrofollicular, trabecular, diffuse, gyriform, water silk, and insular [Schumer, Cannistra, 2003; Bitterman et al., 2022].

It was assumed in a study that the patients with synchronous primary ovarian and endometrial cancers, were nulliparous, younger, and premenopausal patients as against the metastatic group [Oranratanaphan et al., 2008]. Commonly, the specific symptomatology in women with synchronous tumors is the abnormal uterine bleeding [Oranratanaphan et al., 2008], accompanied by abdominal or pelvic pain, abdominal distension, and abdominal mass

[Ottolina et al., 2015]. Another study added the finding that synchronous primary ovarian carcinoma are usually multilocular solid and unilateral [Moro et al., 2019].

There are described several pathologic criteria to distinguish the synchronous primary ovarian and endometrial cancer, from endometrial carcinoma with ovarian metastasis [Scully et al., 1998]. In this regard, endometrial carcinoma with metastasis in the ovaries characterizes by: ovarian tumor bilaterality, multilocular tumors, myometrial vascular space invasion, deep myometrial invasion, the absence of ovarian endometriosis, endometrial carcinoma spreading pattern, similar molecular genetics, without ovarian surface implants, hilar or vascular localizatios [Scully et al., 1998; Abuali et al., 2022].

Despite their importance in the normal development and ovary biology, the role of estrogen and ER in the functionality of the granulosa cells is not completely understood [Jamieson, Fuller, 2012]. Some studies emphasize that among the two ER, ER alpha and ER beta, the latter represents the principal ovarian type [Drummond et al., 1999], and also the most expressed type in GCTs [Chu et al., 2000], while others consider that ER alpha is mainly expressed in the gonads, including ovary, and at lower levels in other tissues and ER beta is especially secreted in colon, bone marrow, vascular endothelium, lung, bladder, and brain [Cui et al., 2013]. Genetically engineered mice have been used to demonstrate that the granulosa tumor cells in ER beta-/- mice produce estrogen and have also a significant expression of ER alpha [Fan et al., 2010]. Other studies with knockout mice found an important correlation between the distribution and functions of ER alpha and ER beta, ER alpha being responsible for proliferation and ER beta for differentiation [Fuller et al., 2004]. However, ER alpha exhibits a crucial role in the neuroendocrine and reproductive systems, and it is the only receptor mandatory for negative feedback regulation of gonadotropin-releasing hormone cells [Couse, Korach, 1999; Christian et al., 2008]. Despite these findings, both ER alpha and ER beta are necessary for normal ovarian function [Cui et al., 2013].

Although it is widely accepted that estrogens manifest mitogenic effects in endometrial and breast carcinomas [Clemons, Goss, 2001; Plaza-Parrochia et al., 2017], and apoptosis inhibition in normal granulosa cells [Billig et al., 1993], their influence upon AGCT proliferation is not very clear [Haltia et al., 2020]. The estradiol effects are mediated by estrogen nuclear receptors alpha (ERalpha) and beta (ERbeta), both present in AGCTs [Chu et al., 2000; Ciucci et al., 2018]. Furthermore, it was described in AGCT another estrogen receptor, the membrane-bound G-protein linked (GPER1), through which estradiol can react [François et al., 2015; Haltia et al., 2020]. Several studies observed in AGCT a lower ERalpha expression comparative with ERbeta, which seems to be the main estrogen receptor in AGCT [Chu et al., 2000; Alexiadis et al., 2011], but the alpha form was increased by the FSH stimulation, which suggest a possible role in some of the AGCT [Haltia et al., 2020]. However, it was revealed, in prostate and breast cancer, that ERalpha is tumorigenic, whereas ERbeta acts as a tumor suppressor, inhibiting proliferation and stimulating apoptosis [Lazennec, 2006; Thomas, Gustafsson, 2011; Bonkhoff, 2018; Haltia et al., 2020].

It was also demonstrated that the transcription factor nuclear factor-kappaB (NF-κB) inhibits ER signaling, which has impact on ER-mediated transcription pathway, thus E2 is not functional even though the receptors are expressed in GCTs [Chu et al., 2004; François et al., 2015]. On the contrary, other data support the idea that ER signaling pathways provide a protective mechanism by ER alpha mutations, associated with the loss of both ERs [Kim, 2016].

These data are in agreement with our study results, which revealed ER alpha positivity in only nine (44.44%) cases, exhibiting a moderate extent of immunostaining. This feature could be related to the lack of thecal cells [Jamieson, Fuller, 2012] in nine (44.44%) tumors from our study group. The direct contribution of estrogen and ERs in GCT oncogenesis is controversial. In vitro studies using granulosa-like tumor cell lines suggested that ER beta is

likely to have an anti-proliferative role in GCT [Chu et al., 2004]. Moreover, it was demonstrated that ER beta represents an anti-proliferative factor in colon, breast, and prostate cancers [Zhao et al., 2008], and if estrogen action is significant for tumorigenesis, it is possible to involve the alpha subtype, which is known to be expressed at lower levels in GCT [Chu et al., 2000]. This speculation has led to the hypothesis that estrogen is likely to target stromal tissue and angio- genesis, rather than granulosa tumor cells, leading to huge implications in GCT treatment [Jamieson, Fuller, 2008; Kottarathil et al., 2013].

Inhibin represents a dimeric glycoprotein formed of an alpha subunit, covalently bound with a beta A and a beta B subunit, forming two heterodimers named inhibin A and inhibin B, respectively [Hohmann et al., 2005; Jamieson, Fuller, 2012]. In the ovary, inhibins are mainly secreted by granulosa cells, participating in the functional dynamics of the ovarian cycle, due to the particularities of their secretion. Therefore, inhibin A is considered the predominant type secreted during the late follicular and luteal phases of the ovarian cycle, while inhibin B is mainly produced in the early and midfollicular phases [Roberts et al., 1993; Hohmann et al., 2005]. It is also stated that beta subunits are primarily characteristic for granulosa cells, with beta A positive in all follicle stages and in the theca cells of the dominant follicle as well as corpus luteum, while beta B expression is limited to primary follicles [Roberts et al., 1993; Jamieson, Fuller, 2012].

Besides the normal inhibin production of granulosa cells and Sertoli cells, extragonadal inhibin expression was demonstrated in the adrenal gland, pituitary gland, liver, and placenta [Crawford et al., 1987; Meunier et al., 1988; McCluggage et al., 1997a; McCluggage et al., 1998a; McCluggage et al., 1998b]. Several studies described inhibin expression in almost all juvenile and adult GCT, Sertoli and Leydig cell tumors, sex cord stromal tumors (SCST) with annular tubules, steroid cell tumors, and gynandroblastomas [McCluggage et al., 1997b; Matias-Guiu, Prat, 1998; Matias-Guiu et al., 1998; Kommos et al., 1998; Zheng et al., 2003], less expression in fibrothecomas, fibromas, unclassified SCST, and complete absence in fibrosarcoma [Matias-Guiu et al., 1998; Rathore et al., 2017].

Inhibin has two main biological roles, suppressing pituitary FSH secretion and representing a potent growth factor for granulosa cells [Jamieson, Fuller, 2012]. In postmenopause, due to the exhaustion of the ovarian follicles, inhibin levels decrease dramatically [Burger et al., 1996]. This aspect has a clinical relevance in patients with GCT, where the inhibin levels are high [Jamieson, Fuller, 2012]. Numerous studies have revealed that granulosa cell tumors secrete elevated levels of inhibin, the role of potential marker for GCT in premenopausal and postmenopausal women being attributed to this hormone [Kottarathil et al., 2013], as an indicator of its biologically active phenotype [Lappöhn et al., 1989; Healy et al., 1993; Jobling et al., 1994]. Although a wide range of studies demonstrated that mainly inhibin A is an important immunohistochemical marker for granulosa cell tumors, recent data showed that inhibin B is the predominant form secreted in GCT, a feature which supports its value as an accurate marker for the detection of this tumor type [Mom et al., 2007; Kottarathil et al., 2013]. Other studies revealed that not all GCT express inhibin and thus, loss of inhibin expression can be correlated with higher tumor grade [Ala-Fossi et al., 2000].

In our study, inhibin A was positive in 14 (66.66%) cases, the cytoplasmic staining being predominantly moderate to severe and diffuse. These results are in agreement with other studies, reporting a variable expression and possible correlation of lack of expression with the poorly differentiated GCT [Ala-Fossi et al., 2000]. This pattern of expression can be also be related to the predominant type B of inhibin secreted by granulosa tumor cells [Mom et al., 2007; Kottarathil et al., 2013].

Considering that other types of ovarian tumors, especially mucinous variant of epithelial tumors, secrete inhibin, this hormone is not specific for GCT [Kottarathil et al.,

2013]. However, this marker is useful to differentiate GCTs and most sex cord stromal tumors from other epithelial neoplasms with similar histology. Usually, in these cases, the immunostaining is limited to non-tumoral stromal theca cells, and the intensity is weaker than the strong expression found in ovarian sex cord stromal tumors [McCluggage, 2000].

Calretinin immunoexpresion can be useful in the diagnosis of sex cord cell tumors, including granulosa cell tumors, according to numerous studies [McCluggage, Maxwell, 2001; Cao, Jones, 2001; Mayr et al., 2001; Moyahedi-Lankarani, Kurman, 2002; Yousefi et al., 2009]. Calretinin represents a 29-kDa calcium binding protein, which was initially discovered in neuronal tissue and afterwards within ovary in theca lutein and theca interna cells, hilar cells, mesothelial cells [Bertschy et al., 1998; Deavers et al., 2003; Lugli et al., 2003], and several ovarian SCST types [Bertschy et al., 1998; Cao, Jones, 2001; Fine, Li, 2003; Jones et al., 2010]. Calretinin, initially considered a specific marker for mesothelioma, calretinin has proved to be very sensitive but less specific than inhibin for ovarian SCST [Rathore et al., 2017].

The finding that inhibin is not expressed in all GCTs lead to the recommendation of other markers evaluation, e.g., calretinin, which has even a higher sensitivity than inhibin in AGCTs [Kommoss, Schmidt, 2007; Yousefi et al., 2009].

Although specific and sensitive for granulosa cell tumors [McCluggage, Maxwell, 2001; Cao, Jones, 2001], calretinin was found to have a stronger immunoexpression in fibromas and fibrothecomas compared with GCTs [Moyahedi-Lankarani, Kurman, 2002; Deavers et al., 2003]. This feature has a useful impact for the differential diagnosis between this tumor group and endometrial stromal sarcoma with fibroma-like phenotype [Young et al., 1984; Deavers et al., 2003].

In our study, calretinin presented positive immunoreactivity in 16 (77.77%) GCTs, with mostly strong and diffuse cytoplasmic staining of the tumor cells. Our results are consistent with the literature data, which revealed that calretinin sensitivity is superior to its specificity in GCTs [Shah et al., 2003, Kaspar, Crum, 2015]. As compared with inhibin, we found that calretinin demonstrated a slightly higher specificity and sensitivity than inhibin in AGCTs. This is in agreement with one report, which showed that calretinin was expressed in 100% of GCT cases whereas inhibin was identified in 73.9% of these tumors [Yousefi et al., 2009], while another survey demonstrated that calretinin and inhibin exhibited a comparable immunophenotype in GCTs, regarding the percentage of positive tumor cells and the degree of expression [Deavers et al., 2003].

Because other tumors, which can be part of differential diagnosis for GCTs, occasionally express inhibin and calretinin, it is recommended to be used within a larger panel of immunomarkers in difficult cases [Deavers et al., 2003; Sekkate et al., 2013]. In agreement with other studies, we emphasize that calretinin appears to be useful in the differential diagnosis, because of its higher sensitivity compared with inhibin in adult granulosa cell tumors [Shah et al., 2003; Kaspar, Crum, 2015].

The assessment of the rate of cell growth using Ki67 nuclear protein has long been studied in different types of tumors and its prognostic value has been demonstrated along with other clinicopathological factors [Miller et al., 2001; Rajagopal, Ramesh, 2016]. In ovarian tumors, Ki67 represents a useful proliferation parameter, which can predict the clinical course of GCTs [Rajagopal, Ramesh, 2016].

In our study, Ki67 was expressed in 12 out of 21 (57.14%) cases. The immunopositive cells showed a heterogeneous immunoreactivity, ranging from weak and focal to strong and diffuse. Ki67 index was high in five cases, being correlated with the higher stages of the tumors (pT1c2, pT2a, and pT2b), and one case with lymphovascular invasion, nuclear atypia, and more than two mitotic figures per 10 HPFs. These results are in agreement with the literature data, where Ki67 index was found to be higher in adult granulosa cell tumors with

an aggressive phenotype [Jozwicki et al., 2011; Sonoyama et al., 2015; Rajagopal, Ramesh, 2016], and less than 40% in typical GCTs [Mayr et al., 2001; Kondi-Pafiti et al., 2010]. In nine cases, Ki67 was negative, with no nuclear expression.

According to our findings, we can emphasize, in agreement with other studies, that in ovarian GCTs, Ki67 index is correlated with the clinical behavior and the stage of the tumor, the higher level being associated with higher tumor stage, and thus with a worse prognosis, and the lower Ki67 index with a better tumor prognosis [Sonoyama et al., 2015; Rajagopal, Ramesh, 2016].

Although most GCTs have a favorable prognosis, their behavior is unpredictable, with late recurrences and thus the patients need a thorough long-term follow-up. The histopathological assessment itself is not enough for a complete prediction of the clinical course. Together with other prognostic factors, like age, tumor size and capsule integrity, bilaterality, mitotic activity, atypia, Ki67 repre- sents a useful parameter for the assessment of the tumor clinical outcome. The finding of a recent study coordinated by Rajagopal & Ramesh [Rajagopal, Ramesh, 2016] of a superior Ki67 index value correlation with the clinical tumor stage compared to the differentiation grade demonstrates its value as a predictive marker of tumor behavior.

The immunohistochemical markers used in our study have proved to be reliable tools in the diagnosis and assessment of the prognostic of ovarian GCTs, along with other clinicopathological parameters. In agreement with other studies [Deavers et al., 2003; Yousefi et al., 2009], we concluded that calretinin and inhibin represent accurate markers for the diagnosis of GCTs. However, due to their different immunopatterns, their association is mandatory, as calretinin has proved to be more sensitive than inhibin A, whose specificity is higher in this tumor category.

However, because of still unexplained features of the ovarian tumors behavior, sometimes reflected in the controversial immunoexpression of these markers, additional studies with a higher number of cases and a larger IHC panel are necessary to better characterize these tumors and to build an appropriate algorithm for a better shortand long-term GCTs follow-up.

1.6.5. FINAL REMARKS

ER and calretinin immunoexpression can help in identification of the cell components of AGCT. Our results regarding Ki67 expression emphasize the potential utility of this marker in tumor behavior prediction. Although nonspecific, inhibin A immunopositivity has an important value in AGCT diagnosis in association to the other evaluated markers.

Additional studies are needed to identify new specific and sensitive markers for AGCT or, at least, a panel of markers which may contribute to a more accurate characterization of these tumors.

CHAPTER 2.
OBSTETRICAL PATHOLOGY

2.1. STATE OF THE ART

Placenta is a transient endocrine organ, representing the interface between fetus and mother during pregnancy, mediating important functions such as gas exchange, nutrients supply, waste products removal [Tsai et al., 2021; Aires, Dos Santos, 2015]. The most significant placental cell population is trophoblast, with various functions necessary for the formation, development and functioning of the placenta [Tsai et al., 2021; Lager, Powell, 2012]. Trophoblastic abnormalities negatively affect pregnancy, causing various complications, such as intrauterine growth restriction (IUGR), preterm delivery (PTD), gestational diabetes mellitus (GDM), and preeclampsia (PE) [Tsai et al., 2021].

IUGR, an obstetrical complication encounterd in about 10% of pregnancies, is associated with an increased risk of fetal morbidity and mortality, including different complications like low birth weight (under 10th percentile), cerebral palsy, perinatal hypoxia and asphyxia, newborn persistent pulmonary hypertension [Mejia et al., 2016; Bahr et al., 2014; Arroyo, Winn, 2008]. Moreover, other studies reported a risk of 44% of PTD correlated with IUGR, as well as longterm sequelae for IUGR, such as diabetes, heart disease, adult hypertension, or stroke [Tsai et al., 2021; Arroyo et al., 2008; Delpisheh et al., 2008].

mTOR (the mammalian target of rapamycin) protein, a serine/threonine protein kinase, responsible for cell growth regulation and protein transcription [Jansson et al., 2006; Wullschleger et al., 2006], is found to be located in human syncytiotrophoblast cells, suggesting its involvement in trophoblast proliferation and invasion [Tsai et al., 2021; Knuth et al., 2014; Arroyo et al., 2009]. Furthermore, studies demonstrated that mTOR inhibition decreases, in vitro, the trophoblast invasion, as well as active mTOR protein was decreased in PE and IUGR placentas, these findings being concordant with the nutrient dependence of mTOR protein activity [Tsai et al., 2021; Arroyo et al., 2009].

There are different macroscopic and microscopic features associated with IUGR placentas, which reflect various pathogenic mechanisms resulting from maternal-fetal interplay [Biron-Shental, 2016; Aviram et al., 2010].

Numerous studies highlight the connection between various telomere alterations and IUGR-associated placental insufficiency, damage of telomere homeostasis being able to affect IUGR pathophysiology, and thereby, the impair of different intrauterine pathways that will lead to the appearance of some diseases in adults [Biron-Shental., 2016; Biron-Shental et al., 2014]. In this regard, several studies, which used cultured cells, Southern blot analysis, quantitative FISH or PCR, revealed different telomere abnormalities length-related, like placental telomere shortening, due to decreased activity of human telomerase reverse transcriptase (hTERT) or decreased TERC (telomerase RNA component) gene copy, various number of telomere aggregates, enhanced senescence explained by a higher percentage of senescence associated heterochromatin foci (SAHF), and increased telomere capture [Toutain et al., 2013; Biron-Shental et al., 2011; Biron-Shental et al., 2010; Biron-Shental et al., 2010; Barker, 1995]. These findings confirm that telomere length is influenced by factors characteristic for placental insufficiency [Biron-Shental et al., 2016]. Another study found more pronounced lower levels of telomerase and short telomeres in trophoblasts from IUGR and PE complicated pregnancies,

suggesting a connection between telomere homeostasis, placental stress, and clinical features [Biron-Shental et al., 2016; Biron-Shental et al., 2010; Sukenik-Halevy et al., 2009].

IUGR placentas are characterized by a reduction in agiogenesis, due to alteration of protein expression of anti-angiogenic factor sVEGFR-1/sflt-1 (soluble receptor for Vascular Endothelial Growth Factor) and angiogenic factors PlGF/VEGF (Placental Growth Factor/Vascular Endothelial Growth Factor) [Gremlich et al., 2014; Arroyo, Winn, 2008]. Another study revealed that apoptosis, identified in normal syncytiotrophoblast and necessary for placental barrier integrity, was increased in IUGR placentas, this process being accompanied by Bcl-2 reduction and p53 and Bax increase [Borzsonyi et al., 2013; Heazell et al., 2011; Scifres, Nelson, 2009; Heazell, Crocker, 2008]. Moreover, the decrease in placental size in IUGR was assumed to be related to the inhibition of protein synthesis because of endoplasmic reticulum (ER) distress [Burton et al., 2009].

Starting from the fact that IUGR is associated with several serum maternal proteins, recent studies demonstrated that a specific fetal single nucleotide polymorphism (SNP) in the MMP2 (matrix metalloproteinase2) gene as well as high levels of IGFBP3 (insulin-like growth factor binding protein 3) in amniotic fluid were both correlated with an increased risk of developing IUGR, the latter appearing at the onset of the second trimester of pregnancy, while the risk of developing IUGR decreases with a fetal VNTR (variable number tandem repeat) polymorphism of IGF-I gene [Gremlich et al., 2014; Murisier-Petetin et al., 2009; Gremlich et al., 2007]. Furthermore, it was demonstrated that a long nuclear restricted non-coding RNA, named nuclear paraspeckle assembly transcript 1 (NEAT1), is elevated in villous trophoblast of IUGR term placentas, suggesting an increase in paraspeckles subnuclear structures, which control the nuclear hyperedited mRNAs retention and which increase in the villous trophoblast could explain the placental dysfunctions that characterize idiopathic IUGR [Gremlich et al., 2014; Clemson et al., 2009].

Placenta accreta represents a partial or total abnormal trophoblast invasion into the myometrium [Usta et al., 2005], the pathologic adherence ranging between direct adhesion to the muscular wall, without a decidual interface, and total invasion of the uterine wall thickness, including serosa or beyond it, extra-uterine structures may also be interested [Cahill et al., 2018].

The first detailed histological classification of this condition dates from 1966, when Lukes et al. took in consideration the extension degree of the villous penetration in the myometrium. Thus, according to the invasion depth, they described three classes of abnormal placental adherence: placenta adherent or creta (PC), placenta increta (PI), and placenta percreta (PP), as villi attach directly to the myometrium, villi penetrate the muscular wall, or they invade the full thickness of the myometrium and serosa, sometimes exceeding it and involving nearby organs and structures [Jauniaux et al., 2021; Luke et al., 1966]. Moreover, the researches found usually a heterogenous villous penetration, all the presented classes can coexist in the same specimen [Jauniaux et al., 2021; Luke et al., 1966]. Due to these findings, experts have introduced the term “placenta accreta spectrum” (PAS) to include all the degrees of abnormal placentation, PAS representing the landmark of a new proposed FIGO (Federation International of Gynecology and Obstetrics) classification [Jauniaux et al., 2021; Jauniaux et al., 2019].

PAS, this high-risk condition, whose prevalence has increased due to the rising incidence of cesarean section (CS), associates serious maternal morbidities, therefore it is recommended for the management of these patients, an experienced multidisciplinary team, to manage all the obstetrical complications [Jauniaux et al., 2021; Cahill et al., 2018].

Genetic abnormalities, which may affect the product of conception or the parents, lead to a predisposition for sporadic or recurrent pregnancy loss (RPL). The conceptus abnormalities can be represented by mutations, aneuploidy, single gene disorders, copy number changes, or skewed X inactivation. The best-studied genetic abnormality among parents with RPL is

balanced chromosomal translocations. With the exception of the latter, which still can be detected by karyotype, the other genetic abnormalities are detected by chromosomal microarray [Blue et al., 2019].

Roberts syndrome (RBS) represents a rare autosomal recessive genetic disorder caused by discrepancies of the ESCO2 (cohesion 1 homolog 2) gene [Zhu et al., 2022; Vega et al., 1993]. RBS is characterized by craniofacial abnormalities, tetraphocomelia, and prenatal and postnatal growth retardation, although other anomalies, such as hand, ear, cardiac or kidney abnormalities may occasionally affect individuals. Although the most severe cases have a reserved prognosis, the evolution being dominated by stillbirth, abortion or death in the first month [da Costa Almeida et al., 2020], the mildly afected patients can live to adulthood, but with severe degrees of intellectual disability [Zhu et al., 2022; Zhou et al., 2018].

Triploidy is a sporadic genetic disorder, without known risk factors or recurrence probability, being considered a lethal numeric chromosomal abnormality due to an extra haploid chromosome set [Oliveira et al., 2021; Kolarski et al., 2017]. The frequency of this chromosomal aberration is 17% from all spontaneous abortion, which appear during first trimester of pregnancy [Kolarski et al., 2017].

Most triploidy pregnancies end in spontaneous miscarriage during the first 17 weeks of pregnancy [Oliveira et al., 2021; Kolarski et al., 2017; Gardner et al., 2012; Huang et al., 2005]. There are described numerous birth defects which accompany this genetic disorder, like single umbilical artery, severe fetal growth or placental problems, hydatiform molar findings. Moreover, infants with this condition suffer from IUGR, multiple birth defects as craniofacial dysmorphism represented by cleft lip, micrognatia, heart or neural tube defects (spina bifida), serious kidney, limbs, and umbilical cord defects [Kolarski et al., 2017; Khong, George, 1992].

My interest for this scientific direction has materialized in the following achievements:

Articles

Butureanu T, **Balan RA***, Socolov R, Ioanid N, Socolov D, Gafitanu D. Retained placenta percreta with acquired uterine arteriovenous malformation - case report and short review of the literature. *Diagnostics* 2022; 12(4): 904.

Vişan V, **Balan RA***, Costea CF, Cărăuleanu A, Haba RM, Haba MSC, Socolov DG, Mogoş RAM, Bogdănici CM, Nemescu D, Tănase DM, Turliuc MD, Cucu AI, Scripcariu DV, Toma BF, Popovici RM, Ciocoiu M, Petrariu FD: Morphological and histopathological changes in placentas of pregnancies with intrauterine growth restriction. *Rom J Morphol Embryol* 2020; 61(2): 477-483.

Balan R, Radu VD, Giuşcă SE, Costache C, Ristescu C, Puia D, Onofrei P, Onofriescu M, Tănase A, Popovici R, Socolov D, Căruntu ID. A rare cause of massive hematuria: placenta percreta with bladder invasion. *In Vivo* 2021; 35(6): 3633-3639.

Socolov RV, Andreescu NI, Haliciu AM, Gorduza EV, Dumitrache F, **Balan RA**, Puiu M, Dobrescu MA, Socolov DG. Intrapartum diagnostic of Roberts syndrome - case presentation. *Rom J Morphol Embryol* 2015; 56(2): 585-588.

Socolov D, Mihălceanu E, Popovici D, Gorduza EV, **Balan R**, Martiniuc V, Socolov R. Prenatal diagnosis of triploidy in second trimester of pregnancy: a series of 4 cases over an eleven-year period. *Rev Rom Med Lab* 2015; 23(2): 213-220.

2.2. PLACENTA AND ITS RELATIONSHIP WITH INTRAUTERINE GROWTH RESTRICTION

2.2.1. INTRODUCTION

Intrauterine Growth Restriction (IUGR) or fetal growth restriction (FGR) represents a multifactorial disease and refers to the incapability of a fetus to achieve the appropriate estimated growth, with expected fetal weight below the 10th percentile calculated for its gestational age [Gremlich et al., 2014; ACOG, 2021]. Proper development and fetal growth are heavily influenced by the maternal oxygen supply and the necessary nutrients crossing the placenta. The different possible causes that impedes the fetus to reach its genetically potential weight are still under investigation and several theories have been put forward [Maulik et al., 2006]. Placental factors and hypoxemia are considered essential elements, which influence the intrauterine growth restriction (IUGR) and fetal death [Pardi et al., 2002].

This condition, which is the consequence of a combination of placental, maternal, and fetal factors, increases on the one hand the risk of neonatal morbidity or mortality and intrauterine fetal death, and the probability of developing metabolic syndrome in adulthood, on the other hand [Gremlich et al., 2014; Biron-Shental et al., 2016].

The main cause of IUGR is abnormal placentation, which causes placental insufficiency, with later consequences for the fetus [Salafia et al., 1995; Biron-Shental et al., 2016; ACOG, 2021]. Intrauterine injuries can affect the normal fetal structure and development, with long-term consequences that may occur in adulthood in the form of stroke, coronary artery disease, type 2 diabetes mellitus, and metabolic syndrome [Aviram et al., 2010; Hales, Barker, 2001; Barker, 2006; Pallotto, Kilbride, 2006; Zohdi et al., 2012; Biron-Shental et al., 2016].

Different placental structures and functions may be involved and this leads to anatomical, physiological, vascular and other morphological abnormalities [Maulik, 2006]. Various gross morphological changes of the placenta have been mentioned, such as low placental weight [Heinonen et al., 2001; Fox, 2003; Biswas, Ghosh, 2008] or modified fetal/placental weight ratio [Sornes, 2000; Biswas, Ghosh, 2008], umbilical cord abnormalities, such as insertion type, anomalies of length and thickness, knots, and infarcts [Salafia et al., 1995; Biron-Shental et al., 2016; Peesak, 2017]. Histological changes, such as chronic villitis, avascular villi, perivillous fibrinoid deposition, cytotrophoblast hyperplasia and basement membrane thickening, have all been associated with FGR, explaining chronic placental ischemia caused by feto-maternal blood supply anomalies, as well as inhibition of protein synthesis due to endoplasmic reticulum stress [Park et al., 2002; Egbor et al., 2006; Aviram et al., 2010; Benirschke et al., 2012; Biron-Shental et al., 2016].

In order to enhance current clinical knowledge of the etiology of IUGR, anatomopathological examinations of placentas could be extremely revealing [Vedmedovska et al., 2011].

Aim

This study aimed to assess the macroscopic and histopathological features, as well as the immunoexpression of CD31 (cluster of differentiation 31) and collagen IV in placenta specimens from pregnancies complicated with IUGR, in order to establish any clinicomorphological correlations, which could explain the mechanisms that characterize FGR.

2.2.2. MATERIAL AND METHODS

The study included third-trimester pregnant patients, admitted to the “Cuza Vodă” Clinical Hospital of Obstetrics and Gynecology, Iaşi, Romania, between 2017-2020. The

research was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy and "Cuza Voda" Clinical Hospital of Obstetrics and Gynecology, based on the patients' informed consent on the usage of their biologic material leftover after diagnostic testing, in accordance with the ethical standards of Helsinki declaration.

Patients

We collected 42 placentas soon after delivery, divided in two groups. Of these, 32 samples came from previously diagnosed cases with IUGR and were included in the first study group. Ten other placentas were selected from normal pregnancies, representing the control group.

The main macroscopic parameters that were assessed included: placental dimensions, membranes appearance, umbilical cord insertion, number of umbilical cord vessels, umbilical cord diameter. These parameters where afterwards corroborated with the histopathological and immunohistochemical characteristics.

We systematically evaluated the following morphological features of the placentas: vasculitis, villous infarction, chronic villitis, villous hypoplasia, intervillous thrombi or hematomas, perivillous and basal plate fibrin deposition, cytotrophoblast proliferation, thickening of trophoblastic basal membrane, stromal fibrosis, and multifocal chorangiomatosis.

The fetal related exclusion criteria were the existence of a chromosomal or congenital anomaly, hydrops fetalis or multiple pregnancies. The maternal exclusion criteria were diabetes mellitus, pre-eclampsia and eclampsia, genitourinary infections, sepsis, antenatal hemorrhage (vasa praevia and placenta praevia, abruption placentae) and systemic disorders.

Fetal biometric measurements obtained through ultrasonography (femur length, abdominal circumference, biparietal diameter, head circumference and estimated weight) were used in order to establish the diagnosis of IUGR.

Histopathological exam

Sections obtained from placental specimens were investigated by routine histopathological examination, being fixed in buffered formalin for 24 hours and processed for paraffin embedding. For microscopic assessment of the above-mentioned placental morphological aspects, 4–5 μm serial sections were stained with Hematoxylin–Eosin (HE) (both study and control group) and Alcian Blue/Periodic Acid–Schiff (PAS) (study group), and furthermore prepared for immunohistochemical (IHC) examination (study group).

Immunohistochemical exam

IHC examination with type IV collagen mouse monoclonal antibody (CIV22, Cell Marque), CD34 mouse monoclonal antibody (QBEND10, Cell Marque), and CD31 mouse monoclonal antibody (JC70, Cell Marque) used automated IHC BenchMark ULTRA Protocol with ultraView Universal 3,3'-Diaminobenzidine (DAB) Detection Kit (Tabel 2.1). The counterstaining was performed using Hematoxylin II-Ventana.

Tabel 2.1. The antibodies used for immunohistochemical assessment

ANTIBODY	MANUFACTURER	CLONE	CLASS	LOCALIZATION
CD34	Cell Marque, USA	QBEND10	Mouse monoclonal	Cell Membrane
CD31	Cell Marque, USA	JC70	Mouse monoclonal	Cell Membrane
Collagen IV	Cell Marque, USA	CIV22	Mouse monoclonal	Basement membrane

Positive controls were performed in order to verify the technique accuracy.

For semi-quantitative assessment of the antibodies, we considered the following classification of the immunoexpression: 0 for no staining, 1+ for weak staining, 2+ for moderate staining, and 3+ for strong staining.

Statistical analysis

We used Microsoft Excel to create our database. Data were assessed using Statistical Package for the Social Sciences (SPSS) v. 20, and considered statistically significant when the p value was less than 0.05. The results of the univariate analysis were reported as mean ± standard deviation (SD) for continuous variables.

2.2.3. RESULTS

Clinical aspects of patients from study and control groups

Clinical characteristics of patients included in this study are illustrated in Table 2.2.

There was no significant age difference between the mothers included in the two groups. Gestational ages, fetuses' weights at birth, and measured placental dimensions were significantly different in the two groups ($p<0.05$).

As expected, we observed a much higher incidence of premature birth, defined as birth before 37 weeks of pregnancy, in the study group (70.2%), while in control group only 2.77% were cases of premature births. We acknowledged a birth weight in the study group of 2059.45±669.87g, compared to 3277.94±379.03g in control group. Mean gestational age of the fetuses diagnosed with IUGR was lower at birth (35.75±3.2 weeks) than in the control group (38.14±0.85 weeks).

Table 2.2. The clinical characteristics in study and control groups (data given as numbers)

CLINICAL FEATURES	STUDY GROUP	CONTROL GROUP
Mothers' age (years, +/- SD)	29.03+/-5.664	29.24+/-6.030
Birth gestational age (weeks, mean +/- SD)	35.75+/-3.2	38.14+/-0.85
Birth weight (gram, mean +/- SD)	2059.45+/-669.87	3277.94+/-379.03
Premature birth (< 37 weeks)	70.2%	2.77%

Macroscopic features of the placentas and the umbilical cords

Macroscopic examination of placenta and umbilical cord from the study group revealed that these placentas tend to be smaller than those collected for the control group (Table 2.3).

Table 2.3. Placentas dimensions

MEASURED DIMENSIONS	STUDY GROUP	CONTROL GROUP	P
Length (cm)	15.75+/-6.22	21+/-2.3	<0.05
Width (cm)	12.12+/-2.23	16+/-3.6	<0.05
Depth (cm)	1.2+/-0.38	2.3+/-0.3	<0.05

Noteworthy is the higher incidence of umbilical cord anomalies from the placentas belonging to study group, including velamentous attachment to the chorionic disc (34.2%) or eccentric insertion (19.2%), abnormal number of vessels or even ruptured vessels (3.125%), and torsion of the umbilical cord (6.25%) (Table 2.4). The placentas in the control group showed no abnormalities of the umbilical cord.

Table 2.4. Macroscopic features of the study group umbilical cords (percentage)

TYPE OF UMBILICAL CORD	STUDY GROUP
Umbilical cord attachment to the chorionic disc	
Central	46.6% (15)
Eccentric	19.2% (6)
Velamentous	34.2% (11)
Number of umbilical cord vessels	
2	21.87% (7)
3	71.13% (25)
Ruptured umbilical cord	3.125% (1)
Umbilical Cord Torsion	6.25% (2)

Histopathological examination of the placentas

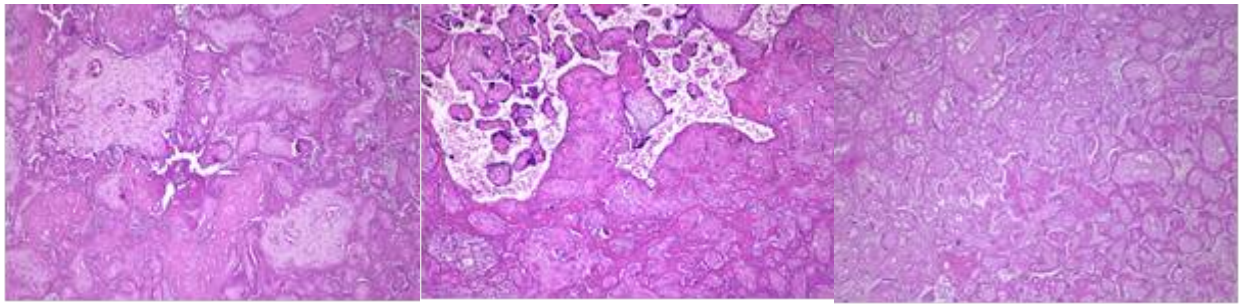
The most common histopathological aspects identified and illustrated by HE and Alcian Blue/PAS stainings were as follows: villous infarction, parietal vascular thrombosis, perivillous and basal plate fibrinoid deposition, syncytiotrophoblastic knots, thickening of trophoblastic basal membrane, stromal fibrosis, multifocal chorangiomatosis, and villous hypoplasia (Table 2.5).

Table 2.5. Histopathological features of the placentas

MICROSCOPIC FINDINGS	STUDY GROUP (%)	CONTROL GROUP (%)
Infarction	28 (87.5%)	5 (5%)
Thrombosis	25 (78.12%)	4 (40%)
Perivillous fibrinoid	32 (100%)	6 (60%)
Calcifications	20 (62.5%)	2 (2%)
Basement membrane thickening	28 (87.5%)	3 (3%)
Stromal fibrosis	28 (87.5%)	3 (30%)
Chorangiomatosis	18 (56.25%)	-
Villous hypoplasia	26 (81.25%)	2 (2%)
Placental hematoma	16 (50%)	-
Syncytiotrophoblastic knots	24 (75%)	2 (2%)

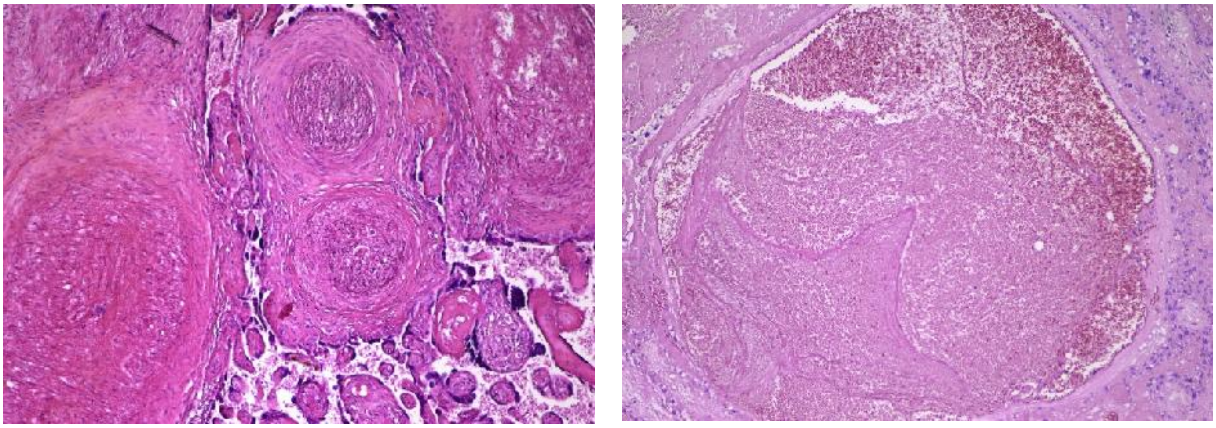
Areas of infarction, sometimes associated with distal villous hypoplasia (rare, small, sometimes filiform villi), and intervillous thrombosis were more numerous in placental cotyledons coming from study group fetuses (Figure 2.1 – a-c; Figure 2.2 – a, b).

During the examinations, we were able to identify different stages of the process, from new, small infarcted areas, to old, extensive regions. It was observed that the incidence of villous infarction was higher in the study group (87.5%) than in the control group (50%). The most frequent histopathological feature linked with infarction or thrombosis was chronic villitis, among perivillous fibrinoid deposits. Perivillous fibrin deposition (Figure 2.3 – a-c; Figure 2.4 – a, b) was more frequently present within the IUGR group (93.75%) than in the control group (60%).



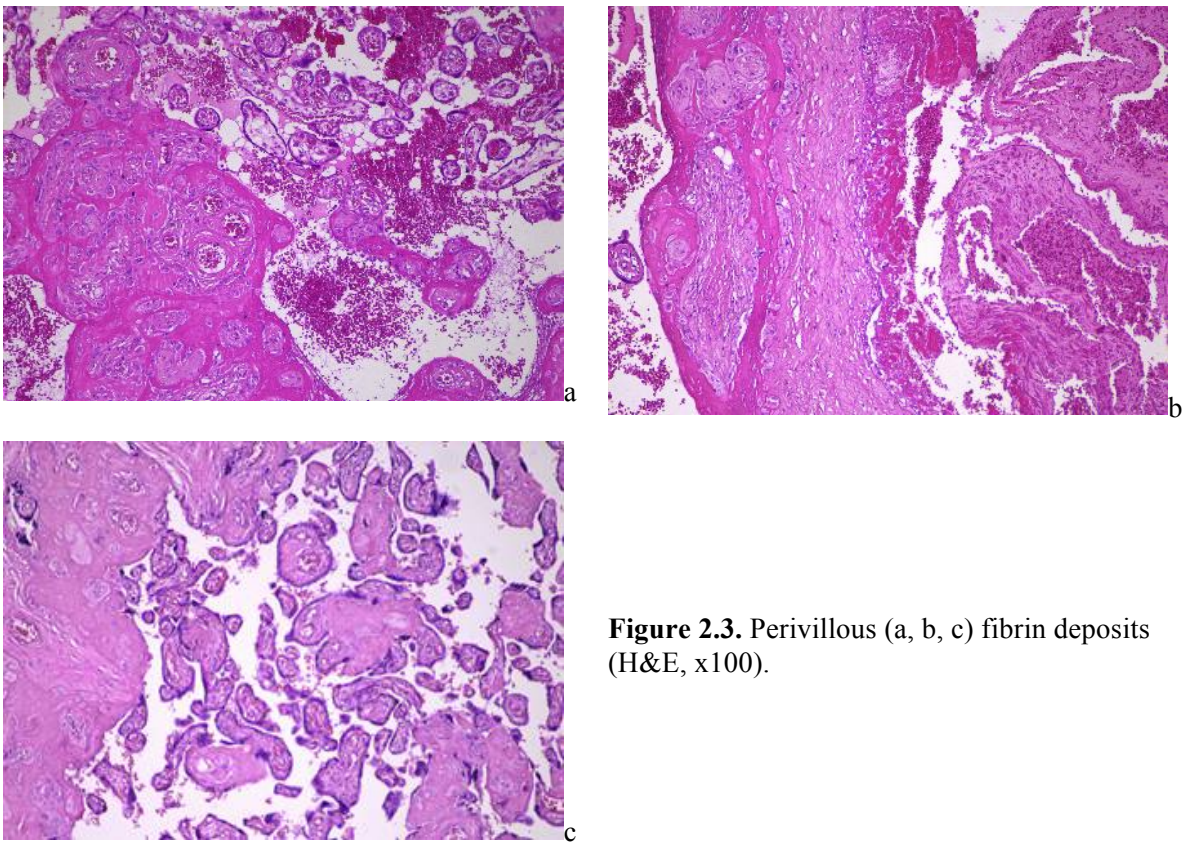
a b c

Figure 2.1. Subacute and chronic placental infarction (a and b) with distal villous hypoplasia (c) (H&E, x100).



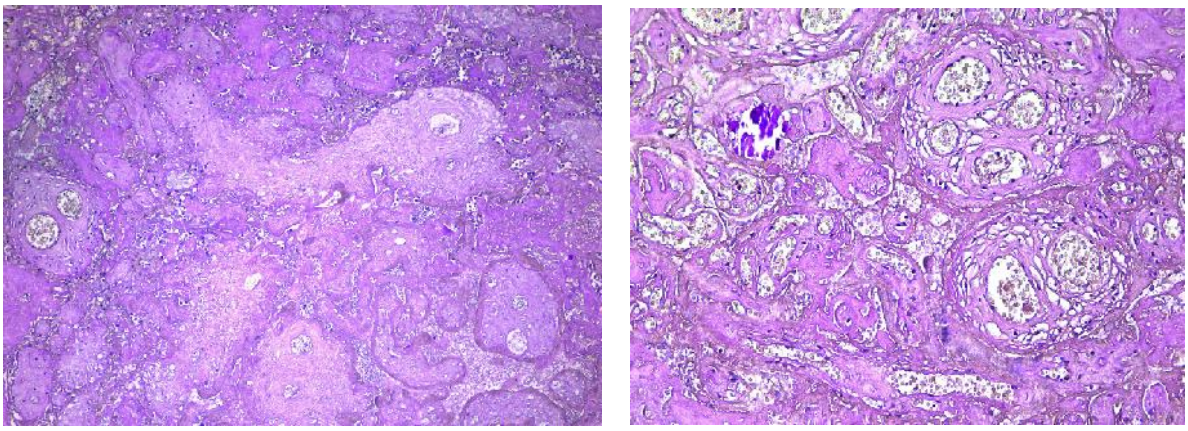
a b

Figure 2.2. Parietal vascular thrombosis (H&E, x100).



a b c

Figure 2.3. Perivillous (a, b, c) fibrin deposits (H&E, x100).



a b

Figure 2.4. Massive perivillous fibrinoid deposition (a) and calcifications (b) (Alcian Blue/PAS: a x100; b x200).

Placental hematoma (Figure 2.5 – a, b) as well as calcifications (Figure 2.6 – a) were also encountered in most cases of the study group (81.25% versus 0%; 62.5% versus 2%), affecting the uteroplacental circulation. Stromal fibrosis, multifocal villous chorangiomatosis, syncytiotrophoblastic knots (Figure 2.6 – a, b), as well as basement membrane thickening (Figure 2.4 – a, b) were also observed more frequently in the study group than in the control group (Table 2.4).

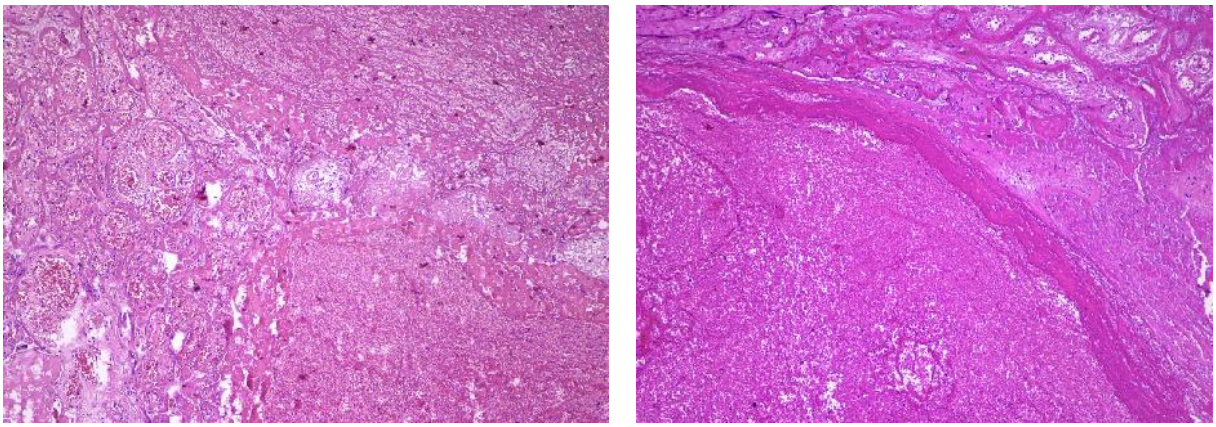


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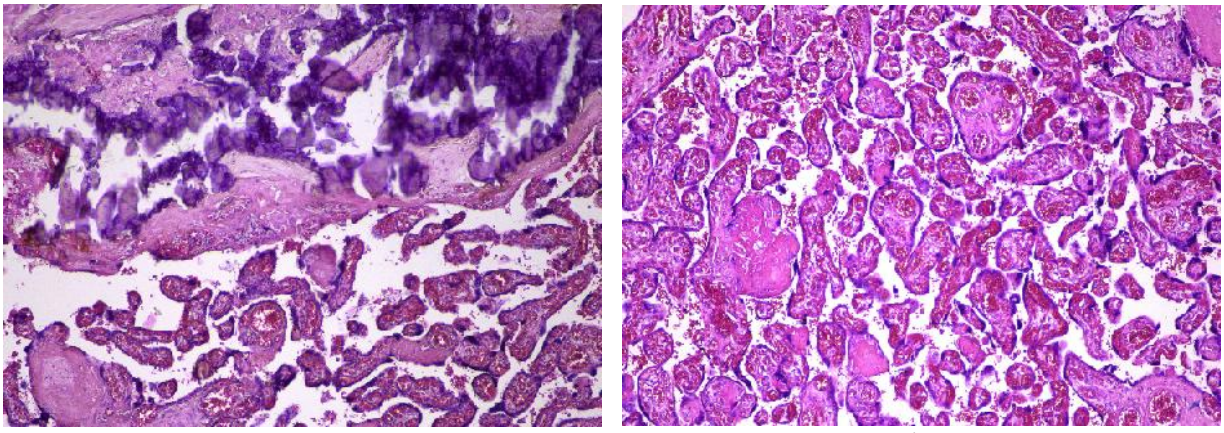
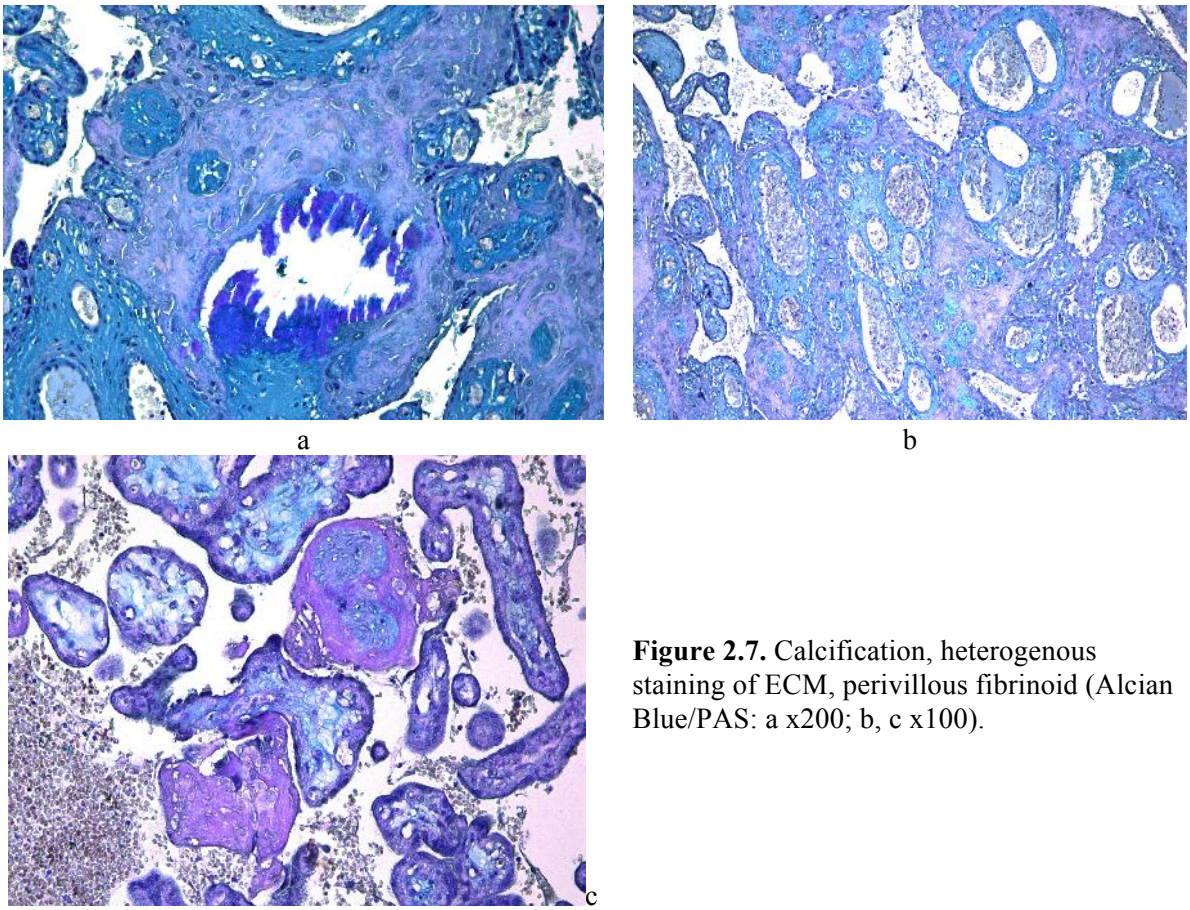
Figure 2.5. Intraplacental hematoma (H&E, x100).



a b

Figure 2.6. Chorangiomatosis (a, b) and extensive calcifications (a) (H&E, x100).

A particular aspect, highlighted by the Alcian Blue/PAS staining, was the tinctorial heterogeneity observed in the villous extracellular matrix (ECM), in the basement membrane, as well as in the vascular walls, expressed through various shades of magenta and blue, due to the staining of fibrinoid and of placental specific glycosaminoglycans (GAGs) and proteoglycans (PGs), with variable ECM and perivascular localization (Figure 2.7 – a, b, c; Figure 2.4 – a, b).



a b c

Figure 2.7. Calcification, heterogenous staining of ECM, perivillous fibrinoid (Alcian Blue/PAS: a x200; b, c x100).

Immunohistochemical exam

IHC evaluation highlighted and confirmed the histopathological findings.

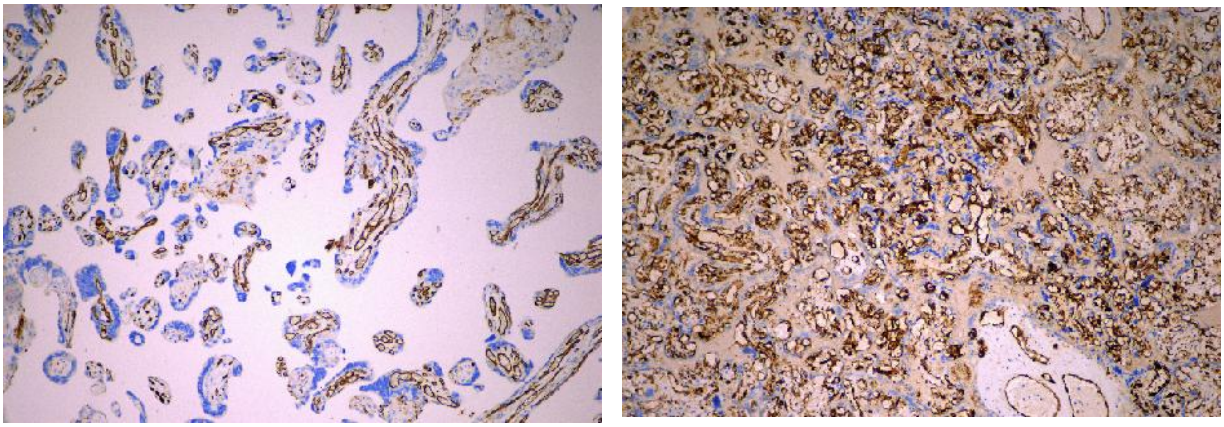
In this regard, the IHC assessment of CD31 presented a moderate to strong positive immunoexpression in endothelial cells from villous capillaries (Figures 2.8 – a, b), highlighting also the areas with chorangiomatosis, and a weak to negative immunoexpression in the infarcted areas and those exposed to massive perivillous fibrinoid deposition (Table 2.6).

Table 2.6. CD31, CD34, and Collagen IV immunoexpressions in the study group placentas

SEMI-QUANTITATIVE IMMUNOSCORING	CD31 IMMUNOSCORE	CD34 IMMUNOSCORE	COLLAGEN IV IMMUNOSCORE
0	-	-	4 (12.5%)
1+	2 (6.25%)	2 (6.25%)	1 (3.12%)
2+	4 (12.5%)	3 (9.37%)	2 (6.25%)
3+	26 (81.25%)	27 (84.37%)	25 (78.12%)

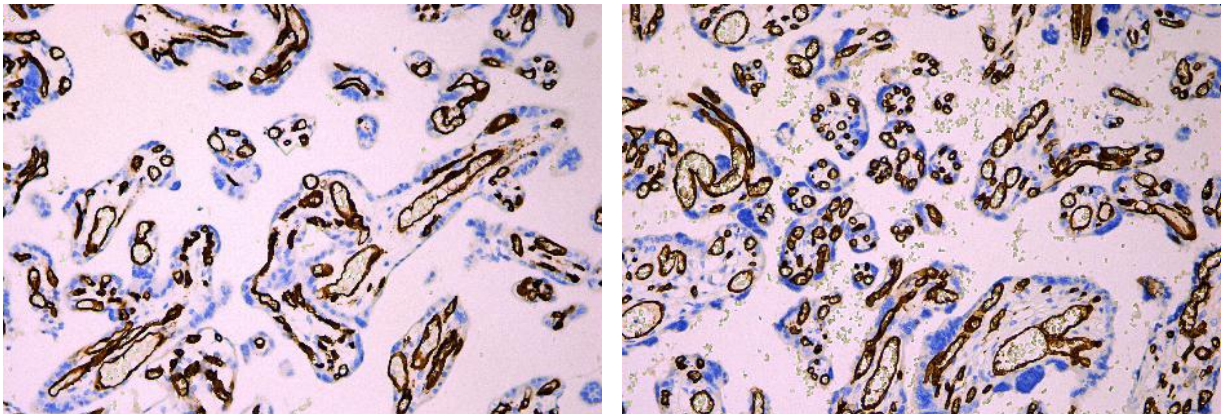
CD34 immunoexpression was also strong in endothelium of villous capillaries, confirming villous chorangiomatosis (Figures 2.9 – a, b).

Most of the cases from the study group presented a strong immunoreaction of the collagen IV, marking the thickening of the villous basement membrane (Figures 2.10 – a, b; Table 2.6).



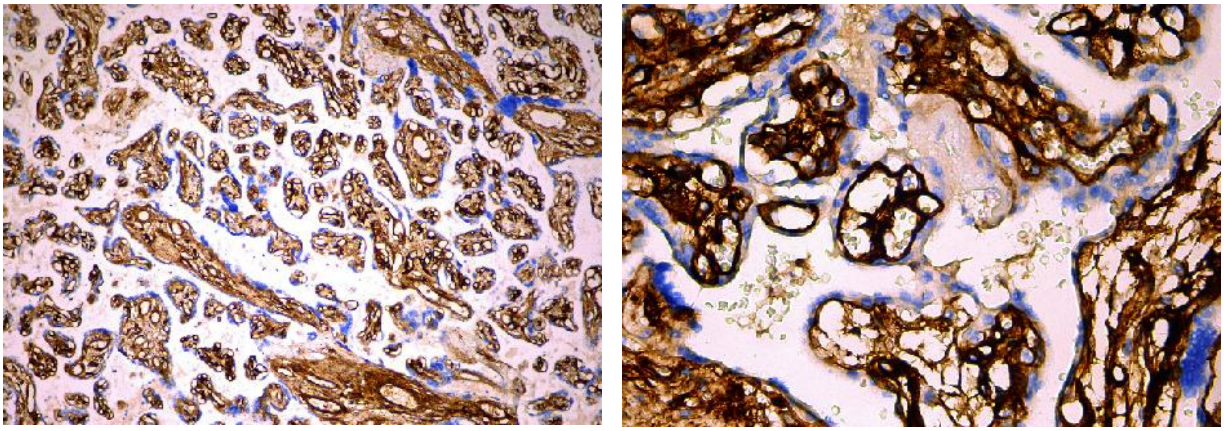
a b

Figure 2.8. a) Moderate and strong CD31 immunoexpression in terminal and stem villi, with numerous syncytiotrophoblastic knots; b) strong CD31 immunoexpression highlighting villous chorangiomatosis (anti-CD3, a x100; b x100).



a b

Figure 2.9. a) Strong CD34 immunoexpression in terminal villi, with numerous syncytiotrophoblastic knots; b) Strong CD34 immunoexpression highlighting villous chorangiomatosis (anti-CD34, a x100; b x200).



a b

Figure 2.10. Strong Collagen IV immunoexpression of the trophoblast basement membrane and ECM in the terminal and stem villi – a, b (anti-collagen IV, a x100; b x400).

2.2.4. DISCUSSIONS

Our study provides a comprehensive analysis of anatomopathological aspects in pregnancies that resulted in IUGR. We focused on both macroscopic and microscopic aspects of the lesions from the collected specimens, as well as the immunoreaction of CD31 and CD34 endothelial markers and collagen IV, trying to identify any correlation with clinical and ultrasound parameters that could explain the evolution of these complicated pregnancies.

In this study, we managed to show that there is a significant correlation between the placental dimensions, including the placental volume, and the birthweight of the fetuses.

In order to have a homogenous classification, we defined villous infarction as a localized area of ischemic villous necrosis. An intervillous thrombus was characterized as a contained clot found in the intervillous space [Kraus et al., 2004]. We considered chronic villitis an infiltration of the stroma by maternal T-lymphocytes [Redline, 2007] and perivillous fibrin deposition as the presence of fibers with a measured thickness less than 10 mm, and specific cross-striation of the filaments [Benirschke et al., 2012]. Trophoblastic basal membrane thickening was defined as an increased thickness of villi, suggestive of an elevated secretion of basal lamina molecules [Benirschke et al., 2012].

Previous studies showed that the placental weight best correlates with FGR [Vedmedovska et al., 2011]. However, Jakó et al. revealed that the placenta volume measured at the time of delivery presented a stronger correlation with birthweight, compared to the association between placental weight and birthweight [Jakó et al., 2019]. This encourages the necessity of developing reliable methods for a more accurate examination of the placental dimensions *in utero*, in order to better evaluate the growth restriction. Furthermore, our study shows significant differences between the smallest placental diameter of the two groups of patients, with IUGR and the control group. This parameter together with the other placental dimensions are feasible to measure antenatally through echography, considering them as prognostic factors. Ultrasound can be used to evaluate the placental diameters and volume more precisely than placental weight [Larsen et al., 2016].

Current research focuses intensely on investigating also the utility of magnetic resonance imaging (MRI) in assessing the placental volume and shape. Consequently, MRI may provide additional information on the morphological characteristics of the placenta. Studies showed that placentas in IUGR pregnancies present more oval and thicker aspect in MRI acquisitions than placentas of normal pregnancies, which have a circular outline [Dahdouh et al., 2018]. The morphological measurements we obtained are in line with these findings. Isakov et al. tried to identify and characterize a percentile curve for the placental volume. According to multiple systematic performable measurements, they proposed novel diagnostic parameters for the clinical practice [Isakov et al., 2018]. Regarding the trimester in which placental measurements are relevant, three-dimensional ultrasonographical measurements in the first trimester can be moderately correlated with fetal weight. On the other hand, measurements taken in the second trimester are associated with good predictive values (up to 45%), can be applied in a clinical setting for screening high-risk pregnancies [Quant et al., 2016]. As for the umbilical cord, our study reveals that almost 2/3 of the specimens that we examined showed either an eccentric or a velamentous insertion and were correlated with IUGR ($p<0.05$). Similar findings were reported by many other studies [Cai et al., 2006; Kent et al., 2011; Manolea et al., 2015], while Brouillet et al. showed that noncentral cord insertion (paracentral, velamentous) can be correlated with FGR and low birth weight in single fetus pregnancies [Brouillet et al., 2014]. Regarding the relationship between peripheral cord insertion and IUGR, we might assume, based on mathematical and geometrical principles, that a central umbilical cord insertion is required in order to provide an equal distribution and exchange of blood.

Although placental infarction appears in both normal and abnormal pregnancies, this lesion presents clinico-morphological significance when it affects at least an average of 10–20% of the placental volume. The relationship between fetal hypoxia, subsequent IUGR and the presence of multiple placental infarction lesion has already been reported in literature [Günyeli et al., 2011]. In the present study, different degrees of placental infarction were detected in 28 (87.5%) cases of the study group, results similar with those previously published [Novac et al., 2018]. In our study group, we reported 25 cases with villous thrombosis, highlighting the literature results, which suggest that vascular fetal thrombotic disorders are commonly found in women with adverse pregnancy outcomes [Arias et al., 1998].

The histopathological evaluation of the placenta also reveals in the study group other morphopathological changes, most of them in a higher proportion than in the control group: intervillous thrombi, diffuse calcifications, avascular terminal villi, chronic villitis, villous hypoplasia, syncytiotrophoblastic knots, thickening of the basement membrane, multifocal chorangiomatosis.

Although not specific to IUGR pregnancies, research reveals that these lesions are linked with IUGR, systemic autoimmune disorders, intrauterine infection and sepsis, genetic disorders, toxic substances, abnormal interaction between host and the placenta, and confined placental mosaicism [Katzman, Genest, 2002; Syridou et al., 2008]. Redline et al. revealed that the massive deposition of intervillous fibrin presented strong correlation with placental and fetal weight [Redline et al., 2004]. Based on existing knowledge, diffuse calcification often occurs in mature placentas and reflects placental senescence, but there is a relationship between preterm placental calcification and adverse pregnancy outcome [Chen et al., 2011]. İskender-Mazman et al. found no relationship between IUGR and diffuse dystrophic calcification [İskender-Mazman et al., 2014].

The composition and level of endometrial glycosaminoglycans (GAGs) and proteoglycans (PGs) change during pregnancy, according to the hormonal variations. GAGs are necessary for uterine and placental development, as well as during birth and postpartum uterine involution, due to the regulatory functions of EMC macromolecules [de Oliveira et al., 2015]. GAGs and PGs are also important in the fertilization and implantation processes, as well as in the decidualization. At birth, the concentration of hyaluronic acid (HA) decreases, but increase progressively during pregnancy [de Oliveira et al., 2015]. At the same time, high levels of matrix metalloproteinases (MMPs) contribute to tissue remodeling processes and postpartum uterine involution [de Oliveira et al., 2015].

Placental maturation brings with it changes in the composition and molecular structure of GAGs, resulting in varying proportions in EMC. These structural changes can affect the molecular transport in the extracellular matrix, affecting the fetal growth rate, with variations during pregnancy [de Oliveira et al., 2015].

At the placental level, two types of PG are described: one type with heparan sulfate (HS) and the other one with chondroitin sulfate (CS) and dermatan sulfate (DS). The first category is represented by syndecan and perlecan, and the second category contains decorin and biglican [Yang et al., 2005; Chen et al., 2007; Said, 2011]. Studies have shown that syndecan 1 is present in syncytiotrophoblast (ST) [Rajaraman, 2009], while decorin in the stroma and around the villous blood vessels [Swan et al., 2010]. Biglican was evidentiated in endothelium and muscle fibers from the vascular wall [Murthi et al., 2010]. Other studies have shown that in IUGR, the levels of decorin and biglican are low [Said, 2011]. As these two molecules, which have significant anticoagulant functions, are located close to blood vessels, it may be stipulated that local anticoagulant activity can be reduced in IUGR placenta [Giri, Tollefsen, 2006; Said, 2011]. Moreover, decorin inhibits trophoblast migration and proliferation, leading to another mechanism of IUGR pathogenesis [Iacob et al., 2008; Said, 2011].

Given the dynamics of placental EMC macromolecules during pregnancy, which also characterize the matrix of placenta from different pregnancy complications such as IUGR, the observed heterogeneous appearance given by the different tinctoriality of villous EMC, in our study, possibly reflects the pathogenic mechanisms, which lead to the described functional alterations. A wider panel of antibodies targeting placental specific EMC molecules could help decipher the altered placental proteoglycans homeostasis characteristic to IUGR.

The immunohistochemical evaluation of CD31 and CD34 endothelial markers in the study group revealed a strong immunoreaction in villous endothelial cells, emphasizing its importance as placental endothelial markers [Novac et al., 2018; Stanek, Abdaljaleel, 2019], as well as a complementary IHC parameter in the diagnosis of villous chorangiomatosis or other fetal vasculopathy associated with IUGR. The strong immunoreactivity of the anti-collagen IV antibody in almost all cases of the study group suggests and completes the already published studies, which demonstrate the existence of type IV collagen outside the basement membrane, being described also in the mesenchyme of the placental villi [Oefner et al., 2015]. This accumulation of collagen IV in the villous extracellular matrix could support and explain, besides the invasive character of the trophoblast at the implantation site, the impairment of the maternal–fetal inductive relationships, with possible restriction of nutrient circulation, which can lead to fetal IUGR.

2.2.5. FINAL REMARKS

Our study revealed a correlation between the placental diameter and volume and the presence of IUGR. Furthermore, the remark suggesting that the localization of umbilical cord insertion is correlated with the fetus weight is in concordance with literature data.

The most common histological finding in our study group was placental infarction, which was linked to IUGR, but a certain causality could not be demonstrated, as this finding was also present in normal pregnancies. The tinctorial variability given by the dynamics of PGs and GAGs in placental EMC could be a key to elucidating the mechanisms at the maternal-fetal interface leading to IUGR.

CD31 and CD34 positive immunoexpressions of the villous endothelial cells can represent supplementary parameters in the diagnosis of chorioangiomatosis or other fetal vasculopathy associated with IUGR. The strong immunoexpression of collagen IV inside or outside the basement membrane of the placental villous structures could explain the disturbance of the specific maternal–fetal inductive relationships from IUGR pregnancies. Further larger studies with extended panel immunomarkers are needed in order to determine and complete the role of these morphological changes in the development of IUGR.

2.3. MULTIFACETED PLACENTA – PARTICULAR ASPECTS

2.3.1. INTRODUCTION

Placenta percreta represents the most severe form of abnormal trophoblastic adherence beyond the decidua basalis, among the three representatives of the placenta accreta spectrum (PAS), a rare condition with reported incidence of 1/500 to ½/500 pregnancies [Wu et al., 2005; Konijeti et al., 2009]. PAS includes 75-80% cases of placenta accreta vera (less than 50% myometrial invasion by the trophoblast), 17% cases of placenta increta (more than 50% myometrial invasion by the trophoblast), and 5% cases of percreta (invasion of uterine serosa and neighboring pelvic organs) [Konijeti et al., 2009; Piñas Carrillo, Chandraharan, 2019]. Moreover, the abnormal adherence can be complete (throughout the entire placenta), partial (limited to only one or more cotyledons), or focal (in isolated areas) [Gersell, Kraus, 2020]. In

this regard, the placenta percreta is no longer able to detach spontaneously after delivery, leading to serious complications, such as massive life-threatening maternal hemorrhage, which often requires a hysterectomy surgical approach [Oppenheimer, 2007; Wortman, Alexander, 2013; Kohi et al., 2015; Zhong et al., 2017; Morlando, Collins, 2020].

More than 2 caesarean sections (CS), including shorter intervals between previous CS and current pregnancy (less than 2 years), and concurrent placenta praevia (in 75% of cases) are the most common major risk factors, followed by advanced maternal age, multiparity, endometritis, hypertension, assisted reproductive technology, submucosal leiomyomas, other uterine surgeries and anomalies (poor quality of scarring and CS performed on long time ruptured membranes leading to chorioamniotitis), smoking [Abbas et al., 2000; Bartels et al., 2018; Paniza et al., 2018; Piñas Carrillo, Chandraharan, 2019; Okunowo et al., 2019; Kyozuka et al., 2019]. Because of the rise in the numbers of CS performed in recent years, the risk of PAS disorders is increasing up to 10 times in the last 50 years [Smith et al., 2014; Koukoura et al., 2017].

The information regardless of treatment of the placenta percreta with bladder invasion are limited, because of the exceptional rarity of this event. Approximatively 70 cases are reported as case presentation, the largest series comprising 54 patients [Washecka, Behling, 2002; Jain et al., 2020; Zhao et al., 2021]. The suspicion or the early diagnosis of PAS disorders is based on the theoretical knowledge of the risk factors in relation with the patient's obstetrical and general medical history. Supplementary, the ultrasonography or magnetic resonance imaging (MRI), when ultrasound cannot provide specific data, contribute to the clinical diagnosis. The ultrasound diagnosis, with combined grayscale and color Doppler images, is the main choice for the assessment of antepartum or postpartum hemorrhage due to abnormal placentation or retained placenta [Abramowicz, Sheiner, 2007; Kohi et al., 2015]. Early diagnosis is crucial in order to prevent shortterm and long-term maternal complications [Sellmyer, 2013; Kohi et al., 2015].

Most placenta accreta are asymptomatic to term and if diagnosed by ultrasound during pregnancy, can be scheduled in special centers and resolved in multidisciplinary teams [Konijeti et al., 2009; Piñas Carrillo, Chandraharan, 2019; Tillu et al., 2019]. The review of the literature shows that it is mandatory for the placenta percreta to be diagnosed early during pregnancy, because of the serious fetal and maternal implications of this condition. These pregnancies can be complicated at birth, during the detachment of the placenta from the uterus, producing massive hemorrhage and trauma to neighboring organs in which the invasion occurred (bladder and rarely the ureters, rectum, iliac vessels). Uterine rupture and placental abruption represent the differential diagnosis for bladder bleeding induced by placenta percreta [Zhao et al., 2021]. After birth, postpartum hemorrhage manifested by vaginal bleeding [Sijanović et al., 2011; Smith et al., 2014] and more rarely hematuria [Aho et al., 1985; Abbas et al., 2000; Washecka, Behling, 2002; Takai et al., 2005; Koukoura et al., 2017] can appear. Therefore, an optimal therapeutic strategy is mandatory [Tillu et al., 2019; Piñas Carrillo, Chandraharan, 2019].

Life-threatening hematuria, a fatal complication, manifests in the 25% of the cases of placenta percreta, in the second trimester, when the placenta invades the maternal bladder wall, which requires premature evacuation, leading to challenges in surgical management [Konijeti et al., 2009; Silver, Barbour, 2015; Piñas Carrillo, Chandraharan, 2019; Tillu et al., 2019]. The morphological substrate of bladder bleeding consists in: (i) abnormal hyperplasia of disordered blood vessels, affected by abnormal placentation, characterized by high efficiency and low endurance, which can easily rupture, causing severe bleeding; (ii) placental villi completely infiltrate the uterine anterior wall and the urinary bladder posterior wall, with their continued development, without normal protective muscle tissue leading to hemathuria. [Zhao et al., 2021].

Although the evidence of the urinary bladder involvement is mostly intraoperatively confirmed, during the delivery [Konijeti et al., 2009], the gold-standard certification comes only after the histopathological examination [Piñas Carrillo, Chandraharan, 2019].

Uterine arteriovenous malformation (AVM), a rare condition, but frequently undiagnosed, can be congenital or acquired [Kwon, Kim, 2002; O'Brien et al., 2006; Kim et al., 2016; Mekaru et al., 2017]. The etiology of acquired AVM comprises uterine trauma [Chang et al., 2014 ; Mekaru et al., 2017], spontaneous abortion [Mishina et al., 2014; Mekaru et al., 2017], dilation and curettage [Mishina et al., 2014; Lee et al., 2014; Mekaru et al., 2017], endometrial carcinoma [Masood et al., 2022] or gestational trophoblastic disease [Touhami et al., 2014; Mekaru et al., 2017].

The therapeutic strategy for abnormal placentation includes conservative or surgical management, considering the degree of placental invasion or other maternal medical contexts, such as infections, the hemodynamic state or preserving fertility [Oppenheimer, 2007; Eller et al., 2009; Zhong et al., 2017].

The association of retained placenta or product of conception with PAS may also need surgery [Schoolmeester, Bakkum-Gamez, 2020].

Numerous congenital anomalies and miscarriage are the result of chromosomal abnormalities in humans [Goddijn, Leschot, 2000; Wellesley et al., 2012; Brady et al., 2014]. In this regard, chromosomal aneuploidies may be the consequence of modified copy number of a chromosome (monosomy X, trisomy 13, trisomy 18, trisomy 21), whose frequency increases significantly with maternal age [Wellesley et al., 2012], or of partial changes in chromosomal structure (duplications and deletions), less common but with an improved detection rate due to use of microarray-based cytogenetic techniques [Breman et al., 2012; Wapner et al., 2012]. When pregnancy loss or congenital malformations display multiple phenotypes, it is difficult to find out the large number of genes related to various structural abnormalities [Chen et al., 2017].

Roberts syndrome (synonyms: hypomelia-hypotrichosis-facial hemangioma syndrome, pseudothalidomide syndrome, Appelt–Gerken–Lenz syndrome, SC syndrome) [National Organization for Rare Disorders, 2012], an autosomal recessive disorder, is related to the mutations in the ESCO2 (establishment of the cohesion 1 homologue 2) gene, the only known gene whose alterations are associated with Roberts syndrome [Tuuli, Odibo, 2018]. ESCO2 mutations lead to a slower cell division, with aneuploidy daughter cells, resulting in the characteristic malformations encountered in Roberts syndrome, as pre and postnatal growth delays, and most typically malformations of the arms and craniofacial region [Gordillo et al., 2013; Tuuli, Odibo, 2018]. Although with an unknown incidence, no more than 150 cases were described in the literature [Gordillo et al., 2013].

This disease was first described in 1919 by John Roberts, but earlier reports of tetra phocomelia and facial anomalies are cited since 1670s [Van Den Bergh, Francke, 1993].

Roberts syndrome was previously referred as "pseudothalidomide syndrome", based on the similarities regarding limb malformations with thalidomide syndrome patients [Jurenka, 1976]. There is no genotype–phenotype correlation established to date, with a potential role of epigenetic factors in the clinical manifestations of the syndrome.

Numeric chromosomal anomalies – aneuploidy and polyploidy – represent a frequent pathology during pregnancy, as an estimated 25% of embryos at conception have this typeof anomaly. The majority of these embryos are spontaneously aborted during the first trimester of pregnancy (numeric chromosomal anomalies cause 50-60% of miscarriages in the first trimester of pregnancy) [Kolarski et al., 2017]. Triploidy, characterized by the presence of three haploid sets of chromosomes, is a severe numeric chromosomal anomaly associated with negative prognosis. Thus, prenatal cytogenetic studies indicate that triploidy has a prevalence of 1:3500 at 12 weeks of pregnancy and only 1:30,000 at 16 weeks of pregnancy [Snijders et al., 1995].

It is estimated that >99% of triploidies are spontaneously aborted during the first trimester [McKinlay et al., 2004], while the discovery of triploidies during second and third trimester is a rare event [Wick et al., 2013].

The additional set of chromosomes leads to numerous significant birth defects, severe fetal growth complications, and placental pathology. Due to intrauterine growth retardation (IUGR), infants are usually small, presenting neural tube defects (spina bifida), facial abnormalities, cleft lip, micrognatia, heart defects, or other important birth defects of the umbilical cord (single umbilical artery), kidney, and limb [Kolarski et al., 2017; Khong, George, 1992; Doshi et al., 1983].

2.3.2. COMPLICATIONS AND MALFORMATIONS ASSOCIATED WITH PLACENTA PERCRETA

In this section, I present two particular cases of placenta percreta, one complicated with massive hematuria due to maternal bladder invasion, with the surgical protocol performed and with excellent outcome, and another case of retained placenta percreta associated with acquired uterine AVM mimicking a gestational trophoblastic disease, surgically treated by hysterectomy, with a very good outcome.

2.3.2.1. Placenta percreta with urinary bladder involvement

Case presentation

A 33-year-old woman, at 27 weeks gestational age, secundiparous, presented into the emergency room of the Urology Department – "Dr. C. I. Parhon" Clinical Hospital with intermittent macroscopic hematuria and acute urinary retention. The COVID-19 test was negative.

The case history revealed no specific follow-up of the pregnancy, due to a low socio-economical status of the gravida. The ultrasound obstetrical examination confirmed a good evolution of the fetus but described a solid mass in the bladder of 13.68/9.74 cm, without Doppler vascularization (Figure 2.11 and Figure 2.12). The problem of differential diagnosis between a solid bladder tumor and a clot has been raised, but the localization of the placenta praevia, with anterior extension to the uterine scar and the absence of Doppler vascularization in the intravesical solid mass, supported the second hypothesis.

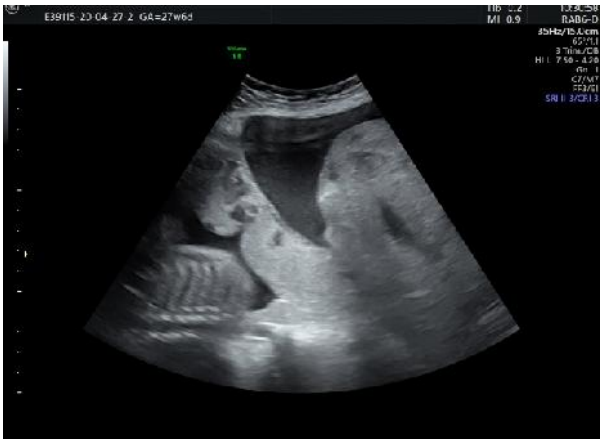


Fig. 2.11. Ultrasound examination of placenta praevia percreta: on the right side, a blood clot; on the left side, fetus with visible costal grid; on the center, placenta.

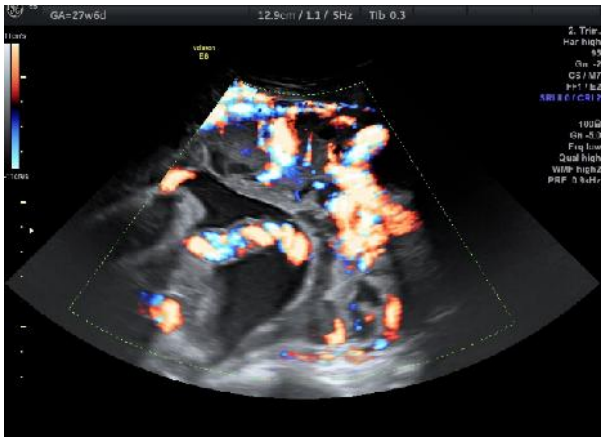


Fig. 2.12. Ultrasound Doppler examination: absence of vascularization in the intravesical; solid mass.

Hemoglobin (Hb) at admission was 11.4 g/100 ml. In the first hours after admission, the patient developed massive hematuria, with urinary acute retention due to intravesical clots, with a drop of Hb to 5g/100ml. Emergency cystoscopy and clots evacuation were performed. Venous ectasia with active bleeding were observed on the posterior wall of the bladder and were subsequently coagulated. Urine became clear on urethral Foley catheter, with no need of bladder irrigation. Four units of isogroup, iso-Rh were administrated. After 48 h, the Foley catheter was removed due to clear aspect of the urine. The patient developed once again, with spontaneous urination, gross hematuria, with a drop of Hb from 7 to 5.3 g/100 ml. A new cystoscopy was performed, with clots extraction, coagulation of the same venous ectasies of the posterior wall and administration of three units of blood. The urine cleared and a Foley catheter was reinserted. The multidisciplinary team composed of gynecologists, urologists, anesthesiologists and a neonatologist decided to perform the cesarean section after 72 h from the last intervention. Initially, bilateral 7CH ureteral double J stents were inserted, after which cesarean section was performed. The child was born with 980 grams and Apgar 7, with a good postoperative evolution in our tertiary neonatal department.

Intraoperatively, placenta was attached to the posterior wall of the bladder, and hemorrhage started during placenta detachment from the uterus, vagina and urinary bladder, necessitating hemostatic hysterectomy and bilateral ligature of the internal iliac arteries. During hysterectomy, the bladder wall was opened, to permit the extraction of the placenta with a portion of invaded bladder wall (Figure 2.13 and Figure 2.14). After thorough hemostasis at the vaginal stump, broad ligament and the bladder, cystotomy was sutured with running uninterrupted 2-0 Vicryl, in one plane. In the end, the tightness of the suture was verified with methylene blue. Intraoperatively 8 units of blood were necessary. Forty-eight hours after the operation, Hb was 7.6 g/100 ml. Postoperative evolution was uneventful, the patient was discharged in the 8th postoperative day, the Foley catheter was removed ambulatory in the 14th postoperative day.

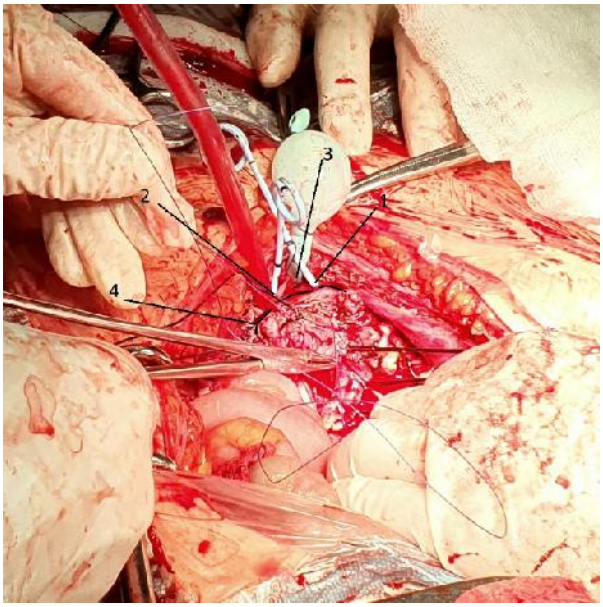


Fig. 2.13. Intraoperative aspects illustrating the surgical procedure: 1) right ureteral double J stent; 2) left ureteral double J stent; 3) bladder neck; 4) ruptured bladder wall.

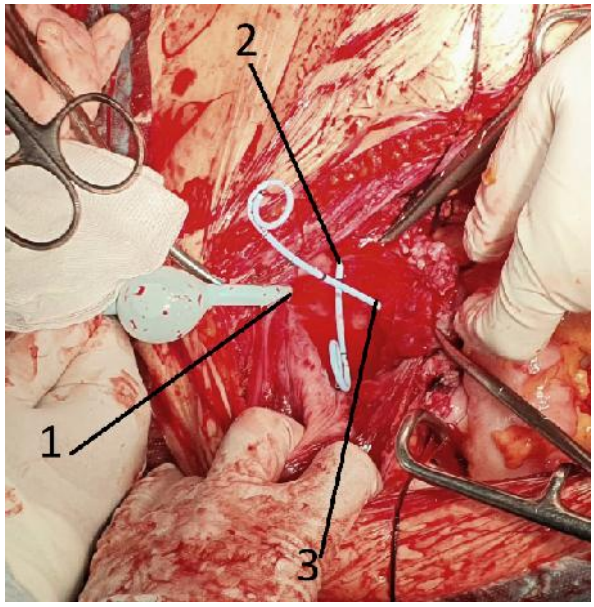


Fig. 2.14. Detailed image of the bilateral 7CH ureteral double J stents: 1) bladder neck; 2) right ureteral double J stent; 3) left ureteral double J stent.

The placenta percreta was documented on the hysterectomy specimen, which was grossly and microscopically examined, revealing stem and terminal placental villi completely

invading the whole thickness of gestational myometrium, which was globally thinned, as well as the uterine serosa, penetrating focally the external surface. The placental villi were attached to myometrial layer, and decidua basalis was reduced or absent (Figure 2.15). Intermediate trophoblast dissected the myometrium, involving also the arterial wall and contributing to their remodeling. Two of the examined fragments presented villi and intermediate trophoblast lining the serosa of the urinary bladder, reaching the external half of the bladder muscularis propria, with focally involvement of the vascular walls (Figure 2.16). The placenta praevia was pathologically diagnosed by the trophoblast invasion in the lamina propria and fibromuscular layer of the isthmus and endocervical internal os (Figure 2.17).

Maternal and fetal mortality in cases of percreta with hematuria is high, especially because of intraoperative and postoperative complications (up to 5% maternal deaths) [Washecka, Behling, 2002], but our case was successfully resolved by cesarean section – hysterectomy with bilateral ligation of hypogastric arteries.

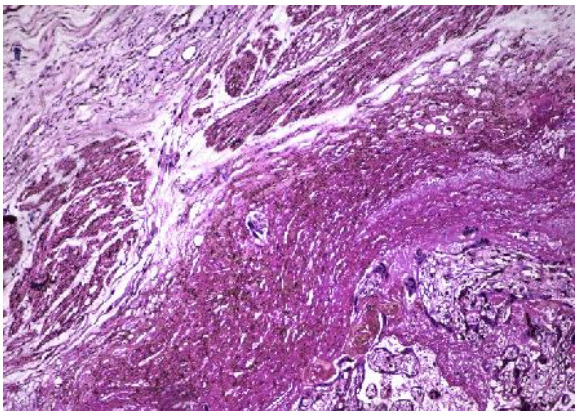


Fig. 2.15. Myometrial involvement by the placental villi (H&E, x100).

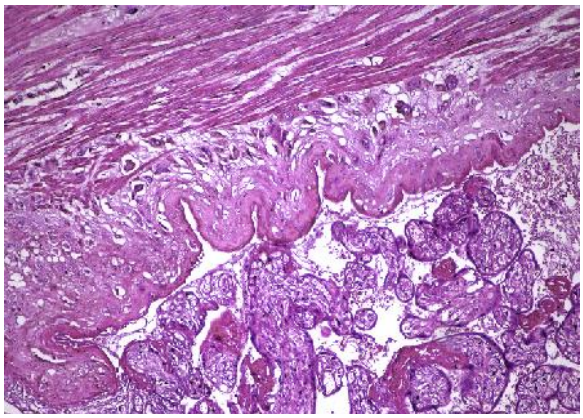


Fig. 2.16. Bladder wall involvement by villi and intermediate trophoblast (H&E, x100).

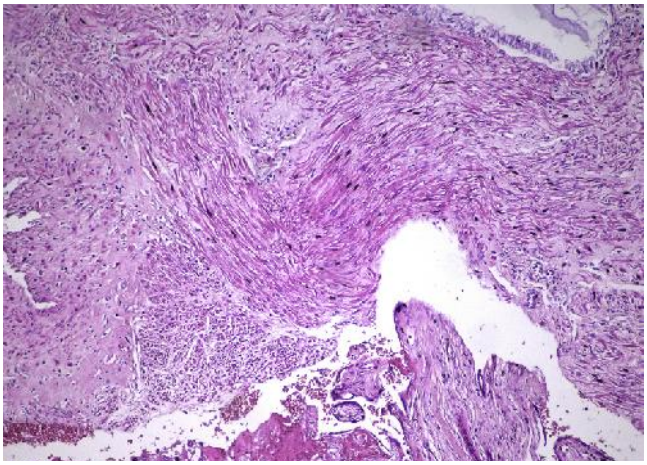


Fig. 2.17. Endocervical involvement by the trophoblast (H&E, x100).

2.3.2.2. Retained placenta percreta and associated uterine arteriovenous malformation

Case presentation

A 29-year-old woman with three previous births presented into the emergency room of the Gynecological Department with heavy bleeding and the absence of menstruation for 14 weeks. The COVID-19 test was negative. The patient's history revealed three previous cesarean sections, without any other pathological medical history.

It is worth mentioning that the patient was previously admitted to another Gynecological Department for the same symptomatology when β-HGC was 160 mUI/mL, and the histopathological diagnosis was consistent with an incomplete abortion.

The ultrasound transvaginal examination performed in our clinic described a 72/55 mm uterus, with an inhomogeneous structure. A heterogeneous area of 30/24 mm, with a color Doppler signal in the entire thickness of the anterior wall, extended into the endometrial cavity (Figure 2.18). The image raised suspicion of arteriovenous malformation, with a multicystic appearance and intense vascularization in the color Doppler flow. There was no echogenic chorionic ring, a specific ultrasound marker of the gestational sac. The adnexa presented a normal sonographic appearance, with suspicion of postoperative adhesions, because of the reduced and painful mobility of the left ovary. The diagnosis was improved with the help of the Doppler ultrasound system, which showed increased vascularization with different flow speeds, arterial and venous (Figure 2.19).

Pulsatile Doppler specified the hemodynamic characteristics of the arteriovenous shunt, as well as the alternation between predominantly systolic arterial flow and venous diastolic flow (Figure 2.20 – a, b).

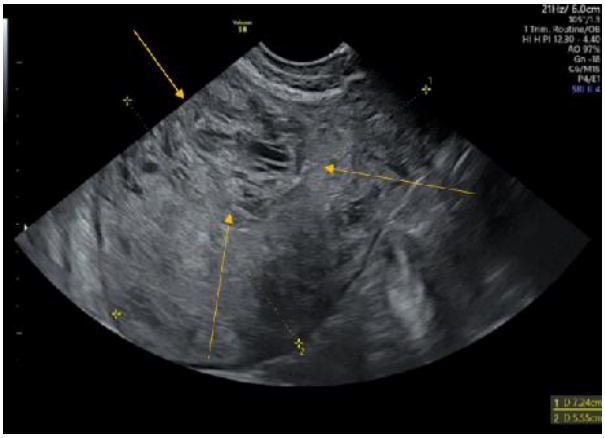


Fig. 2.18. Abnormal echostructure of the uterine anterior wall.

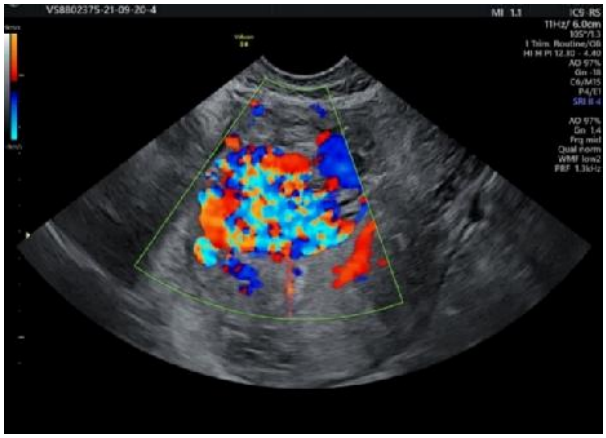
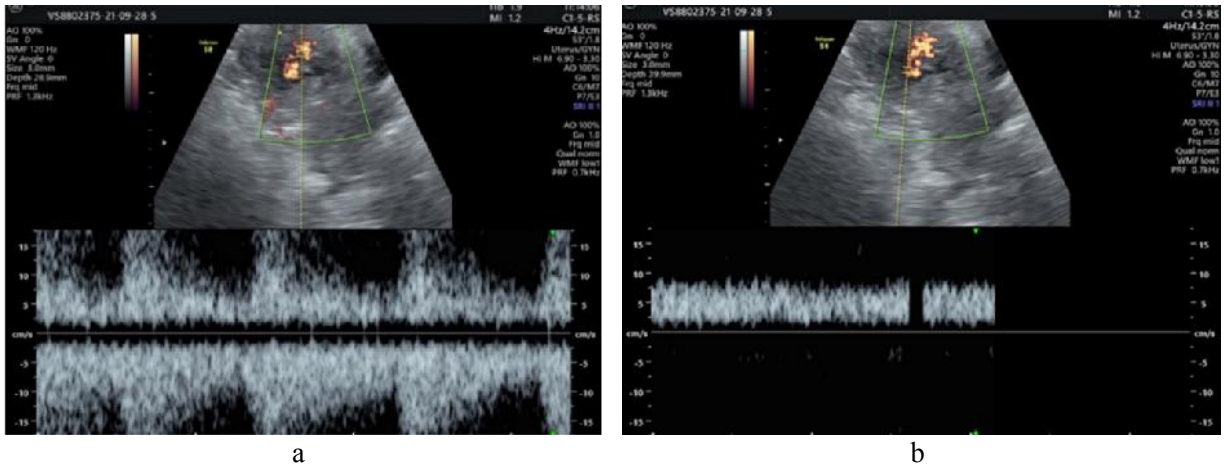


Fig. 2.19. Uterine vascularization with mixed vascular Doppler signal.



a b

Fig. 2.20. Pulsatile Doppler ultrasound: (a) Arterial flow; (b) Venous flow.

At admission, hemoglobin (Hb) was 6 g/dL. Because the iron therapy only slightly increased the hemoglobin levels at 8 g/dL, after decreasing again to 6.7 g/dL, the patient was referred for transfusion in order to perform surgery. In our clinic, the first β-HGC value was 40 mUI/mL, with no other modified biochemical and hematological parameters. Because, during

hospitalization, the gonadotrophin value continued to increase to 427 mUI/mL, a 48 mg methotrexate treatment was instituted. Subsequently, β-HGC decreased to 90 mUI/mL, and a second course of methotrexate of 40 mg was administered.

The patient underwent a second ultrasound examination, which revealed the persistence of the intensely vascularized area from the anterior uterine wall (Figure 2.21). The absence of the gestational sac, the moderate values of β-HGC and the persistence of the highly vascularized area in the anterior uterine wall at ultrasound examination represented the reasons why she was referred to an abdomino-pelvic MRI examination, which described an expansive heterogeneous lesion, with numerous tubular vascular structures and indistinct borders, measuring 51/48/48 mm. The lesion distorted the junctional layer and protruded into the uterine cavity, creating a mass effect. The images were suggestive of a hypervascularized variant of gestational trophoblastic disease, raising suspicion of a placental trophoblastic tumor or choriocarcinoma.

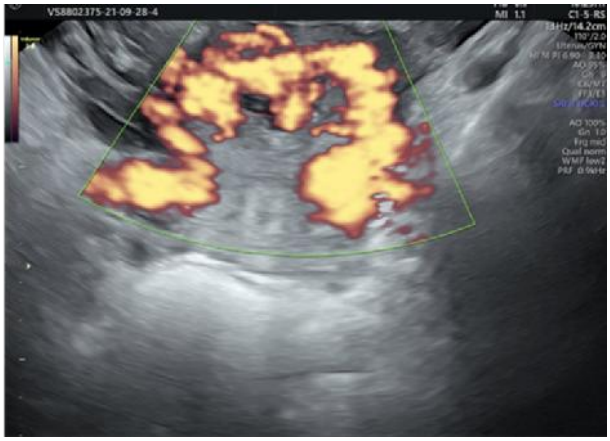


Fig. 2.21. Intensely vascularized area; communication with the uterine vascularization.

The biopsy curettage performed in our clinic revealed only a few cells with suspected atypical morphology, embedded in massive blood clots.

A new ultrasonography was performed, which revealed a uterus of 86/50 mm, with a heterogeneous structure, and the same inhomogeneous area of 31/32 mm on the anterior wall, with increased arteriovenous vascularization, maintaining the suspicion of intrauterine AVM. The adnexa had also a normal ultrasound aspect.

Considering the MRI suspicion of diagnosis and the persistent bleeding, a multidisciplinary team composed of gynecologists, an oncologist, and anesthesiologists decided to perform a total hysterectomy. The surgical procedure involved a total hysterectomy, bilateral salpingectomy, left oophorectomy, with right external iliac lymph node sampling and extended adhesiolysis. The post-operative evolution was uneventful, with the patient requiring only a new transfusion.

The surgical specimen was submitted to the Pathology Department, where it was grossly (Figure 2.22) and microscopically examined (Figures 2.23-2.26). The retained placenta percreta was documented by histologic examination, through extensive sampling, which revealed, in the endometrium and myometrium, large areas of necrosis and hemorrhage, focal microcalcifications, diffuse mixed inflammatory infiltrate, a few areas of decidua and bundles of predominantly necrotic muscle fibers, infiltrated by fibrotic and degenerating chorionic villi, sometimes lined by a discontinuous trophoblast involving the entire uterine wall (Figures 2.23 and 2.24). Arteriovenous malformation was also histologically documented by numerous myometrial irregularly shaped and dilated blood vessels, containing well-organized thrombi, with a highly branched fibrin network and abnormal communication between arteries and veins (Figures 2.25 and 2.26). These aspects intersected the entire uterine wall, toward the isthmus, where the glandular epithelium presented syncytial and hobnail-type reactive changes.

Morphological aspects compatible with histiocytic mixed follicular hyperplasia in the right external iliac lymph node completed the diagnosis.

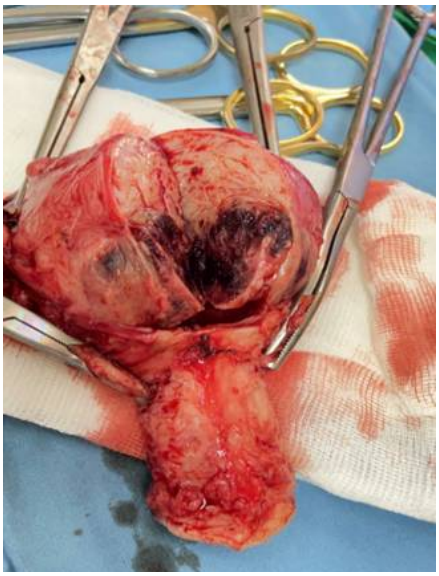


Fig. 2.22. Placenta percreta with abnormal vascularization reaching the lower uterine segment.

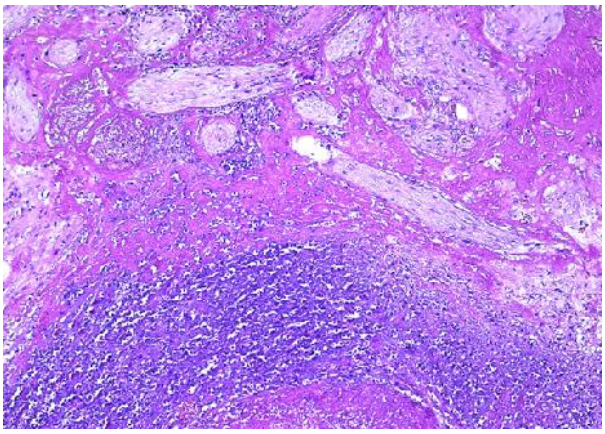


Fig. 2.24. Fibrotic chorionic villi infiltrating the myometrium (H&E, x100).

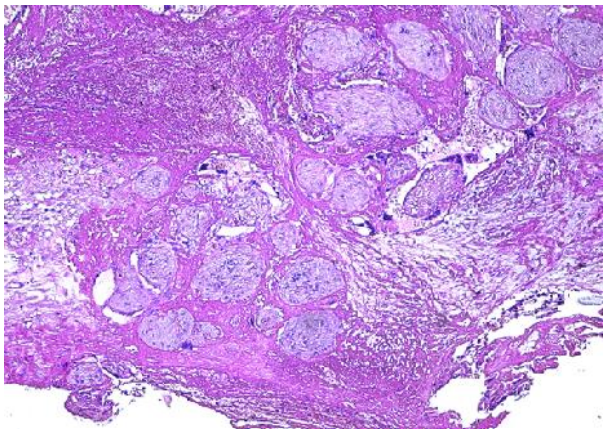


Fig. 2.25. Marked fibrosis of chorionic villi, which invade the full thickness of the uterine wall (H&E, x100).

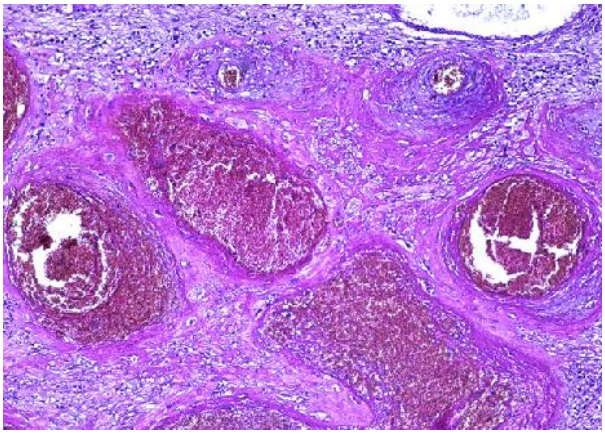


Fig. 2.26. Dilated irregularly shaped blood vessels and thrombosis (H&E, x100).

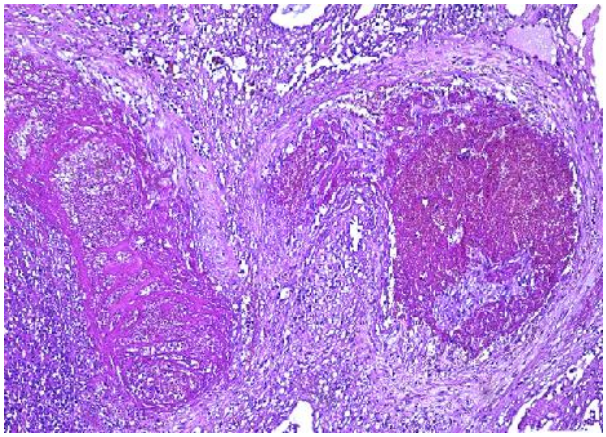


Fig. 2.27. Vascular malformation and thrombi (H&E, x100).

2.3.2.3. Discussions

Unlike normal pregnancies, in which trophoblastic invasion ceases when the cells reach the spongiosum layer of the decidua (Nitabuch's layer) [Silver, Barbour, 2015], in placenta accrete spectrum (PAS) the abnormal adherence of the trophoblastic cells produces damage of the decidua basalis, with thoroughly gradual invasion of the uterine wall [Carrillo, Chandraharan, 2019].

Although placenta percreta is the least frequent form of PAS and can sustain a full-term pregnancy with normal fetal development, it is considered the most severe of the three conditions, sometimes causing premature labor or heavy peripartum bleeding, and moreover, greater complications if diagnosed intraoperatively or if trophoblastic invasion involves neighboring organs or structures [Gersell, Kraus, 2019; Okunowo et al., 2019].

The placenta accreta spectrum presents several controversies, namely (i) the definition discordance regarding clinical, ultrasound and histopathological criteria [Jauniaux et al., 2016; Morlando, Collins, 2020]; (ii) the inclusion in the same spectrum of diseases of both categories, placental adhesion abnormalities and placental abnormal invasion, each of them with different histopathological aspects; (iii) potential confusion between a retained placenta and an abnormally adherent placenta [Jauniaux et al., 2018; Jauniaux et al., 2019; Morlando, Collins, 2020].

In this regard, placental retention cannot be included in the same category as PAS because this condition manifests when the placenta remains inside the uterus after delivery or evacuation, even if it separates from the uterine wall [Morlando, Collins, 2020; Schoolmeester, Bakkum-Gamez, 2020]. Although a retained placenta or product of conception is rare, it has an increasing prevalence due to the growing number of cesarean sections and abnormal placentation [Oppenheimer, 2007; Wortman, Alexander, 2013; Pather et al., 2014; Perez-Delboy, Wright, 2014; Zhong et al., 2017; Mullen et al., 2019; Schoolmeester, Bakkum-Gamez, 2020]. Both the retention of placental fragments or product of conception and placenta percreta have a risk of postpartum bleeding, with a frequency of 3–5% after vaginal delivery [Kohi et al., 2015]. Given that most similar cases are resolved by conservative treatment, the corresponding case reports do not present the histopathological examination for diagnostic certification [Wortman, Alexander, 2007; Oppenheimer, 2007; Kohi et al., 2015; Yoon et al., 2016; Zhong et al., 2017; Morlando, Collins, 2020].

The invasion of the urinary bladder with subsequent life-threatening hematuria represents a rare complication of placenta percreta.

To the best of our knowledge, only Washecka et al. [Washecka, Behling, 2002], in 2002, reported a series of 54 cases after a review of the literature, in which 17 (31%) patients presented hematuria, reporting 3 maternal and 17 fetal deaths. There are also few case reports that present peculiar aspects of the surgical management of placenta percreta with bladder wall involvement [Aho et al., 1985; Abbas et al., 2000; Takai et al., 2005; Smith et al., 2014; Koukoura et al., 2017]. Thus, our first presented case revealed a successful treatment of a placenta percreta associated with placenta praevia and complicated with massive hematuria, with a very good outcome.

It is mandatory that, in case of patients with a history of multiple pregnancies, frequent CS or abortions and hysteroscopies, to perform imaging investigations (ultrasound, MRI) for a correct pregnancy diagnosis, and once detected complications (cesarean scar pregnancy, placenta praevia, placenta accreta spectrum, bladder invasion) to evaluate the optimal therapeutic method [Zhao et al., 2021]. For example, in case of cesarean scar pregnancy, urgent completion of pregnancy may be necessary [Zhao et al., 2021]. Practically in such cases, is better that the delivery not to be performed later than 34–35 weeks of gestation, because of an increased risk of severe hemorrhage [Jain et al., 2020]. Another recommended investigation is

cystoscopy, which can reveal the presence of atypical hyperplastic blood vessels or of placenta invasion, but with limited use in the diagnostic of suspected placenta percreta or bladder wall invasion, because of increased risk of hemorrhage [Zhao et al., 2021].

The best therapeutic option is still controversial [Paniza et al., 2018; Tillu et al., 2019;]. In case of placenta percreta with bladder wall involvement, the elective approach is cesarean hysterectomy, followed by cystotomy or partial cystectomy, with the risk of urinary fistula and prolonged catheterization [Koukoura et al., 2017; Carrillo, Chandraharan, 2019; Tillu et al., 2019]. In our presented case, primary hysterectomy was mandatory, as reported in other studies [Aho et al., 1985; Konijeti et al., 2009; Sijanović et al., 2011; Smith et al., 2014; Carrillo, Chandraharan, 2019], because uterus preservation is associated with the hemorrhagic risk, even months after CS. Moreover, our multidisciplinary team proposed, for a good hemostasis, bilateral internal iliac artery ligature, strategy already reported in the literature [Okunowo et al., 2019]. To avoid intraoperative ureteral injuries, there were inserted bilateral, ureteral double J stents, as previously reported [Smith et al., 2014; Tillu et al., 2019]. The multidisciplinary team composed of gynecologists, urologists and neonatologists achieved a good postoperative outcome, with a progressive recovery of mother and the improvement of the newborn health.

Because of the increasing frequency of short-term and long-term consequences due to radical surgical techniques used in the management of maternal complications of the PAS, the surgical strategy has progressively evolved towards more conservative techniques, such as: the “triple P procedure” (ultrasound perioperative placental localization, pelvic devascularization, and placental non-separation), intentional placental retention or partial myometrial excision [Carrillo, Chandraharan, 2019; Tillu et al., 2019]. The in situ maintenance of the placenta adherent to the uterine and urinary bladder wall, respectively, implies the use of methotrexate for placental involution (spontaneously resorbed in 9-12 months), as well as a careful follow-up at least 20 weeks because of the high risk of sepsis, delayed hematuria and massive hemorrhage, with subsequent peripartum hysterectomy [Washecka, Behling, 2002; Carrillo, Chandraharan, 2019; Tillu et al., 2019]. If the abnormal placental penetration is partial or focal, a possible surgical option is one-step conservative surgery, which consists in the resection of myometrium and bladder tissues invaded by placenta, concomitantly with the reconstruction of the affected areas [Carrillo, Chandraharan, 2019; Tillu et al., 2019]. However, this procedure presents several disadvantages, namely: sepsis, multiple organ failure, intravascular disseminated coagulopathy, and vesicovaginal fistula [Carrillo, Chandraharan, 2019; Tillu et al., 2019].

Another approach to therapeutic strategies considers hematuria before or after birth. Before delivery, the indicated method for placenta percreta complicated with bladder bleeding consists in ultrasound and MRI examinations, together with personalized surgery timely performed, represented by cesarean section, accompanied by cystotomy, partial cystectomy, or even hysterectomy, sometimes with in situ preservation of placenta, the operation being individualized according to the patient status, associated related pathology, and complications [Zhao et al., 2021; Jain et al., 2020].

When hematuria associated to placenta percreta occurs after delivery, the following therapeutic options may be considered: (i) electrocoagulation hemostasis cystoscopic-guided, with limited and careful use, as neoformation blood vessels have a thin media and cannot be electrocoagulated; (ii) interventional embolization, with gelatin sponge or microspheres, being performed for atypical hyperplastic and blood supplying vessels, in case of previous treatment failure; (iii) exploratory laparotomy, considered in case of serious hemorrhage, or failure of the previous embolization [Zhao et al., 2021].

The key particularity of our first presented case is the existence of only one previous CS, these varieties of placenta percreta appearing on multiscar uteri, the risk increasing geometrically with the number of scars. The presence of the placenta praevia, as an important

risk factor of placental accretion, associated in our case with the placenta percreta is also remarkable, considering that previous CS was more than two years apart (5 years ago) and without scarring defects. Moreover, no data of pregnancy evolution were documented, as patient addressed directly to the Urology service for intermittent hematuria and acute urinary retention. Another challenge of this case was featured by the fact that abdominal ultrasound, which revealed a solid intravesical mass of 13.68/9.74 cm, had to perform the differential diagnosis between a solid bladder tumor and a giant blood clot. The conservative therapeutic option could not be applied in our case, because it would not have stopped the hematuria. It is worth mentioning that the attempt to coagulate by cystoscopy the bladder mucosa vessels developed by the abnormal placental invasion failed, by the hematuria recurrence, after the first suppression of the Foley catheter. A distinct feature of our report consists in the histological documentation of the abnormal placentation into the muscular layer of the urinary bladder, as the literature provides few typical microscopic images for this condition.

Arteriovenous uterine malformation (AVM) is a female genital pathology with specific descriptive imaging elements due to macroscopic abnormal communications between arteries and veins, without the intervention of the capillary network [Masood et al., 2022]. Ultrasonography may highlight these characteristics [Mungen et al., 1997]. At the same time, diagnostic ultrasounds can detect limited and painful mobility of the organs due to adhesions formation [Moro et al., 2014; Butureanu, Butureanu, 2014], as it was in our second presented case, where the reduced movement of the left ovary raised the suspicion of postoperative adhesions.

In this second case, for the initial ultrasound, transabdominal route was used, through which an area of 31/32/28 mm was pointed out, with a heterogeneous echostructure, in the anterior uterine wall, concordant with similarly reported data in the literature [Zhu et al., 2018; Nakasholo et al., 2021].

At this stage, the differential diagnosis may take into account the following uterine pathologies: (i) uterine fibroids, which usually have a capsule, absent in our case; (ii) adenomyosis, which modifies the echostructure by altering the endo-myometrial line, with intrauterine characteristic ectopic foci near the endometrium, or in our case, the abnormal structure involved uterine serosa; (iii) isthmocele, where the hernia of the cesarean scar has a triangular appearance, characteristic of the uterine isthmic region (Table 2.7) [Kohi et al., 2015]. Moreover, the preoperative imaging examination can also take into account a differential diagnosis of leiomyosarcoma or degenerating fibroids [Lollie et al., 2020].

The case management could be guided by following the peak systolic velocity (PSV) of the flow inside the malformation. When PSV is higher than 0.83 m/s, the cases respond better to permanent surgical treatment, while those with PSV under 0.4 m/s may respond to conservative treatment [Timmerman et al., 2003].

Table 2.7. Differential diagnosis for heterogenous uterine wall echostructures

DIFFERENTIAL DIAGNOSIS	CHARACTERISTIC ULTRASOUND FEATURES
Uterine fibroids	Capsule
Adenomyosis	Alteration of endo-myometrial line Intrauterine characteristic ectopic foci Location near the endometrium
Isthmocele	Triangular appearance of cesarean scar hernia

If β-HGC dynamics is considered, the differential diagnosis of arteriovenous malformation includes pathologies with increased vascularization where it can be assumed a neoangiogenesis process, possible in a gestational context [Fox et al., 2009]. Thus, the relatively common forms of gestational trophoblastic disease (GTD), invasive mole and choriocarcinoma may present vascular arteriovenous abnormalities, but in these cases, β-HGC values are higher than the ones seen in our patient [Kido et al., 2003; Soper et al., 2004]. A particular situation represents the placental site trophoblastic tumor (PSTT), which is a variant of the gestational trophoblastic disease with an arteriovenous shunt, but with lower β-HGC values [Ju et al., 2015; Zeng et al., 2015]. Not least, retained products of conception (RPC) may show increased vascularization predominantly of the systolic arterial type and mainly with endometrial involvement [Yoon et al., 2016; Zhu et al., 2018]. In these situations, β-HGC values are moderately increased (Table 2.8) [Chen et al., 2016].

Table 2.8. Differential diagnosis of arteriovenous malformation, according to β-HGC dynamics

DIFFERENTIAL DIAGNOSIS	CHARACTERISTIC ULTRASOUND FEATURES	B-HGC VALUES
Invasive mole Choriocarcinoma	Arteriovenous abnormalities	Higher Over 50.000 mUI/ml With rapid increase
PSTT	Arteriovenous shunt	Lower Over 100 mUI/ml With variable increase
RPC	Increased systolic arterial type vascularization	Moderately increased Over 100 mUI/ml With slight decrease

In our second presented case, the arteriovenous malformation was acquired, as long as the patient did not have any menstrual disorder as a marker of abnormal vascularization. Moreover, uterine curettage and previous cesarean sections also represented conditions for its occurrence, in accordance with those reported in the literature [Kim et al., 2016; Mekaru et al., 2017].

Historically, the first case of uterine AVM was reported in 1926 [Fleming et al., 1989; Zhu et al., 2018]. The acquired form of uterine AVM occurs more frequently in women of fertile age [Zhu et al., 2018]. Although the mechanism is still unknown, several studies mention, along with myomectomy, uterine dilation and curettage (D&C), cesarian section, the existence of pregnancy, along with β-HGC variations, which may play a role in the development of an otherwise latent AVM [Yoon et al., 2016; Zhu et al., 2018; Masood et al., 2022]. Moreover, these surgical procedures, along with gestational trophoblastic disease (GTD) or endometrial carcinoma, can cause uterine trauma in the presence of an arteriovenous malformation, which makes curettage—the specific treatment in the case of uterine bleeding—contraindicated, in the context of an AVM [Yoon et al., 2016; Masood et al., 2022]. This could possibly justify the alteration of the patient's condition through persistent severe hemorrhage after the performed diagnostic curettage. Arteriovenous malformation is usually identified in multiparous patients with abundant and intermittent bleeding, usually due to high vascular flow across the lesion because of the different pressure degrees between the arterial and venous systems [Yoon et al., 2016]. Literature data report that approximately 50% of patients with acquired AVM need

transfusions, a situation encountered in our case as well [Peitsidis et al., 2011; Masood et al., 2022].

Moreover, a history of recurrent miscarriage indicates an increased risk of AVM, as patients may remain asymptomatic [Yoon et al., 2016].

Histological examination of the hysterectomy specimen revealed the irregular and dilated blood vessels in the myometrium, which confirm the uterine AVM, corresponding to similar features reported in the literature [Lollie et al., 2020]. Sometimes, in this context, one can note an atypical aspect of bland endothelial cells of the malformed arteries and venules with abnormal vascular dilation and blunt changes in their media thickness, such as papillary endothelial hyperplasia in the lumen of thrombosed vessels, but without presenting atypia, mitosis, or necrosis [Zhu et al., 2018; Lollie et al., 2020].

Another distinct aspect of our report was the histopathological confirmation of the retained product of conception, revealed by the presence of fibrotic placental villi in the uterine wall, considering that the microscopic certification of persistent trophoblast or placental tissue by detecting chorionic villi represents the characteristic feature that certifies this condition [Sellmyer et al., 2013].

The difficult cases, which rule out malignant or other benign tumors, can be supplementary highlighted by special stains or immunohistochemical techniques [Lollie et al., 2020]. In this context, the concern of a malignant condition also requires extensive sampling of the surgical uterine specimen [Lollie et al., 2020].

The particularities of the presented case consist of the association of the abnormal placentation, in the form of a retained placenta percreta, with an acquired uterine AVM leading to a radical surgical approach. Radical surgery made the histopathological examination possible, as generally, the diagnosis and treatment of AVM do not require extensive surgery that can provide microscopical certification of injuries. To the best of our knowledge, there are only a few reported cases in the literature with similar diagnostic associations as in our presentation, as well as with a radical therapeutic decision due to the occurrence of massive bleeding and suspicion of gestational trophoblastic disease [Barber et al., 2011; Soeda et al., 2014; Roach, Thomassee, 2015; Takeda, Koike, 2017; Schoolmeester, Bakkum-Gamez, 2020; Clark et al., 2021].

Conservative treatment methods [Barber et al., 2011; Soeda et al., 2014; Roach, Thomassee, 2015; Yoon et al., 2016; Takeda, Koike, 2017; Calzolari et al., 2017; Masood et al., 2022] were also considered in this reported case, but the medical context of our patient led to the choice of radical surgical therapy, with a hysterectomy being preferred when the patient does not want to preserve fertility [Oppenheimer, 2007; Eller et al., 2009; Zhong et al., 2017].

Operative hysteroscopy [Calzolari et al., 2017] was also taken into account in the presented case, with this method being described in the literature as a good variant fertility-sparing technique in the context of AVM [Calzolari et al., 2017]. When there is significant hemorrhage, which can mask the visualization of the cavity and cauterization of the malformation, added to other diagnostic suspicions (placental site trophoblastic tumor or placenta accreta), the treatment should be radical to avoid a possible life-threatening risk [Calzolari et al., 2017].

Moreover, hysterectomy is the therapy of choice in the context of complications due to abnormal placentation, retained product of conception and arteriovenous malformation, all of which lead to acute and severe bleeding, events that can endanger the patient's life [Timmerman et al., 2003; Kido et al., 2003; O'Brien et al., 2006; Oppenheimer et al., 2007; Eller et al., 2011; Touhami et al., 2014; Perez-Delboy, Wright et al., 2014; Carrillo, Chandraharan, 2019; Morlando, Collins, 2020; Schoolmeester, Bakkum-Gamez, 2020]. A similar context was with our second case, in which the surgical therapeutic decision was also justified by the suspicion of gestational trophoblastic disease and the lack of the patient's fertility desire. Therefore, as

the patient declined additional gestational planning, a total hysterectomy, bilateral salpingectomy, left oophorectomy, with right external iliac lymph node sampling and extended adhesiolysis were performed.

Our multidisciplinary team decided on adnexectomy due to extended adhesiolysis followed by intraoperative bleeding, which made it difficult to preserve the left ovary. Because of the suspected throphoblastic tumor following MRI examination and biopsy results, lymph node sampling was performed. Our therapeutic strategy was in accordance with data reported in the literature [Lan et al., 2010; Gadducci et al., 2019].

An increasingly used therapeutic approach is endovascular transcatheter uterine artery embolization (TCUE), which, since its appearance in the late 1980s, has proven to be a safe and less-invasive method, especially for patients who want to preserve their fertility [Masood et al., 2022; Yoon et al., 2016]. Thus, in the presence of a uterine AVM, the unilateral or bilateral variant of TCUE must be taken into consideration [Yoon et al., 2016]. However, the literature data show that the second follow-up embolization has a higher success rate compared to the first embolization [Yoon et al., 2016]. Moreover, published studies have not led to clear treatment guidelines for cases in which the initial embolization did not succeed, in order to compare the effectiveness of a second embolization with that of hysterectomy or medical therapy, in the case of persistent hemorrhage [Yoon et al., 2016].

Another study presents the therapeutic results of 62 patients who received TCUE associated with pelvic angiography, with a failure rate of 29%, where the persistent uterine hemorrhage was resolved at different intervals by a secondary embolization, hysterectomy or bilateral laparoscopic ligation of the uterine artery. Although beneficial in treating arteriovenous fistula and bleeding cessation, the embolization method remains controversial in maintaining the patient's fertility [Zhu et al., 2018].

Given the aspects presented above, as well as the reported increased maternal morbidity, in the case of placenta accreta treated with TCUE [Sentilhes et al., 2010], together with the specific medical context of our patient, the multidisciplinary team decided to perform a hysterectomy instead of TCUE.

A special technique reported in one study was the use of ureteral stents, in the case of a cesarean hysterectomy for the placenta accreta, to avoid involuntary urinary tract injury, but results showed that the method does not reduce these risks [Crocetto et al., 2021].

The two presented cases of placenta percreta are similar in the existence of some similar major risk factors, which favored the accretion. Moreover, each of them was associated with either the placenta praevia and complicated with the uterine bladder wall involvement, or with an acquired uterine arteriovenous malformation, both of which producing serious symptoms, such as hematuria and hemorrhage, which necessitated emergency radical surgery. However, although the surgery required hysterectomy, the operating technique was individualized and personalized for each case.

Due to the particular conditions and potential risks, none of the patients benefited from conservative treatment, which can sometimes have serious consequences. Radical surgery facilitated the histopathological certification of the complex diagnosis in both cases, this being another particular common element of the presented cases. In both situations, the patient's outcome was favorable.

2.3.2.4. Final remarks

Placenta percreta associated with bladder invasion is a rare but serious, life-threatening problem. The urologist should consider a massive hematuria of a pregnant woman as key diagnostic feature for complicated placenta percreta.

Retained placenta percreta associated with an acquired arteriovenous malformation is also a rare but sometimes life-threatening condition. Besides other benign or malignant diseases, the gynecologist should include in the differential diagnosis the uterine AVM in the presence of a massive and persistent unexplained uterine hemorrhage, associated with significant β-HGC variations and specific Doppler imaging in a premenopausal woman.

Total hysterectomy is taken into consideration when the acquired AVM is associated with abnormal placentation and the patient does not want to maintain fertility. In this rare context, a histopathological examination may provide a definite diagnosis for these conditions.

2.3.3. RARE CHROMOSOMAL ANOMALIES

This section comprises two examples of chromosomal abnormalities, both associated with a negative prognosis, in the form of a case report and a series of four cases.

2.3.3.1. Roberts syndrome

Case presentation

This is a case diagnosed at the moment of birth in the Clinical Hospital of Obstetrics and Gynecology "Elena Doamna" from Iasi.

The mother of the propositus was a 17-year-old primiparous presenting at 38 weeks of gestation, with intrauterine fetal demise. No obstetrical or familial risk factors were detected. It should be mentioned that the pregnancy was not followed due to patient socioeconomic and family situation, and no prenatal ultrasound was made.

After a normal length labor, the patient delivered a deceased male fetus with the following anthropometric features: weight 2650 g, length 53 cm, head circumference 37 cm, thoracic circumference 32 cm. During the delivery, a tight double umbilical abdominal loop was found. The postpartum evolution of the mother was uneventful.

The deceased fetus was referred to the Pathology Department for the autopsy. The stillborn baby had multiple congenital anomalies, and the clinical evaluation showed a craniofacial dysmorphism with dolichocephaly, hypoplastic inferior maxilla with micrognathia (Figure 2.28), downslanted palpebral fissures, prominent beaked nose, pterygium colli (Figure 2.29), abnormal and lower implanted ears. Congenital anomalies of the limbs were superior limbs phocomelia (superior limbs had a total length of 10 cm) (Figure 2.30), syndactyly at lower right limb (toes IV and V) and oligodactyly with tetradactyly in all the other limbs. The stillborn baby had also a thoracic asymmetry with absence of ossification, and bilateral cryptorchidism.

Fig 2.28. Macroscopic appearance of dolicocephaly, low implanted ears and micrognathia.

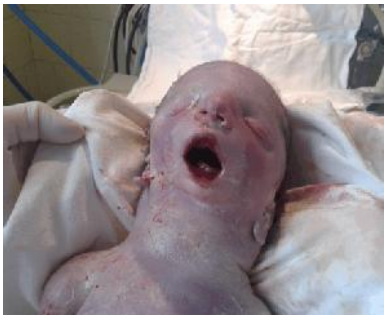


Fig. 2.29. Antimongoloid palpebral slant and pterygium colli.

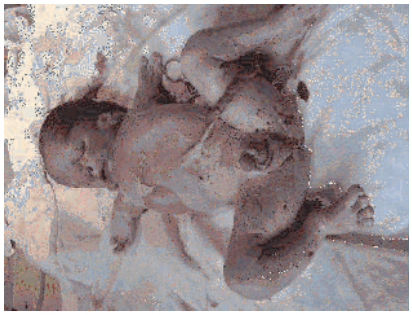


Fig.2.30. Superior limb phocomelia, tetradactyly, syndactyly.

The external examination revealed several abnormal findings. Overlapping sutures, small anterior fontanella, parieto-occipital meningeal hemorrhage, flattened circumvolutions were observed at the examination of the skull.

Thorax examination revealed only 11 pairs of unossified and loose ribs, with the last pair of ribs floating. An interatrial communication of 0.2 cm and ductus arteriosus were observed in the heart. The lungs were dark purple, with no crepitations, with incomplete lobulation between the middle and lower lobe of the left lung, and incomplete lobulation between the upper and inferior lobe of the right lung. Examination of the digestive tract revealed pancreas agenesia.

On gross examination, the placenta measured 18/12/4 cm, presenting few hemorrhagic areas; the umbilical cord measured 20/2 cm and contained three blood vessels.

Histopathological examination of the placenta and fetal tissues was carried out, the specimens being fixed for 24 hours in buffered formalin and processed for paraffin embedding. Serial sections of 4–5 μm were stained with Hematoxylin–Eosin (H&E) and trichromic Masson's technique.

The microscopic examination confirmed the clinical diagnosis. The lung tissue revealed slightly thickened interalveolar septa, alveoli with eosinophilic granular debris and desquamated cells, rare macrophages (Figures 2.31 and 2.32). The placental tissue revealed blood vessels with ectasia and thrombosis in the chorionic plate (Figure 2.33), perivillous and basal plate fibrin deposition, intervillous thrombi, microcalcifications (Figures 2.34 and 2.35).

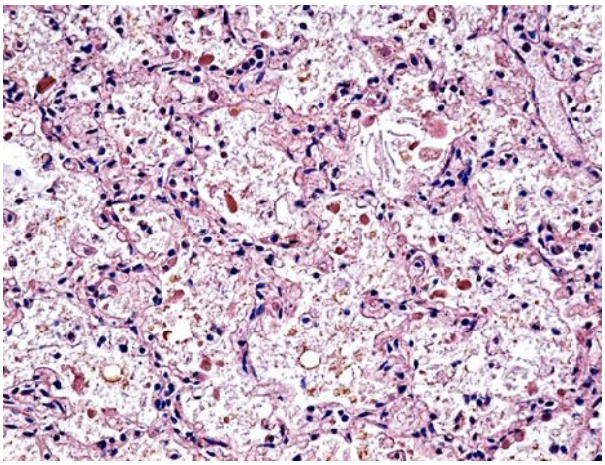


Fig. 2.31. Pulmonary alveolar proteinosis with interstitial thickening of the alveolar septa (H&E, x400).

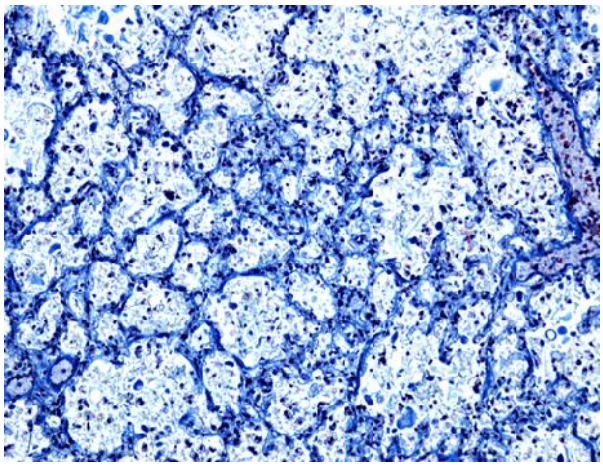


Fig. 2.32. Pulmonary alveolar proteinosis with interstitial thickening of the alveolar septa (Masson's, x400).

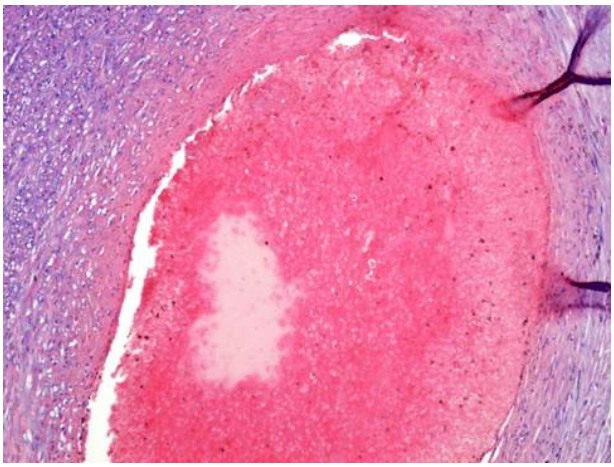


Fig. 2.33. Chorionic plate thrombosis. Recent thrombus (H&E, x400).

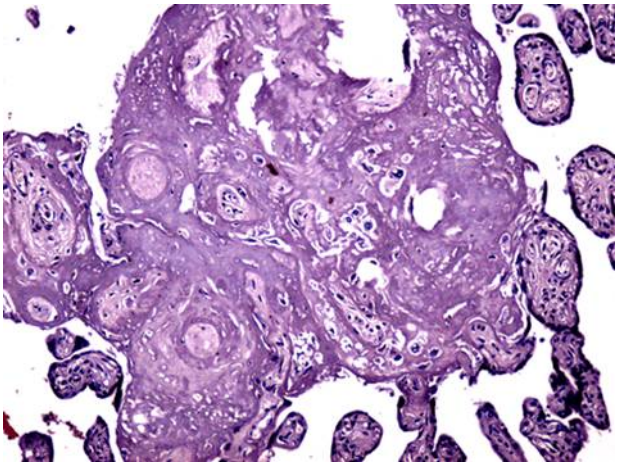


Fig. 2.34. Placental infarct. Avascular villi enmeshed in fibrinoid (H&E, x400.)

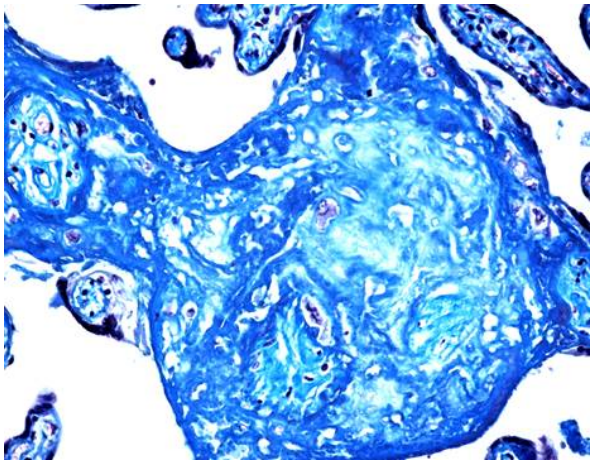


Fig. 2.35. Placental infarct. Avascular villi enmeshed in fibrinoid (Masson's, x400).

For genetic analysis, genomic DNA was extracted from the paraffin-embedded tissues stored after the histopathological examination. Full gene sequencing of the ESCO2 gene for the baby and their normal parents was performed. In the case of the stillborn baby, homozygosity for the c. 745_746delGT mutation of ESCO2 gene was identified. Parents were heterozygous, presenting one normal variant and the c. 745_746delGT mutation of ESCO2 gene.

Based on the clinical findings corroborated with the anatomopathological examination and with genetic analysis, the final diagnosis was established for this stillborn fetus and confirmed the initial suspicion of Roberts syndrome.

Discussions

Roberts syndrome is characterized by pre- and postnatal growth retardation (mild to severe), craniofacial anomalies (microcephaly, dolichocephaly, cleft palate and lip, micrognathia, premaxillary prominence, microbrachycephaly, downslanted palpebral fissures, widely spaced eyes, exophthalmos, corneal clouding or cataract, ear malformations, intracranial aneurisms, capillary hemangioma), limb malformations (bi-/tetra-phocomelia, hypomelia, oligodactyly with thumb aplasia, syndactyly, clinodactyly), more severe to the upper limbs [Wang et al., 2011; Gordillo et al., 2013; Borjas-Lucio et al., 2017]. There are also described heart congenital anomalies, like patent ductus arteriosus, interventricular communication as well as cystic kidneys or enlarged genitalia [Borjas-Lucio et al., 2017]. It is considered that 49% of the cases present parental consanguinity [Borjas-Lucio et al., 2017].

In our case, the stillborn baby presented multiple congenital abnormalities, the clinical exam revealing a craniofacial dysmorphism with dolichocephaly, hypoplastic inferior maxilla with micrognathia, downslanted palpebral fissures, prominent beaked nose, pterygium colli, abnormal and lower implanted ears. The congenital anomalies of the limbs were superior limbs phocomelia (superior limbs had a total length of 10 cm), syndactyly at lower right limb (toes IV and V) and oligodactyly with tetradactyly in all the other limbs. It was also described a thoracic asymmetry with absence of ossification, and bilateral cryptorchidism.

Moreover, the external examination of the autopsy revealed several other abnormal findings, such as overlapping sutures, small anterior fontanella, parieto-occipital meningeal hemorrhage, flattened circumvolutions were observed at the examination of the skull.

The histopathological examination revealed various lesions in lungs (thickened interalveolar septa, alveoli with eosinophilic granular debris and desquamated cells, rare macrophages) and placenta (vascular lumens with ectasia and thrombosis, perivillous and basal plate fibrin deposition, intervillous thrombi, microcalcifications).

In less severe cases, altered physical and intellectual development is frequent. In severe cases, as the one depicted in this case report, death occurs prenatally or immediately after birth [Gordillo et al., 2013].

Regarding its pathogenesis, the Roberts syndrome is caused by mutations in ESCO2, a gene which is located at 8p21.1, and encodes a protein with function of acetyltransferase essential in establishing sister chromatid cohesion during S phase and mitosis [Goh et al., 2010]. While the essential role of the cohesin complex in chromosome segregation has been well characterized, the protein codified by ESCO2 gene plays additional roles in DNA damage repair, chromosome condensation, and gene expression [Xu et al., 2013]. The normal ESCO2 gene product also has an important role in sister chromatid cohesion during mitosis [Williams et al., 2003]. The chromosomal analysis made in cases of Roberts syndrome revealed a characteristic premature centromere separation and separation of the heterochromatic regions in most chromosomes [Gordillo et al., 2008]. The molecular genetic testing allowed identification of 26 different gene mutations in ESCO2 gene [Williams et al., 2003]. The ESCO2 gene mutations can lead to loss of acetyltransferase activity, by truncation in the protein, or single amino acid changes in the protein [Williams et al., 2003; Schüle et al., 2005; Vega et al., 2005]. ESCO2 mutations are correlated with lack of cohesion at heterochromatic regions impairing the mitotic cell division and affecting the cell proliferation. During embryogenesis, the ESCO2 mutations determine different structural defects by loss of genitor cells in several organs explaining the clinical manifestations of the syndrome.

Recent studies tried to explain the effect of the ESCO2 gene mutation on the embryogenesis [Williams et al., 2003; Xu et al., 2013]. Roberts syndrome is considered a cohesinopathy, having similar pathogenic changing with Cornelia de Lange syndrome (CdLS) [Xu et al., 2013]. Cornelia de Lange syndrome and Roberts syndrome have some overlapping phenotypic features but the differential diagnosis is easy done between those two syndromes as they have well described phenotypes, that can be easily recognized [Dorsett, 2007]. The different transcriptional changes specific for Roberts syndrome are the result of translational defects and it was suggested that Roberts syndrome might be partially attributed to defects in translation [Xu et al., 2013]. Different clinical manifestations of Roberts syndrome were found to be present in several disorders associated with defects in ribosome biogenesis. A similar pathogenic pathway, implying the involvement of p53 and mTOR, was described in Treacher Collins syndrome [Dauwerse et al., 2011], 5q- syndrome [Pellagatti et al., 2008], Diamond–Blackfan anemia (DBA) [Choesmel et al., 2007], Shwachman–Bodian–Diamond syndrome (SBDS) [Boocock et al., 2003], and dyskeratosis congenital [Pereboom et al., 2011], diseases that encompass some clinical features with Roberts syndrome: craniofacial, urogenital, cardiac and limbs anomalies. By our knowledge, the ESCO2 gene mutation 745_746delGT was previously reported only in two cases [Resta et al., 2006]. Resta's report presented two fetuses, which exhibit severe malformations and growth retardation, first was aborted at 22 weeks and the second at 15 weeks of gestation [Pereboom et al., 2011]. For the first fetus, autopsy was not done. The second fetus was aborted at early gestational age and presented hypertelorism, low-set ears, bilateral cleft lip, and palate, micrognathia, hypomelia of the upper extremities, oligodactyly and clitoris hypertrophy [Resta et al., 2006]. In Resta's report was showed that a mutated full length mRNA was present in both fetuses [Resta et al., 2006]. Following that finding, it was performed Western blot analysis, which revealed the lack of the ESCO2-truncated protein in cells derived from those fetuses [Resta et al., 2006]. Apart from the cases reported previously by Resta et al., in this case, the fetus was delivered at 38 weeks of gestation and a better characterization of the phenotype was possible [Resta et al., 2006].

Genetic testing of the carrier status for the parents was indicated in order to calculate the risk of having another child with Roberts syndrome for a future pregnancy. As the genetic analysis revealed the both parents are carriers of the c. 745_746delGT mutation of ESCO2 gene,

genitors were informed that they have a 25% risk of having another affected child and were discussed options regarding a future pregnancy.

Final remarks

Our report presents a very rare case and brings information about the phenotype but also the histological anomalies associated with Roberts syndrome. We consider relevant to present the histopathological findings documented for this case, in order to add new information regarding the specific phenotype of Roberts syndrome.

The particularities of our case are the presence of agenesis of several ribs and pancreas associated with absence of cleft palate/lip. Only two other cases exhibiting the same gene mutation were previously reported. In our case, the lack of prenatal follow-up delayed the diagnosis, although Roberts syndrome have some features that could be easily identified by prenatal ultrasonography. Thus, we believe that this case is an argument towards compulsory introduction of ultrasound screening to all pregnancies.

2.3.3.2. Triploidy

This report includes a series of 4 cases of triploidy diagnosed prenatally during one year, which is part of a larger study that evaluated the chromosomal anomalies over an 11-year period (2003-2013), carried out in the Clinical Hospitals of Obstetrics and Gynecology from Iasi. The cases were selected based on the results of karyotyping and the availability of ultrasound data.

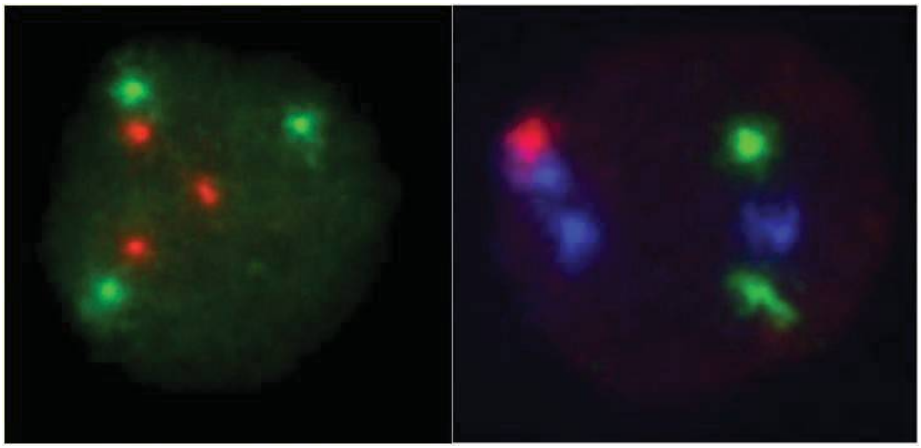
All pregnant women agreed to participate in the case study report and signed an informed consent (approved by the Bioethics Commission of Clinical Hospital of Obstetrics and Gynecology “Cuza Vodă” Iaşi).

The majority of analyses were performed after amniocentesis and only few analyses followed a chorionic villus sampling. The cytogenetic analysis was made using FISH (Fluorescence In Situ Hybridization) or complete chromosomal analysis.

For the FISH method, the fluid was centrifuged, and the cells of the supernatant were hybridized with Aneuvision® probes for prenatal diagnosis. There were used centromeric probes (CEP) CEP 18, CEP X, and CEP Y for chromosomes 18, X, and Y, and locus specific (LSI) probes LSI 13 and LSI 21 for chromosomes 13 and 21, respectively. The hybridization analysis was performed with a Zeiss Axiomot 2 epifluorescent microscope, and the images were processed with Isis software. For each case, a minimum of 100 cells were analyzed. In all cases, the epifluorescent microscope evaluation showed three blue signals (corresponding to chromosome 18), three red signals (corresponding to chromosome 21), and three green signals (corresponding to chromosome 13).

When chromosomal formula was 69,XXY, two green signals (corresponding to the X chromosome), and one red signal (corresponding to the Y chromosome) (Figure 2.36) were identified. The chromosomal analysis with the formula 69,XXX was characterized by three green signals (corresponding to the X chromosome) and the absence of red signal (corresponding to the Y chromosome).

The embryonic cells were harvested in vitro using AmnioMAX® medium (Gibco) for 14 days. After this period, the following were performed: the addition of colcemid to block the mitotic activity, the trypsin treatment, KCl (0.56 M) hypotonisation, methanol and acetic acid (3:1/v:v) fixation, microscopic slides preparation, and finally, the chromosomes analysis by conventional method using G banding. The metaphases were analyzed using a Zeiss Axiomot 2 microscope with direct lighting. For each case, 64 cells were assessed, and 12 cells were karyotyped. The karyotype indicated a 69,XXX chromosomal formula (Figure 2.37) in two cases and a 69,XXY chromosomal formula in the other two cases.



a b

Fig. 2.36. FISH features in a case of triploidy 69,XXY. (a) three LSI signals for chromosome 21 (red) and chromosome 13 (green); (b) CEP signals for chromosome 18 (blue) X (green) and Y (red)

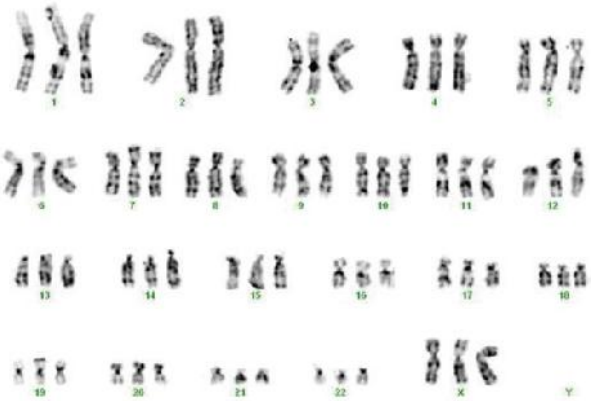


Fig. 2.37. Karyotype 69,XXX (G banding technique; 200 bands resolution)

Case Series

Case 1

A 25-year-old patient, with 19 weeks of amenorrhea, whose fetal ultrasound indicated the presence of: IUGR, complex cardiac abnormality (interventricular septum (IVS) defect and transposition of great vessels), cheilopalatoschisis (Figure 2.38a). The placenta had a normal aspect. The amniocentesis was performed and the karyotype was 69,XXX. Pathological examination confirmed the ultrasound description.

Case 2

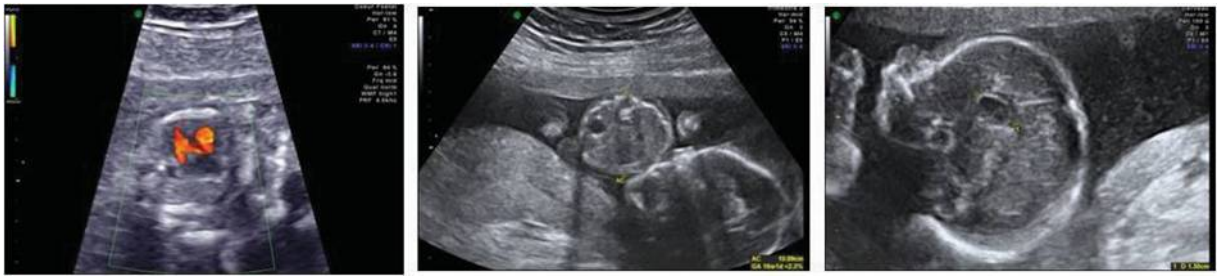
A 33-year-old patient with 18 weeks pregnancy presented at the ultrasound examination the following fetal anomalies: intrauterine growth retardation (IUGR), corpus callosum hypoplasia, bilateral cerebral ventriculomegaly, mandibular hypoplasia, large vessels anomaly with interventricular septum defect but without placental changes. The karyotype showed a 69,XXY chromosomal formula. Because the chromosomal anomaly was incompatible with survival, the parental couple decided to interrupt the pregnancy. The clinical examination at autopsy indicated: low implanted ears, dolicocephaly, retrognathia, right tetradactyly and bilateral syndactyly at upper limbs and bilateral syndactyly in lower limbs. The external examination of internal organs indicated: hypoplasia of the corpus callosum, agenesia of the thymus, and interventricular septum defect (Figure 2.38b).

Case 3

A 23-year-old women with 21 weeks pregnancy, presented the following fetal morphological aspects at ultrasound examination: IUGR, macrophtalmia, double-outlet left ventricle (Figure 2.38c), left lung agenesis, single umbilical artery, congenital varus equinus, and oligoamnios, but with absence of placental modifications. Amniocentesis was performed and the karyotype was 69,XXY. The autopsy examination confirmed the morphological anomalies identified by ultrasound scan.

Case 4

A 21-year-old woman with 22 weeks of amenorrhea, presented at ultrasound exam of the fetus only oligoamnios and common arterial duct. Amniocentesis was performed and the karyotype was 69,XXX. The autopsy examination showed: cranio-facial asymmetry, low-implanted ears, bilateral syndactily in upper limbs, and common arterial duct.



a b c

Fig. 2.38. The main ecographic features in cases with triploidy: (a) cheilopalatoschisis; (b) hypoplasia of the mandible and corpus callosum; (c) double-outlet left ventricle.

The anomalies described in these four cases are summarised in Table 2.9, with their confirmation at the autopsy examination (Table 2.10).

Table 2.9. Review of the main features encountered in the presented cases

PARAMETER	CASE 1	CASE 2	CASE 3	CASE 4
Age of patient	25	33	23	21
Chronological gestational age	19	18	21	22
Biometrical gestational age	16	15	17	18
Cephalic	Cheilopalatoschisis	Hypoplasia of corpus callosum, retrognathia, Low implanted ears	Macrophtalmia	Low implanted ears
Limbs	-	Tetradactily, syndactily	Varus equinus	Bilateral syndactily
Heart	IVS defect, transposition of large vessels	IVS defect	Double-outlet ventricle	Common arterial duct
Placenta	Normal	Normal	Normal	Normal
Other	-	Thymus agenesia	Oligoamnios	Oligoamnios
Karyotype	69,XXX	69,XXY	69,XXY	69,XXX

Table 2.10. Main features encountered at autopsy examination

PARAMETER	CASE 1	CASE 2	CASE 3	CASE 4
Cephalic	Cheilopalatoschisis	Hypoplasia of corpus callosum, retrognathia, Low implanted ears	Macrophtalmia	Low implanted ears
Limbs	-	Tetradactily, syndactily	Varus equinus	Bilateral syndactily
Heart	IVS defect, transposition of large vessels	IVS defect	Double-outlet ventricle	Common arterial duct
Other	-	Thymus agenesia	Oligoamnios	Oligoamnios

Discussions

Triploidy is a numerical chromosomal anomaly, characterized by presence of three haploid set of chromosomes. The extra chromosomal set could have maternal (digyny) or paternal (diandry or dispermy) origin [McFadden, Robinson, 2005]. This anomaly is relatively frequent in the first steps of ontogenesis, with an estimate frequency of 4.5-12.4% at conception [Egozcue et al., 2002]. Majority of cases are spontaneously aborted, thus frequency of triploidy in miscarriages is estimated at 6-15% [McFadden, Robinson, 2005; Wang et al., 2014]. In newborns, the prevalence of triploidy was estimated at 1.26/10,000 [Wellesley et al., 2012]. However, the survival of triploid babies is very short, with less than 10 cases that passed the age of 45 days [Iliopoulos et al., 2005]. Triploidy can be recurrent, with difference between maternally and paternally derived cases, considering that the former live longer than the latter [Kolarski et al., 2017].

Usually, the discovery of a numerical chromosomal anomaly during the prenatal period is the result of a prenatal diagnosis after identification of different congenital anomalies by ultrasound exam for fetal morphology. This ultrasound exam is usually performed at 18-22 weeks of pregnancy. This late discovery of chromosomal anomalies is correlated with many factors like low accuracy of biochemical screening or the relative high risk associated with chorionic villus sampling and amniocentesis. For some aneuploidies (trisomies 21, 13, 18) there are biochemical markers able to establish a high risk and therefore to recommend genetic investigations. In triploidies, a specific association of biochemical markers is absent. In some cases a biochemical association similar those of trisomy 21 (low alpha-fetoprotein (AFP), low unconjugated estriol and high human Chorionic Gonadotrophin (hCG)) was found, while in other cases values characteristic for trisomy 18 (normal alpha-fetoprotein (AFP), low unconjugated estriol and normal hCG) were described. Thus, the best indicator for the presence of triploidy is the identification by ultrasound of some congenital anomalies [Bagherizadeh et al., 2010; Guanciali-Franchi et al., 2012]. Fetuses with triploidy frequently present in the first trimester an increased fetal nuchal translucency [Sehnert et al., 2013; Norton et al., 2013], as well as elevated maternal serum AFP and total and beta-hCG levels, with low maternal serum PAPP-A (pregnancy-assisted plasma protein-A) [ACOG, 2012; Larion et al., 2014; Kolarski et al., 2017].

Our results were similar with the data reported in other studies [Wapner et al. 2012].

The phenotype in triploidy is heterogeneous and depends on the origin of the supplementary set of chromosomes. Thus, one "paternal" and one "maternal" triploid phenotype could be defined. The paternal triploidies could be the result of a diandry (fertilisation of a normal oocyte by a diploid sperm cell, because of spermatogenesis nondisjunction) or, more often, a dispermy (concomitant fertilisation of a normal oocyte by two normal sperm cells) [Kolarski et al., 2017]. In this case, the phenotype is characterized by slight IUGR, and hypertrophic cystic placenta with partial hydatiform mole. There are two groups of triploidy phenotypes, fetal and placental, corresponding to the diandric origin of additional chromosomes set [McFadden, Kalousek, 1991]: (1) well developed fetus with microcephalia or

normal head and large cystic placenta related to diandry; (2) large head fetus with growth restriction and small noncystic placenta digynic-associated [Kolarski et al., 2017]. The maternal triploidy is the result of digyny (fertilisation of a diploid oocyte by a normal sperm cell) and is characterized by severe asymmetric IUGR, with macrocephaly associated with hypoplasia of the other corporeal segments [Baumer et el., 2000; Daniel et al., 2001; Brancati et al., 2003; Dalmia et al., 2005; Falcon et al., 2005; McFadden, Robinson, 2006].

Other anomalies are common to the two forms of triploidy: anomalies of fingers (oligodactily and syndactily of fingers III and IV) different non-pathognomonic heart anomalies, genitourinary defects, lung anomalies and a non-specific facial dysmorphism [Baumer et el., 2000; Daniel et al., 2001; Brancati et al., 2003; Dalmia et al., 2005; Falcon et al., 2005; McFadden, Robinson, 2006; Witters et al., 2011].

Triploid pregnancy presents an increased risk for maternal complications [Lugthart et al., 2020; Massalska et al., 2020]. In this regard, vaginal bleeding represents the most frequent symptom encountered in both digynic and diandric cases [Memtsa et al., 2020; Lugthart et al., 2020; Massalska et al., 2020]. Diandric triploidy can be complicated with severe symptomatology, like preeclampsia and second trimester gestational hypertension [Lugthart et al., 2020; Massalska et al., 2020]. There are also described other maternal abnormalities, such as ovarian theca lutein cysts, symptomatic hyperthyreosis, and hyperemesis gravidarum [Massalska et al., 2020]. In fewer cases can occur partial hydatidiform mole (PHM), histopathologically confirmed [Buza, Hui, 2013; Massalska et al., 2021].

In our cases, we did not found major differences between cases with 69,XXY and those with 69,XXX chromosomal formula. In all situations, we found important intrauterine growth retardation and no changes in the placenta that could be an indirect argument for maternal origin of triploidy. We found different cardiac anomalies and non-specific dysmorphic features in all four cases. Other particular aspect was the presence of fingers anomalies in two of our cases. All ultrasound features were documented at autopsy. Starting from our clinical data, we found several interesting aspects. The fact that all four cases were identified in only one year brings up the question of the real incidence of triploidies, as no explanation is available for specific risks related to triploidy, and the incidence of other aneuploidies is unchanged during the same period.

Heterogeneity of ultrasound features could create difficulties in differential diagnosis, and no specific ultrasound markers could be described, excepting maybe intrauterine growth retardation. The identification of "classical" phenotypes (maternal, paternal) is not possible in ultrasound examination or even in autopsic examination. The positive diagnostic is made by chromosomal analysis, as newer technologies (genomic hybridization, cell-free fetal DNA testing) could not detect triploidy [Lapaire et al., 2007; Chiu, Lo, 2012].

Our case series is limited, although comparable to the literature on the same topic [Wick et al., 2013]. The weakness comes from the unusual presentation of our cases in the second trimester, while the majority of triploidies end in first trimester miscarriages. Regarding the ultrasound scan, we could not identify any specific sign for triploidy, although some common features (intrauterine growth retardation) were mentioned. This could also be related to the small number of cases.

Final remarks

Although a rare event in prenatal diagnosis, more reports are necessary for a better description of the phenotypes that could ameliorate the detection rate of triploidy on ultrasound scanning. Thus, it is important to perform morphological examination at 18-22 weeks of gestation to search for specific features, especially intrauterine growth retardation and cardiovascular anomalies associated with triploidy. Therefore, in all cases with fetal anomalies amniocentesis is required, followed by prenatal chromosomal analysis. No specific sign to identify the type of triploidy (maternal/paternal) has been found. Although described in the literature, this aspect was not certified by our case series, and further larger studies are needed for confirmation.

CHAPTER 3.
DERMATOPATHOLOGY

3.1. STATE OF THE ART

Autoimmune bullous or blistering dermatoses (ABD) represent a rare and heterogeous group of diseases characterized by autoantibodies directed against various molecules from the skin and/or different mucosa, comprising two major categories: (i) ABD with enzymes-linked autoimmunity (dermatitis herpetiformis) and (ii) ABD with structural proteins-linked autoimmunity (anti-desmosomal, antidermal-epidermal junction, and others). Although the autoimmunity switch from physiological to pathological state, together with bullae formation, which characterize ABD, remains a controversial issue, it is considered that malignancy could induce the pathological autoimmunity. In this regard, it was shown the relationship between chronic immunosuppressive therapy and malignancy development [Franks, Slansky, 2012; Pietkiewicz et al., 2013].

The association between ABD and malignancy still raises dissensions among researchers. Different such associations were reported, malignant pathology including various tumors, such as endometrial, ovarian, breast, prostate, renal, bladder, lung, gastrointestinal, pancreatic, laryngeal, cancers, follicular dendritic cell sarcoma or thymoma, lymphoproliferative disorders (non-Hodgkin's lymphoma, Castleman's tumor, chronic lymphocytic leukemia - CLL) [Shannon et al., 2003; Gül et al., 2006; Iwashita et al., 2007; Zhu, Zhang, 2007; Budzińska et al., 2011; Pietkiewicz et al., 2013].

A supposed association is between bullous pemphigoid (BP) and melanoma, this interrelation being still poorly understood [Amber et al., 2017], and also hypothesized by several pathomechanisms, such as: (i) BP180 cell-residual endodomain expression in malignant melanocytes and its absence in benign melanocytic tumors, mainly blue, common, and Spitz nevi [Krenacs et al., 2012]; (ii) high seropositivity of anti-BP230 autoantibodies in melanoma patients, in both incipient and late stages of disease [Shimbo et al., 2010]; (iii) the melanoma progression in a BP180 dysfunctional mouse strain, BP180 presenting an antitumoral effect by modulation of myeloid derived suppressor cells (MDSC) cutaneous infiltration [Hwang et al., 2019; Kridin et al., 2022].

Genetic studies have found a link between human leukocyte antigen (HLA) polymorphisms and melanoma and BP predisposition, a specific allele, HLA-DQB1*03:01, being overexpressed in Caucasian melanoma patients, and, at the same time, representing the most commonly HLA allele associated with BP, interacting with BP180 [25, Bateman et al., 1998; Büdinger et al., 1998; Amber et al., 2010b; Kridin et al., 2022].

A literature review conducted in 2017 highlighted five cases of melanoma association with BP [Amber et al., 2017]. Moreover, some interesting clinical aspects have been reported, being revealed a similar evolution of BP with that of melanoma, as well as a certain improvement in the autoimmune dermatosis after surgical removal of the melanoma or metastatic lymph nodes [Amber et al., 2017; Kridin et al., 2022]. It was also revealed, in a large population-based study, a 1.5-fold increased risk for developing BP in patients already diagnosed with melanoma, especially among males, with lower BMI, or people older than 80 years [Kridin et al., 2022]. The onset of BP after a diagnosis of melanoma could be related to other cofactors, including the existence of PD-1/PDL-1 antagonists' therapy [Kridin et al., 2022].

These checkpoint inhibitors (CIs), initially approved for metastatic melanoma treatment and subsequently expanding its applications in other malignancies, represent a novel category of immunomodulatory drugs, which, by blocking inhibitory signals on effector immune cells, generates strong T-cell mediated immune responses. The following are currently used: ipilimumab, which inhibits cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), pembrolizumab and nivolumab, which inhibit the programmed cell death protein 1 (PD-1), and durvalumab and atezolizumab, which block the PDL-1 (PD-1 ligand) [Callahan et al., 2016; Sadik et al., 2021].

Increasing evidence suggest the correlation between PD-1/PDL-1 antagonists, including administered to patients with metastatic melanoma, and subsequent BP development [Lopez et al., 2018]. In this regard, besides their encouraging anti-cancer activity [Chen et al., 2020], the checkpoint inhibitors immunotherapies, with anti-PD-1/PDL-1 monoclonal antibodies are associated with the so-called "immune-related adverse events" (irAEs), including the onset of BP, with the same morphological and immunopathological phenotypes as the typical BP [Lopez et al., 2018]. Moreover, it was shown that melanoma represents an independent risk factor for bullous pemphigoid, unrelated to PD-1/PDL-1 antagonists' therapy, fact justified by the persistence high risk for BP among patients with melanoma history, after the adjustment of the immune checkpoints treatment [Kridin et al., 2022]. In this regard, it cannot be ruled out that this increased risk of BP after PD-1/PDL-1 antagonists' therapy may be due to an worsening of another preexistent association between BP and melanoma [Kridin et al., 2022].

One such example is nivolumab, which, despite its therapeutic benefits, leads to many irAEs, such as hypophysitis, pneumonitis, thyroid dysfunction, hepatitis, and colitis [Haug et al., 2018]. Among dermatological immune adverse effects, presented as non-specific maculopapular rash with pruritus, lichenoid reactions, exanthemas, Steve-Johnson syndrome, granulomatous reactions, dermatitis, psoriasis, leukoderma, vitiligo, lupus-like skin changes, sarcoidosis, erythema multiforme-like changes, and toxic epidermal necrolysis-like reactions are the most frequent toxicities associated with CIs immunotherapies [Weber et al., 2017; Wang et al., 2019; Sibaud, 2018; Sibaud et al., 2019], [Sibaud, 2018; Heinzerling et al., 2019; Egami et al., 2020; Sadik et al., 2021]. Bullous dermatosis, such as BP, linear IgA bullous dermatosis or bullous lichenoid dermatitis represent rare adverse events, characterize by blisters which interese large cutaneous area, which can lead to discontinuation or interruption of treatment [Siegel et al., 2018; Aggarwal, 2019; Miranda et al., 2020]. However, increasing evidence attest the autoimmune BP as the most frequent pemphigoid disease related to CIs immunotherapy [Jour et al., 2016; Hirotsu et al., 2017; Thomson et al., 2018; Sibaud, 2018; Siegel et al., 2018; Sun et al., 2019; Heinzerling et al., 2019; Sadik et al., 2021].

It was shown that drug-induced BP develops usually in the first 6-8 months of therapy [Lopez et al., 2018; Siegel et al., 2018], presenting the same pathophysiology of conventional BP, which makes it difficult to differentiate them clinically [Lopez et al., 2018]. Although in such cases, BP requires giving up immunotherapy [Anastasopoulou et al., 2018], in the situations where the diagnosis is early, the installation of appropriate treatment with systemic or topical steroids may allow the continuation of CIs therapy [Lopez et al., 2018; Siegel et al., 2018; Anastasopoulou et al., 2018; Miranda et al., 2020].

The BP certification consists in autoimmunity development against type XVII collagen (BP180) [Sadik et al., 2020]. The most characteristic clinical signs are extensive urticarial plaques (prebullous phase), evolving towards blisters and erosions [Sadik, Schmidt, 2019; Egami et al., 2020]. Other aspects include subacute prurigo-like and eczematous skin changes [Feliciani et al., 2012]. Sometimes, BP present only widespread itch, without any other symptom.

Besides this drug-induced BP variant, atypical and non-bullous forms of BP are described, with localized bullae and minimal perilesional inflammation. This heterogeneity of symptoms makes BP require a complex diagnosis [Sadik et al., 2021].

Albeit the vast majority of data report bullous pemphigoid cases [Naidoo et al., 2016; Siegel et al., 2018; Lopez et al., 2018], there is an increase in case reports with mucous membrane pemphigoid (MMP) associated with pembrolizumab therapy [Zumelzu et al., 2018; Haug et al., 2018; Bezinelli et al., 2019].

MMP comprises a heterogeneous group of subepithelial ABD as a consequence of the linear coupling of IgG/IgA or complement throughout the basement membrane [Maderal et al., 2018; Carey, Setterfield, 2019].

Although without an elucidated pathophysiology, it was shown that T cells, mainly Tregs, as well as B cells have important roles in its pathogenesis [Sibaud et al., 2019].

It has been shown that autoantibodies against the hemidesmosomal protein BP180, the most addressed antigen in BP or MMP, occur only after nivolumab or pembrolizumab therapies [Naidoo et al., 2016; Lopez et al., 2018; Siegel et al., 2018; Zumelzu et al., 2018; Haug et al., 2018; Bezinelli et al., 2019; Sibaud et al., 2019].

Another particular aspect is the rare bullous form of melanoma [Woltsche et al., 2015], characterized by subepidermal or intraepidermal blistering lesions [Vogt et al., 2003].

The diagnosis of bullous melanoma uses the same criteria from other melanoma variants (history, dermatological clinical examination, and histopathology), but the blisters makes it difficult to measure the Breslow index [Woltsche et al., 2015; de Almeida et al., 2021].

Considering acantholysis the loss of desmosomes cohesion between keratinocytes, the term bullous melanoma is used instead [Woltsche et al., 2015]. Because tumor cells lose the perilesional intercellular adhesions, probably from cadherins down-regulation as a consequence of melanoma cells pregoression, it is not recommended the use of terms “spongiosis” or “acantholysis”, as they do not describe the bullous melanoma mechanism of development [Hsu et al., 2000; de Almeida et al., 2021].

We may assume that an intercausal relationship between ABD (especially BP) and melanoma can be observed, as the chronic immunosuppressive therapy can lead to malignancy, including melanoma, and on the other hand, specific immunosuppressive therapy with CIs, especially targeting metastatic melanoma, may have as side effects numerous dermatological lesions, often BP or other forms of bullous dermatoses.

My interest for this scientific direction has materialized in the following achievements:

Articles

Balan RA, Lozneanu L, Grigoras A, Caruntu ID, Balan TA, Giusca SA, Amalinei C. Spongiotic reaction patterns in autoimmune bullous dermatoses. *Exp Ther Med* 2021; 22(5): 1334.

Amalinei C, Grigoraș A, Lozneanu L, Căruntu ID, Giușcă SE, **Balan RA**. The interplay between tumour microenvironment components in malignant melanoma. *Medicina (Kaunas)* 2022; 58(3): 365.

3.2. PARTICULAR MORPHOLOGICAL ASPECTS IN AUTOIMMUNE BULLOUS DISEASES

3.2.1. INTRODUCTION

Autoimmune blistering dermatoses represent a broad category of skin diseases defined by autoantibodies - antigens reactions in mucous membranes and in the skin [Witte et al., 2018]. Thus, the connection of autoantibodies to desmosomes interrupts the intraepidermal

adhesions, causing intraepithelial blisters and acantholysis [Tsunoda et al., 2011; Pan et al., 2011; Alhamami et al., 2021].

There are described several groups of autoimmune blistering diseases, according to the blister localization and targeted proteins, such as pemphigus group, with autoantibodies - desmosomal proteins connection, leading to cell adherence loss between keratinocytes, pemphigoid diseases, in which autoantibodies target hemidesmosomal proteins from dermo-epidermal junction, or dermatitis herpetiformis, with the connection between autoantibodies and transglutaminase [Baum et al., 2014].

Because of their rarity and heterogeneity, the diagnosis of these dermatoses often represents a major challenge. In this regard, the gold standard for the confirmation of autoimmune blistering diseases is represented by the detection of circulating and tissue bound autoantibodies, the clinical and histopathological aspects being not sufficient [Baum et al., 2014].

Autoimmune bullous dermatoses present limited spongiotic reactive patterns, the various dermatoses practically exhibiting the same types of spongiosis.

Spongiosis or spongiotic reaction pattern (classical eczematous tissue reaction) represents the histological hallmark of intercellular epidermal edema, translated by cell condensation with corresponding wide spaces betwen keratinocytes, with elongated intercellular bridges (‘spinous processes’), leading to a sponge-like appearance of the epidermis [Trautmann, 2001; Murphy, GrantKels, 2010; Weedon, 2010a; Weedon, 2010b;]. Hematoxylin and eosin (H&E) routine staining highlights this process as clear spaces within the epidermis. Sometimes, when there is a marked intraepidermic edema, spongiosis forms multiloculated vesicles. Moreover, spongiosis includes also intracellular edema.

Although considered a histopathological term, spongiosis has clinical correlations, with the variable degrees of spongiotic reaction leading to different dermatological findings, from mild skin erythema to crusted plaques, oozing papulovesicles or vesiculobullous lesions, with a specific collaret of scale for resolving lesions [Murphy, Grant-Kels, 2010; Weedon, 2010a; Weedon, 2010b]. However, the most common cutaneous lesion associated with spongiosis is eczematous dermatitis [Holden, Berth-Jones, 2004].

Autoimmune bullous dermatoses present limited spongiotic reactive patterns, the various dermatoses practically exhibiting the same types of spongiosis.

This review aimed to provide a summarized overview of the current state of research regarding spongiotic patterns found in autoimmune bullous dermatoses, considering that only few publications have this approach on spongiosis. For this study, a literature search was conducted using the main scientific databases (Web of Science, Scopus, Science Direct, Google Scholar, Cochrane Database for Systematic Reviews, PubMed/Medline) and acquisition was based on a database search using the following keywords: “spongiosis”, “autoimmune bullous dermatoses”, “spongiotic pattern” in all the relevant combinations. In the next step of selection, all the relevant full content English written literature in a period of 20 years was categorized, using as inclusion criteria only autoimmune bullous diseases.

3.2.2. MAIN EVENTS IN SPONGIOSIS PATHOGENESIS

Still controversial and with limited studies, the mechanism of spongiosis assumes the passage of extravasated edema fluid from the dermis into the epidermis [Murphy, Grant-Kels, 2010]. It is assumed that the dermal inflammatory cells, which usually accompany this process, with or without epidermal involvement, secrete substances that regulate the particular tissue fluid dynamic [Murphy, Grant-Kels, 2010]. The classification of the spongiotic reaction patterns, as well as their associated spongiotic dermatitis, take into consideration the type and distribution of these inflammatory cells.

Although widely accepted, the fluid extravasation etiology, as well as the mechanism of dermal extravascular fluid influx into the epidermis, remain to be understood [Trautmann, 2001]. Two main opposing concepts have been developed, one related to the osmotic gradient towards the epidermis, and the other considering that epidermal involvement of edema is due to increased dermal hydrostatic pressure [Trautmann, 2001]. Even though opposite, both mechanisms seem to be involved, as spongiotic reaction represents a dynamic process, with transient vesicles formed in different locations in the epidermis [Trautmann, 2001]. Overlying parakeratosis may occur because of disordered maturation or of an accelerated keratinocyte movement towards the superficial layers. When involved by plasma droplets, parakeratosis is responsible for Collarette scales, which appear in resolving lesions [Trautmann, 2001].

Studies have classically shown that spongiosis is caused by the cleavage of membrane E-cadherins due to interferon (IFN)-γ-producing T cell-mediated keratinocyte apoptosis, which affects the integrity of E-cadherins but retains desmosomal cadherins, in acute eczematous dermatitis [Wolff et al., 2003; Ohtani et al., 2009].

Maintaining intercellular contact only at the desmosomal level confers the sponge-like morphology to the epidermis [Trautmann, 2001; Wolff et al., 2003; Ohtani et al., 2009].

Recent data, which have demonstrated high expression levels of interleukin (IL)-4 and IL-13 in biopsy specimens of allergic contact dermatitis, suggest that IFN-γ is not the only trigger of spongiosis pathogenesis [Neis et al., 2006; Ohtani et al., 2009].

Moreover, Ohtani et al demonstrated that in acute eczema, the intercellular space of spongiotic epidermis contains a high amount of hyaluronan (HA). By histochemistry, they demonstrated that the intercellular space enlargement related to acantholysis is not associated with an abnormal HA quantity, suggesting that, in pemphigus vulgaris, impairment of keratinocyte cohesion does not directly cause excessively accumulation of HA [Ohtani et al., 2009]. At the same time, the study observed that not all skin diseases characterized by lymphocyte exocytosis have more HA in the spongiotic epidermis [Ohtani et al., 2009].

Although dermatomyositis exhibits interface dermatitis in almost all cases, several patients were identified who showed spongiotic dermatitis on skin biopsies, the histological aspect being more correlated with eczematous lesions [Zeidi et al., 2021]. In this regard, one case-control study demonstrated that increased immunohistochemical expression of myxovirus-resistance protein A (MxA), IFN-β, CD11c, and dendritic cell lectin (BDCA2) protein in dermatomyositis-related spongiotic dermatitis could represent a useful differential diagnostic tool between dermatomyositis and eczema [Zeidi et al., 2021].

Studies reveal that for bullous pemphigoid (BP), the main events that lead to blister formation are the autoantibodies targeting hemidesmosomal proteins BP180 (type XVII collagen) and BP230 (intracellular plakin-like protein) [Bagci et al., 2017; Liu et al., 2017]. BP180 represents a transmembrane glycoprotein, which mediates the adhesion between the basement membrane and the epidermis [van Beek et al., 2016]. It was demonstrated that the disease activity was linked to serum IgG antibodies against NC16A domain of BP180, which is considered to represent the main target of pathogenic IgG in BP [Schmidt et al., 2000; Amber et al., 2018]. Besides the demonstrated function of anti-BP180 IgG autoantibody, other factors involved in BP pathogenesis are eosinophils with IgE autoantibodies, anti-inflammatory (IL-10, IL-4) and proinflammatory cytokines (IL-8, IL-6, TNF-alpha), effector cells of mediated autoimmunity [Amber et al., 2018].

3.2.3. CATEGORIES OF THE SPONGIOTIC REACTION PATTERNS AND THEIR MIMICKERS

The epidermis intercellular edema comprises six main spongiotic reaction patterns, each correlated with different skin diseases, the major ones being atopic dermatitis, contact dermatitis, nummular and seborrheic dermatitis.

These entities present psoriasiform hyperplasia, which accompanies spongiosis, sometimes in the same biopsy, with different stages of activity. The areas of spongiosis may vary from microscopic foci to grossly observable vesicles or bullae (Table 3.1) [Weedon, 2010a].

Table 3.1. Skin conditions showing a spongiotic reaction pattern (adapted from Weedon, 2010)

SPONGIOTIC PATTERN						
Clinical Categories	Eosinophilic Spongiosis	Miliarial Spongiosis	Follicular Spongiosis	Pityriasiform Spongiosis	Haphazard Spongiosis	Spongiosis with subepidermal edema
● Pustular psoriasis/Reiter's syndrome ● Prurigo pigmentosa ● IgA pemphigus ● Infantile acropustulosis ● AGEP ● Palmoplantar pustulosis ● STSS ● Neisserial infections ● Dermatophytosis / candidosis ● Beetle (Paederus) dermatitis ● Pustular contact dermatitis ● Glucagonoma syndrome ● Amicrobial pustuloses ● Periodic fever syndromes	● Pemphigus (precursor lesions) ● Herpetiform pemphigus ● Pemphigus vegetans ● Bullous pemphigoid/CP ● Herpes gestationis ● IES ● EPPE ● ACD ● Protein contact dermatitis ● Atopic dermatitis ● Arthropod bites ● Eosinophilic folliculitis ● Drug reactions ● Incontinentia pigmenti ● Drug reactions ● 'Id' reaction ● Still's disease	● Miliaria	● Infundibulo-folliculitis ● Atopic dermatitis (follicular lesions) ● Apocrine miliaria ● Eosinophilic folliculitis ● Follicular mucinosis ● Infectious folliculitides ● Perioral dermatitis	● Pityriasis rosea ● PDR ● EAC ● ACD ● Nummular dermatitis ● Lichen striatus (uncommonly) Gianotti–Crosti syndrome (sometimes)	● ICD ● ACD ● Nummular dermatitis ● Sulzberger –Garbe ● syndrome ● Seborrheic dermatitis ● Atopic dermatitis ● Papular dermatitis ● HDH ● JPD ● VGDD ● Mycosis fungoides ● 'Id' reaction ● Urticarial dermatitis ● Pityriasis rosea ● PAC ● Spongiotic drug reactions ● APD ● Estrogen dermatitis ● CSD ● EAC ● Psoriasis (spongiotic) ● PPD ● LSC ● Herpes gestationis	● Arthropod bites and bite-like reactions in lymphoma ● Cercarial dermatitis/larva migrans ● 'Id' reaction ● ACD ('dermal type') ● Contact urticaria, papular urticarial ● Urticarial dermatitis ● Erysipelas, erysipeloid ● Dermatophytoses ● Prebullous pemphigoid ● Sweet's syndrome ● Wells' syndrome ● Miliaria rubra ● Polymorphic light eruption ● Spongiotic drug reactions (including estrogen/progesterone dermatitis)

AGEP - Acute generalized exanthematous pustulosis; STSS -Staphylococcal toxic shock syndrome; CP – cicatriceal pemphigoid; IES - Idiopathic eosinophilic spongiosis; EPPE - Eosinophilic, polymorphic and pruritic eruption; ACD - Allergic contact dermatitis; PDR - Pityriasiform drug reaction; EAC - Erythema annulare centrifugum; ICD - Irritant contact dermatitis; HDH - Hyperkeratotic dermatitis of the hands; JPD - Juvenile plantar dermatosis; VGDD - Vein graft donor-site dermatitis; PAC - Papular acrodermatitis of childhood; APD - Autoimmune progesterone dermatitis; PPD - Pigmented purpuric dermatoses; CSD - Chronic superficial dermatitis; LSC - Lichen simplex chronicus

The spongiotic reactive patterns include: i) neutrophilic spongiosis, with neutrophils within foci of spongiosis; ii) eosinophilic spongiosis, where the spongiosis foci contain numerous eosinophils; iii) miliarial (acrosyringial) spongiosis, with edema related to the acrosyringium; iv) follicular spongiosis, with edema involving the follicular infundibulum; v) pityriasiform spongiosis, characterized by small vesicles with lymphocytes, histiocytes, and Langerhans cells; and vi) haphazard spongiosis, with no particular pattern (Table 3.2) [Weedon, 2010a]. There is a variant of the haphazard type, considered as a seventh pattern of spongiosis, characterized by an association of epidermal spongiosis and subepidermal edema, with different degrees of severity (Table 3.2) [Weedon, 2010a].

Table 3.2. Types of spongiotic reaction patterns and their histological features

SPONGIOTIC PATTERN	HISTOLOGICAL CHARACTERISTICS
Neutrophilic spongiosis	neutrophils in areas of spongiosis
Eosinophilic spongiosis	eosinophils within areas of spongiosis
Miliarial (acrosyringial) spongiosis	edema correlated with the acrosyringium
Follicular spongiosis	spongiotic foci involving the follicular infundibulum
Pityriasiform spongiosis	small vesicles with lymphocytes, histiocytes, and Langerhans cells
Haphazard spongiosis	no particular pattern
Haphazard variant of spongiosis	epidermal spongiosis with subepidermal edema, mild to severe variants, can evolve towards subepidermal blisters

Although displaying several histological forms, spongiosis shows limited patterns in bullous autoimmune diseases, without a characteristic morphological type.

Considering these aspects, the spongiotic reactions represent one of the most difficult histopathological patterns that can lead to a specific clinicopathological diagnosis [Weedon, 2010a].

There are different reactive processes, specific for other skin disorders, which act as simulants of different spongiotic patterns.

Variants of lupus erythematosus, pityriasis lichenoides, erythema multiforme or fixed drug eruption, with vacuolar changes or lichenoid patterns, may exhibit mild suprabasal spongiosis.

Some viral diseases, such as morbilliform drug eruptions or exanthemas show epidermal basal spongiosis, while others, such as herpes zoster and simplex, display ballooned degenerated keratinocytes followed by acantholysis, changes that obscure the mild spongiotic reaction. Early psoriasis, which is not a spongiotic disease, may show some epidermal spongiosis close to the dermal papillae [Weedon, 2010a].

Another mimicker of spongiosis is a high amount of acid mucopolysaccharides accumulated in the follicular infundibulum of follicular mucinosis, identified with mucin stains. The accumulation of mononuclear cells in spongiotic dermatitis can sometimes simulate the Pautrier microabscesses found in mycosis fungoides. The difference resides, on the one hand, in the specific vase-like shape of intraepidermal inflammatory cell collections, with their extremities situated between granular and keratinized layers, and on the other hand, in the characteristic phenotypes expressed by mononuclear cells: CD1a, CD36, CD38 and S100 protein [Weedon, 2010a].

3.2.4. TYPES OF SPONGIOSIS PATTERNS ENCOUNTERED IN AUTOIMMUNE BULLOUS DERMATOSES

Autoimmune bullous dermatoses represent a group of diseases, which develop autoantibodies against structural adhesion proteins from the epidermis and basement membrane area, with skin or mucosal blisters development [Radoš, 2011; Harrell et al., 2019]. The diagnosis is very complex due to their heterogeneity, always associating different diagnostic methods such as direct or indirect immunofluorescence, immunoblot analysis, immunohistochemistry, or molecular biology to histopathological and clinical assessment [Radoš, 2011; Harrell et al., 2019].

The classification of autoimmune bullous dermatoses takes into consideration the histologic localization of the bullae, dividing this category in intraepidermal and subepidermal bullous dermatoses.

- ***Intraepidermal autoimmune bullous dermatoses***

Intraepidermal autoimmune bullous dermatoses include five subtypes of the pemphigus category: pemphigus vulgaris (PV), pemphigus foliaceus (PF), paraneoplastic pemphigus (PNP), IgA pemphigus, and pemphigus herpetiformis (PH) [Radoš, 2011]. Histopathologically, intraepidermal bullae or pustules characterize these dermatoses, aspects observed also in various diseases with similar histology but different etiopathogeny [Radoš, 2011]. The intercellular or intracellular edema (spongiosis or ballooning degeneration) can cause desmosomes destruction [Radoš, 2011].

Pemphigus vulgaris

PV is a chronic autoimmune intraepithelial bullous dermatosis, characterized by IgG autoantibodies directed against transmembrane cadherin desmoglein3 (Dsg3) [Schmidt et al., 2010; Baum et al., 2014; Harrell et al., 2019]. Classically, the diagnosis considered lesional histopathological assessment and perilesional direct immunofluorescence, but recently, ELISA tests have been identified as the most accurate technique for the diagnosis of PV [Schmidt et al., 2010; Harrell et al., 2019].

The key histopathological feature of pemphigus group diseases is the intraepidermal bullae with acantholysis, which makes clinical correlations and immunofluorescence techniques mandatory for differential diagnosis with other intraepidermal bullous diseases [Radoš, 2011; Laws et al., 2015; Morais et al., 2019]. The bullae contain acantholytic cells with eosinophilic cytoplasm, pyknotic nuclei perinuclear halo, and serum. In older lesions, the dermis shows mild to moderate inflammatory infiltrate with lymphocytes, macrophages, along with eosinophils or neutrophils. The pattern of spongiosis in PV is eosinophilic or neutrophilic and appears in the very early stage of the disease, when acantholysis is absent (Table 3.3) [Megahed, 2004; Hong et al., 2010; Radoš, 2011; Morais et al., 2019]. Subsequently, newly formed vacuoles and foci of acantholysis become confluent, resulting in fissures and bullae. However, eosinophilic and neutrophilic spongiosis, when present, are not specific to PV, the reaction being common to various other diseases [Radoš, 2011; Morais et al., 2019].

Pemphigus foliaceus

In PF, IgG autoantibodies are targeting desmoglein1 (Dsg1). This subtype of pemphigus exhibits characteristic superficial and fragile bullae, which usually disappear in clinical examination, leaving an erosion post-examination. The mature PF lesion shows an intraepidermal bulla within spinous and granular layers. As in PV, the early stage of PF shows neutrophilic or eosinophilic spongiosis (Table 3.3) [Megahed, 2004; Hong et al., 2010; Radoš, 2011]. Sometimes, PF can transform into PV, changing its clinicopathological and immunological profile. The definite diagnosis requires, as in PV, supplementary immunofluorescence techniques as well as ELISA, besides histopathological examination [Radoš, 2011; Harrell et al., 2019].

Paraneoplastic pemphigus

PNP presents IgG autoantibodies targeting cytosolic plakin proteins (desmoplakins1 and 2, periplakin, envoplakin), Dsg1, Dsg3, BP antigen1 (BP230), Dsc1-3, and α-2-macroglobulin-like protein 1 (A2ML1) [Harrell et al., 2019].

Within the spectrum of benign and malignant neoplasms, commonly lymphoproliferative malignancies, the histopathological features of PNP are associated with the lesion morphology, with an overlap of histological patterns. PNP consists of an interface dermatitis with few acanthotic areas and necrotic keratinocytes. This entity includes three histopathological patterns, as follows: i) intraepidermal suprabasal acantholytic bullae; ii) intraepidermal suprabasal acantholytic bullae with basal vacuolar degeneration; iii) nonspecific interface dermatitis with a thinned/hyperplastic epidermis, basal vacuolar degeneration, hypergranulosis, necrotic keratinocytes, and band-like lymphocytes [Radoš, 2011]. Eosinophilic spongiosis may be the initial histological feature in PNP, according to some studies [Gallo et al., 2014; Morais et al., 2019]. However, because of its heterogeneity, the diagnosis requires confirmation by immunofluorescent and immunoblot studies [Radoš, 2011].

IgA pemphigus

IgA pemphigus represents a rare entity of this group of bullous dermatoses [Baum et al., 2014]. According to the site of bullae, there are two types of IgA pemphigus, namely intraepidermal neutrophilic dermatosis (IEN) and subcorneal pustular dermatosis (SPD). IEN is characterized by IgA autoantigens against Dsg3 and Dsg1, while IgA autoantibodies target desmocollin1 (Dsc1) in SPD [Radoš, 2011; Harell et al., 2019].

Clinically, IgA pemphigus shows pustules, and histologically, both types identified show intraepidermal, subcorneal or spinous layer bullae, with hyperplastic epidermis, acantholytic keratinocytes, neutrophilic spongiosis, superficial inflammatory infiltrate with lymphocytes, neutrophils, and eosinophils, features which are also observed in other dermatoses (Table 3.3) [Kopp et al., 2006; Hong et al., 2010; Radoš, 2011]. However, the most accurate diagnostic test remains direct immunofluorescence [Harrell et al., 2019].

Pemphigus herpetiformis

PH is a rare variant with clinical features of herpetiform dermatitis, and histological and immunofluorescent features of pemphigus [Radoš, 2011]. PH shows IgG and C3 deposits, with anti-epithelial and anti-desmoglein1 (Dsg1) antibodies [Fuentes-Finkelstein et al., 2014]. The clinical aspects may vary from erythematous, pruriginous, vesicular or papular lesions, exhibiting herpetiform arrangement. The histopathological picture is nonspecific, showing eosinophilic spongiosis, the most characteristic spongiotic pattern for this dermatosis [Radoš, 2011], as well as neutrophilic spongiosis, or a mixed eosinophilic-neutrophilic spongiosis, with acantholytic cells, intraepidermal vesicles, and papillary neutrophilic microabcesses (Table 3.3) [Duarte et al., 2010; Radoš, 2011; Morais et al., 2019].

Early urticarial stages of PH may also present neutrophilic and eosinophilic spongiosis [Megahed, 2004]. The mature PH lesions show intraepidermal bullae, with various locations. The final diagnosis requires the corroboration of histopathological, clinical, and immunological techniques [Radoš, 2011].

● ***Subepidermal autoimmune bullous dermatoses***

Subepidermal autoimmune bullous dermatoses, with autoantibodies against basement membrane components, represent a heterogeneous group, which include bullous pemphigoid (BP), gestational pemphigoid/herpes gestationis (GP/HG), mucous membrane or cicatricial pemphigoid (MMP/CP), linear IgA dermatosis, and lichen planus pemphigoides, as the pemphigoid group of bullous diseases. Other distinct autoimmune dermatoses are dermatitis herpetiformis (DH) and epidermolysis bullosa acquisita (EBA), as well as anti-p200 pemphigoid, a new entity, considered as part of the subepidermal autoimmune bullous dermatoses by some authors [Radoš, 2011; Baum et al., 2014; Harrell et al., 2019].

Bullous pemphigoid

BP is the most common entity from this group, which affects especially elderly people, being characterized by circulating antibodies against hemidesmosomal proteins, as BP antigen1 (BPAG/BP230) and BPAG2 (BP180) [Langan et al., 2008; Radoš, 2011; Taghipour, Perera, 2017; Harrell et al., 2019]. Direct immunofluorescence represents the gold standard for diagnosis, revealing linear C3, IgG, IgA, IgE or IgM, along the dermo-epidermal junction in perilesional biopsies [Harrell et al., 2019].

The histopathological examination reveals different morphological aspects, according to the stage of the lesion [Rapini, 2012]. In early or urticarial BP, the histological picture draws attention by eosinophilic spongiosis, with dermal edema, pseudovacuolar change, and perivascular inflammatory infiltrate dominated by lymphocytes, histiocytes, and numerous eosinophils (Table 3.3) [Radoš, 2011; Rapini, 2012]. In this regard, one study reported a group of patients with clinical signs suggestive of BP, but with no characteristic histopathological and serological features. Biopsies showed in these cases only eosinophilic spongiosis, demonstrating either a particular form of bullous eczema or a BP with a prolonged incipient stage [Atteh et al., 2021]. BP shows subepidermal bullae with lymphocytes, eosinophils, neutrophils, plasma, and fibrin, in mature lesions [Radoš, 2011].

Gestational pemphigoid/Herpes gestationis

GP represents an autoimmune bullous dermatosis, which can occur during the second half of the pregnancy or in puerperium [Radoš, 2011; Moore, Werth, 2016; Harrell et al., 2019; Patsatsi et al., 2019]. Recent studies have revealed the autoimmune pathogenesis, by identification of IgG autoantibodies against placental BP180 and, rarely, against BP230 [Radoš, 2011; Harrell et al., 2019].

GP shares histological features with BP due to their similarities in the pathogenic mechanism, displaying various morphology according to the lesion age. Thus, there is no difference between these two entities, especially in early urticarial stages, which show eosinophilic spongiosis, subepidermal bullae, marked papillary edema, and dermal inflammatory infiltrate with lymphocytes, eosinophils, and occasional neutrophils (Table 3.3). The bullae have the same content as in BP, with flat or necrotic overlying epidermis [Radoš, 2011; Harrell et al., 2019]. Direct immunofluorescence, ELISA and immunoblotting complement are sometimes necessary to certify the histopathologic diagnosis because of the high degree of heterogeneity and nonspecific morphological features [Radoš, 2011].

Moreover, BP and PG could both represent intraepidermal and subepidermal autoimmune bullous dermatoses because extensive spongiosis can lead to the formation of epidermal bullae [Radoš, 2011].

Mucous membrane pemphigoid/Cicatricial pemphigoid

MMP or CP represents a rare disease which stands out by autoantibodies against multiple basement membrane antigens, such as BP180, type VII collagen (COL7), β4 integrin subunit, laminin5 (laminin332, epiligrin), and laminin6 [Harrell et al., 2019].

The particularity of MMP is the main mucosal localization of bullae, with the skin being involved only in 25% of cases [Zeidi et al., 2021]. The most affected areas are represented by oral mucosa, closely followed by ocular lesions [Radoš, 2011; Rapini, 2012; Harrell et al., 2019].

Because of the numerous CP clinicopathological variants, it is more likely a general term comprising several diseases with similar clinical features and cicatrices, with oral and ocular mucous membrane tropism and with subepidermal bullae formation [Radoš, 2011; Harrell et al., 2019].

Given the histological similarities with BP, CP represents a variant of bullous pemphigoides, although some authors report clinical, morphological, and immunological differences between these two entities [Megahed, 2004; Radoš, 2011]. Subepithelial blisters

dominate the histological picture, with dermal inflammatory infiltrates containing lymphocytes, numerous eosinophils, and neutrophils, sometimes associated with fibrosis. Eosinophilic spongiosis occurs less frequently in CP [Morais et al., 2019]. The histological and immunological heterogeneity make the CP diagnosis complex, always taking into account additional immunological testing [Radoš, 2011; Harrell et al., 2019].

Linear IgA disease

LAD has a tendency to affect children, although an adult type linear IgA dermatosis is also described, which differs by the clinical presentation [Radoš, 2011; Harrell et al., 2019].

According to the immunofluorescent patterns and protein targets, there are two types of linear IgA disease: i) lamina lucida type, with two ectodomains of BP180, LABD97, and LAD-1 as target antigens and ii) sublamina densa type, with COL7, as well as other target antigens [Harrell et al., 2019].

As in other autoimmune subepidermal bullous dermatoses, LAD shows eosinophilic, neutrophilic, or a combined spongiotic reaction pattern, with perivascular inflammatory infiltrate with lymphocytes, neutrophils, and eosinophils, as well as vacuolar degeneration of basement membrane zone, in early urticarial lesions (Table 3.3) [Rao, Hall, 2008; Radoš, 2011]. Although displaying typical formation of subepidermal bulla and a mixed dermal inflammatory infiltrate with neutrophils and eosinophils, the general histopathological picture is almost identical with that seen in dermatitis herpetiformis (DH), with a tendency to form papillary microabcesses [Radoš, 2011].

Lichen planus pemphigoides

LPP represents a heterogeneous disease characterized by basal membrane autoantibodies directed against numerous antigens and the formation of bullous lesions, probably developed as a result of basal keratinocyte hydropic degeneration, in lichen planus [Radoš, 2011].

The histopathological features highlight subepidermal bullae associated with perivascular inflammatory infiltrate with lymphocytes and eosinophils, sometimes with characteristic morphological features of lichen planus, such as acanthotic epidermis with hypergranulosis and a band-like lymphocytic infiltrate. Immunofluorescence, other immunological techniques, and clinical correlations are mandatory for LPP diagnosis [Radoš, 2011].

Dermatitis herpetiformis

DH is an IgA-mediated bullous dermatosis, which forms autoantibodies against epidermal transglutaminase (eTG) and against tissue transglutaminase (tTG) [Harrell et al., 2019]. The histopathological picture demonstrates subepidermal clefting and neutrophils in the dermal papillae, in most cases. The typical evolution of the lesions comprises an early perivascular and interstitial inflammatory infiltrate with lymphocytes, neutrophils, sometimes eosinophils, and papillary dermis edema, followed by fibrin in papillary tips, and even abscesses, in cases with numerous neutrophils. In time, the subepidermal clefts transform into bullae, which are characteristic for mature lesions, while dermal infiltrate contains a high amount of eosinophils [Radoš, 2011].

Because of nonspecific DH histopathology and common morphological changes with other bullous dermatoses, the diagnosis confirmation should comprise direct immunofluorescence and electron microscopy, which highlight the granular IgA pattern, in dermal papillae [Radoš, 2011; Clarindo et al., 2014; Antiga et al., 2019].

Epidermolysis bullosa acquisita

EBA represents a rare and chronic bullous dermatosis, characterized by autoantibodies against COL7, with subepidermal blister formation [Ishii et al., 2010; Harrell et al., 2019]. EBA can exhibit different clinical forms, as well as variable and nonspecific histopathological features [Radoš, 2011].

Eosinophilic or neutrophilic spongiosis, associated with perivascular inflammatory infiltrate with lymphocytes, neutrophils, and eosinophils characterizes only the early urticarial lesions, the findings being similar with those seen in other bullous dermatoses [Megahed, 2004] (Table 3.3). The variable clinical presentations correspond to different histological patterns.

Thus, in classic type, the morphological features are bullae, associated with low perivascular and interstitial lymphocytic infiltrate. Inflammatory variant shows an abundant inflammatory infiltrate, with lymphocytes, eosinophils, and neutrophils, this form being similar with BP or with the mucous form of EBA. Other variants stand out by papillary neutrophilic abscesses, while non-inflammatory type lacks inflammation at the trauma sites [Radoš, 2011; Harrell et al., 2019]. Recurrent forms display fibrosis and milia [Vorobyev et al., 2017]. The diagnosis confirmation requires additional immunological testing and confirmation by electron and immunoelectron microscopy [Radoš, 2011; Kridin, Ahmed, 2019].

Anti-p200 pemphigoid

Anti-p200 pemphigoid represents a distinct subepidermal autoimmune bullous dermatosis, which shares clinical features with linear IgA bullous dermatosis, BP, or DH [Radoš, 2011]. Recent studies have revealed autoantibodies against recombinant lamininγ1 in anti-p200 pemphigoid [Radoš, 2011; Kridin, Ahmed, 2019].

The histopathological picture does not have specific features, as subepidermal bullae, along with inflammatory infiltrate with occasional papillary microabscesses are also seen in dermatitis herpetiformis or linear IgA disease [Rose et al., 2007; Radoš, 2011].

Few studies are also reporting an additional eosinophilic spongiosis [Radoš, 2011; Meijer et al., 2016; García-Díez et al., 2017].

Table 3.3. Spongiosis patterns in autoimmune bullous dermatoses

AUTOIMMUNE BULLOUS DERMATOSES (ABD)	SPONGIOTIC PATTERNS		
	EOSINOPHILIC SPONGIOSIS	NEUTROPHILIC SPONGIOSIS	EOSINOPHILIC AND NEUTROPHILIC SPONGIOSIS
Intraepidermal ABD			
Pemphigus vulgaris (early stages)	+	+	-
Pemphigus foliaceus (early stages)	+	+	-
Paraneoplastic pemphigus	+/-	-	-
IgA pemphigus	-	+	-
Pemphigus herpetiformis	+	+	+
Subepidermal ABD			
Bullous pemphigoid (early urticarial stage)	+	-	-
Gestational pemphigoid (early urticarial stage)	+	-	-
Cicatriceal pemphigoid (early urticarial stage)	+/-	-	-
Linear IgA disease (early urticarial stage)	+	+	+
Lichen planus pemphigoides	-	-	-
Dermatitis herpetiformis	-	-	-

AUTOIMMUNE BULLOUS DERMATOSES (ABD)	SPONGIOTIC PATTERNS		
	EOSINOPHILIC SPONGIOSIS	NEUTROPHILIC SPONGIOSIS	EOSINOPHILIC AND NEUTROPHILIC SPONGIOSIS
Epidermolysis bullosa acquisita (early urticarial stage)	-	-	+
Anti-p200 pemphigoid	-/+	-	-

3.2.5. FINAL REMARKS

Although spongiosis exhibits different reaction patterns, only eosinophilic spongiosis and neutrophilic spongiosis are completing the histological picture of the autoimmune bullous dermatoses. While almost all the intraepidermal bullous diseases display eosinophilic and neutrophilic, or a mixed pattern of spongiosis, in early stages, only half of the subepidermal bullous dermatoses show these patterns of spongiosis in early urticarial lesions. The occurrence of spongiosis limited to the early stages of these diseases emphasizes the transient nature of this process, a feature that can mirror the specific pathogenesis of these autoimmune entities.

Considering these findings, as well as the heterogeneity and non-specificity of the histopathological features of these diseases, the diagnosis is very complex, requiring clinicopathological correlations, as well as additional techniques, such as electron microscopy, immunofluorescence, immunohistochemistry or molecular biology. All of these diagnostic methods may contribute to a thorough understanding of the pathogenic mechanisms, as a fundamental source of possible classification refinement of these autoimmune bullous dermatoses.

3.3. TUMOUR MICROENVIRONMENT COMPONENTS OF MALIGNANT MELANOMA

3.3.1. INTRODUCTION

Normally located in the basal layer of the epidermis and dermis of the skin, by their ability of melanin synthesis, melanocytes cooperate with neighbouring cells, especially keratinocytes, to protect DNA from ultraviolet light (UV)-induced damage. Although malignant melanoma accounts for about 1% of all skin cancers, its incidence has been constantly increasing in the last two decades, mainly affecting light-skinned persons [Richeta et al., 2014; de Menezes et al., 2020]. An estimated 420,000 new melanoma cases per year are registered worldwide [Siegel et al., 2018], representing 5% of all newly diagnosed cancers [Mazurkiewicz et al., 2021] and exhibiting a large spectrum of locations and clinicopathological characteristics.

There are two different molecular pathways leading to melanoma: the first one is associated with sun-exposure, and a second one, an oncogenic pathway, is associated with genetic susceptibility, such as inherited variants of melanocortin-1 or mutations of tumour suppressor genes involved in cellular growth regulation [Elder et al., 2018; Tyrrell, Payne, 2018; Garbe et al., 2020].

Although current histopathological, biochemical, immunohistochemical, and molecular methods provide a deep insight into its biological behaviour and outcome, melanoma is still an unpredictable disease, with poor outcome [Mimeault, Batra, 2012; Belter et al., 2017; Han et al., 2020; Gaudy-Marqueste et al., 2021].

Recent progresses in immunomodulatory therapy have been added to the current arsenal in the fight against melanoma, but there are considerable efforts to identify suitable

biomarkers for early diagnosis, staging, differential diagnosis, prognosis, and tailored therapy [Vereecken et al., 2012; Belter et al., 2017; Carr et al., 2020; Torres-Cabala et al., 2020]. Microscopic analysis of biopsies or of the surgical specimens is important for the establishment of the histopathological diagnosis and prognosis parameters (tumour thickness or Breslow index, mitotic rate, and ulceration), added to specific immunohistochemical markers, playing together a very important role in melanoma management [Belter et al., 2017; Wilson, 2021].

Circulating melanoma cells or melanoma-associated extracellular molecules provide noninvasive analytical access, considering the release of proteins and other molecules into the extracellular fluid, and may be considered potential serum biomarkers [Belter et al., 2017]. Different qualitative and semi-quantitative molecular assays have been used for melanocyte associated monoclonal antibody (MelanA/MART1), Melanoma-associated antigen recognized by T cells 1 (MART1), and Glycoprotein 100 (gp100) detection [Santonocito et al., 2005]. Although debated, blood or serum mRNA levels of tyrosinase, which is involved in melanin synthesis, detected by reverse transcriptase-PCR (RT-PCR), has been significantly correlated with stage progression and the risk of metastatic spread by comparison to other investigated markers [Santonocito et al., 2005; Vendittelli et al., 2009].

Melanoma's "tumour niche" consists of an ensemble of malignant cells associated with other cells, located in the epidermis or dermis, such as keratinocytes, cancer stem cells, cancer-associated fibroblasts, endothelial cells, macrophages, adipocytes, and immune cells, like the antigen Langerhans cells that are immune dendritic cells [Naves et al., 2017].

The most abundant cells from epidermis are keratinocytes, which have several important roles, including the maintainance of skin homeostasis by producing the skin structural protein in the presence of growth factors, the regulation of melanocytes proliferation using E-cadherin, desmoglein-1, and connexins [Li et al., 2003; Naves et al., 2017]. Besides the mentioned heterogeneous cells population, the melanoma microenvironment may contain growth factors produced by cancer cells or stromal cells.

There is a complex interplay between the malignant cells and the surrounding cellular components. In this regard, melanoma cells produce growth factors and cytokines, which target different stromal cells, stimulating the tumour microenvironment, through a direct or indirect process [Naves et al., 2017]. It is widely accepted the difference between the microenvironment of the normal and melanoma skin, which degrades with tumor growth progress. Thus, the melanoma stages are characterized by radial and vertical growth phases, while the metastatic stage is distinguished by the last phase. The direct tumor activation promotes melanoma growth by stimulating stromal cells, while indirect growth is involved in tumor initiation, progression, and metastasis, by altering different stromal cells functions [Wachsberger et al., 2003; Naves et al., 2017]. This microenvironment has an impact on melanoma cell progression and resistance to therapy [Clement et al., 2017].

Aggressive melanoma behaviour may be attributed to its heterogeneity, including a population of cancer stem cells (CSCs) [Hendrix et al., 2017], currently studied in order to identify their specific markers, which may be further exploited considering their prognostic and therapeutic potential [Hendrix et al., 2017].

The dynamic interaction between melanoma cells and adipocytes of the tumour niche may contribute to the establishment of a favourable microenvironment for melanoma growth and progression, especially in obese patients [Clement et al., 2017]. The therapeutic manipulation of this relationship may offer hopeful perspectives in these patients.

During the last decade, the gut and oral cavity microbiota have been considered a key factor of tumour development by its immunomodulatory function [Mitsuhashi, Okuma, 2018]. The unbalanced microbiota may lead to the development of an immune-compromised tumour microenvironment [Mitsuhashi, Okuma, 2018]. Current research is aimed at modifying

patients' microbiota as an adjuvant therapy in melanoma [Mitsuhashi, Okuma, 2018; Gopalakrishnan et al., 2018].

This review of literature is aimed to provide a comprehensive guide of the tumour microenvironment components and their involvement in melanoma prognosis and management, along with updating the knowledge regarding the specific clinicopathological and molecular hallmarks.

3.3.2. HISTOPATHOLOGICAL AND MOLECULAR HALLMARKS

Although magnetic sequential digital dermoscopy added to clinical examinations are used for diagnosis, the confirmation is based on histological analysis [Elder et al., 2018]. Melanoma's presentation may be as melanoma in situ, confined to the epidermis, and infiltrative melanoma, invasive into the dermis. Individuals with multiple atypical nevi (dysplastic nevi) or people with common nevi (types I, II), especially with genetic susceptibility or a family history of melanoma, have a high risk for development of melanoma at an earlier age [Majem et al., 2021].

The most frequent type of melanoma's growth is the radial growth phase (RGP), which is associated with a better prognosis compared to the other type, the vertical growth phase (VGP). RGP is characteristic for early lesions, being manifested as pigmented plaques or patches expanding horizontally in the epidermis, along the rays of a circle, while VGP is specific for progressive lesions as bona fide tumours, which infiltrate the dermis or expand into the epidermis, forming a nodule. Moreover, an early "tumourigenic" VGP is distinguished by a group of cells within the dermis, wide-reaching to the largest epidermal cell cluster, or a lesion which also shows a proliferation pattern from epidermis to dermis, exhibiting a high mitotic activity [Elder et al., 2018; Garbe et al., 2020; Elder et al., 2020].

Table 3.4. Malignant melanoma classification according to sun exposure and general features

MM ASSOCIATED WITH UV EXPOSURE					**MM NOT CONSISTENTLY ASSOCIATED WITH UV EXPOSURE**
Low-CDS (intermittent sun exposure)			**High-CSD (chronic sun exposure)**		mucosal melanoma; melanoma arising in congenital nevi; melanoma arising in blue nevi; Spitz melanoma; acral melanoma; uveal melanoma; nevoid melanoma; nodular melanoma
superficial spreading melanoma	subset of nodular melanoma	lentigo malignant melanoma	desmoplastic melanoma	subsets of nodular melanoma	
RGP; young age; precursor lesion (nevi);	VGP; young age; precursor lesion (nevi);	RGP; Old age; precursor lesion (MM in situ)	VGP; young age; precursor lesion (nevi);	VGP; young age; precursor lesion (nevi);	VGP and RGP; all ages; precursor lesion (nevi)

CSD – cumulative sun damage; MM – malignant melanoma; RGB – radial growth pattern; VGP – vertical growth pattern; UV – ultraviolet light.

Current clinicopathological World Health Organization (WHO) classification comprises four major histopathological subtypes: superficial spreading melanoma (SSM) (41%), nodular melanoma (NM) (16%), lentigo maligna melanoma (LMM) (2.7–14%), also known as melanoma arising in a Hutchinson melanotic freckle, and acral lentiginous melanoma (1–5%) [McGovern et al., 1980; Garbe et al., 2020]. The atypical melanocytes may be restricted to the

epidermis, showing a lentiginous arrangement at the dermoepidermal junction, or may be restricted to the upper parts of the epidermis (pagetoid or superficial spreading), or can grow along the hair follicles [Elder et al., 2018; Garbe et al., 2020]. SSM occurs in low cumulative sun damage (CSD), induced by intermittent sun exposure, while its histological diagnosis is made in the presence of pagetoid growth of single cells and nests, exhibiting severe cytological atypia [Longo, Pellacani, 2016; Elder et al., 2018; Garbe et al., 2020] (Table 3.4).

NM is characterized by an early progression to vertical growth without a radial growth phase and may represent a progression of an acral melanoma or of any other type of melanoma [Elder et al., 2018]. NM diagnostic criteria include complete loss of maturation, deep mitoses, lymphovascular invasion, and satellitosis, being frequently associated with ulceration [Elder et al., 2018]. LMM is a high-grade melanoma, with a high-mutation burden and severe solar elastosis which is mandatory for diagnosis, as well as microscopic features consisting of atypical melanocytes with a confluent growth along the dermoepidermal junction, with dermis invasion or with growth along adnexal structures [Elder et al., 2018; DeWane et al., 2019; Garbe et al., 2020]. Acral lentiginous melanoma can occur in all skin types (palms, soles, and nails), being frequently detected in an advanced stage, while its specific features include: low-mutation burden, epidermal hyperplasia, and characteristic asymmetrical, lentiginous, or nodular pattern of melanocytic cell growth [Goydos, Shoen, 2016; Elder et al., 2018; Garbe et al., 2020].

A spectrum of lesions has to be considered in melanoma clinicopathological differentials, such as melanocytic nevus (junctional, compound, and dermal), blue nevus, and congenital nevus, along with congenital dermal melanocytosis, a group of lesions represented by Mongolian spot, nevus of Ota, and nevus of Ito [Granter et al., 2001; Patel et al., 2013; Helm et al., 2017; Elder et al., 2018; Caccavale et al., 2021; Chua, Pico, 2021]. Sometimes, it is very difficult to differentiate a melanocytic proliferation (genital or oral lentigo) from a non-melanocytic proliferation (basilar epidermal physiological pigmentation) that also displays a strong pigmentation [Garbe et al., 2020]. Additionally, some lesions, such as skin squamous cell carcinoma in situ or Bowen's disease, early or macular pigmented seborrheic keratosis, actinic keratosis, or junctional dysplastic nevus, are very frequently biopsied, due to strong pigmentation, in order to rule out LMM in situ or invasive melanoma [Lützow-Holm et al., 2013]. In some situations, a neurotised melanoma can resemble a neuroid tumour, such as neurofibroma [Su et al., 2014].

In our experience, the differentiation of a primary tumour from a metastatic one sometimes raises issues of differential diagnosis, especially when certain organs do not have melanocytes in their structure and their migration from neural crests is not reported.

Melanoma is stratified according to the American Joint Committee on Cancer (AJCC) staging system (TNM Classification of Malignant Tumors) and WHO classifications, with a direct impact in practice. Thus, both AJCC and WHO systems classify melanoma into five stages (0, IA/B, IIA/B, IIIC, and IV), according to the surgical evaluation of the tumour size and invasion level (0—in situ lesion and stages I–IV), node invasion (stages III–IV), and occurrence of microscopically confirmed distant metastasis (stage IV) [Gershenwald et al., 2017; Keung, Gershenwald, 2018; Elder et al., 2018; Yang et al., 2020; Garbe et al., 2020]. In addition to the AJCC staging system, WHO classification includes genetic, genomic, and epidemiologic features of skin melanomas, based on their mutational signatures [Elder et al., 2018]. Moreover, the molecular data provided by WHO classification result in various histopathologic variants, according to the degree of cumulative solar damage (CSD) of the skin [Elder et al., 2018].

Traditionally, melanomas were grouped into different subtypes, according to their morphological features, such as spindle, epithelioid, balloon, giant, signet ring, clear, and small cells, along with desmoplastic, rhabdoid, and myxoid [Ossio et al., 2017; Elder et al., 2018; Keung, Gershenwald, 2018; Lattanzi et al., 2019; Garbe et al., 2020]. Additionally,

another distinctive histological feature is the regression of primary melanoma, which may be classified according to the variability of the mononuclear infiltrate, melanophages, and fibrotic process, into the following three categories: early, intermediate, and late stages [Aung et al., 2017]. However, reported data regarding the tumour–immune system relationship in melanoma regression are controversial. This relationship is particularly intriguing, taking into account that regression has a potential positive impact upon melanoma prognosis, but especially considering that drugs targeting these pathways have shown significant clinical efficacy in multiple tumour types [Fu et al., 2019]. Recent studies have suggested that the peritumoural inflammatory infiltration can represent a potential therapeutic target, while histological regression stands as an indicator of the immune system's efficiency in melanoma [Ossela-Abate et al., 2019; van den Berg et al., 2020].

The diagnosis of melanoma has been traditionally based on Clark's levels of invasion and Breslow's index (tumour thickness) that informs on the depth of melanoma invasion. There are five Clark's levels (level I - confined to the skin surface and epidermis; level II, III, and IV- dermis invasion; level V- subcutaneous fat invasion) and three levels of Breslow's index (≤1.0 mm - confined to the skin surface and epidermis; >1.0–4.0 mm - dermis invasion; >4.0 mm - subcutaneous fat invasion) [Balch, Buzaid, 2003; Balch, 2009; Elder et al., 2018; Keung, Gershenwald, 2018; Garbe et al., 2020].

AJCC and TNM staging systems and the Union for International Cancer Control (UICC) have improved the accuracy of prognostic prediction scoring, in addition to the presence of histologically recognized ulceration, epidermal defects, mitotic rate (per square millimetre), microscopic satellites, tumour infiltrating lymphocytes (TILs), and lymphatic and perineural invasion, along with tumour regression [Amin et al., 2017; Gershenwald et al., 2017; Elder et al., 2018; Keung, Gershenwald, 2018; Garbe et al., 2020]. Moreover, pure RGP melanomas have a very good prognosis, while VGP tumours are prone to metastasis, with metastasis probability correlated to higher stage criteria such as larger thickness, ulceration, microsatellites, increased mitotic rate, lymphovascular invasion, and lack of or minimal TILs [Elder et al., 2018; Garbe et al., 2020].

Melanomas display immunostaining characteristics of melanocytic differentiation such as protein melan-A (MelanA) or MART1, microphtalmia-associated transcription factor (MITF), Human Melanoma Black (HMB45), SRY-related HMG-box 10 protein (SOX10), and S-100 protein positivity [Elder et al., 2018; Garbe et al., 2020; Szumera-Ciećkiewicz et al., 2020]. Although most types of malignant melanomas exhibit immunopositivity for these markers, desmoplastic melanomas are negative for MelanA/MART1, MITF, and HMB45. These markers cannot discriminate between nevi and melanomas, and they are not expressed only by melanocytes [Prieto, Shea, 2008; Ramos-Herberth et al., 2010; Palla et al., 2013; Elder et al., 2018; Szumera-Ciećkiewicz et al., 2020] (Table 3.5).

Table 3.5. Immunohistochemical markers useful in malignant melanoma diagnosis.

MARKER/ PROTEIN	LOCATIONS	IHC FEATURES	FUNCTIONS
Melan A (MART1)	• normal skin • retina pigmented epithelium • melanocytes • most melanomas	• high sensitivity and specificity for primary and secondary melanoma • positive immunoexpression in clear cell sarcomas, PEComas or angiomyolipomas	• melanocyte differentiation antigen • Pmel expression, processing, traffic, and stability

MARKER/ PROTEIN	LOCATIONS	IHC FEATURES	FUNCTIONS
MITF	• dynamic subcellular location • associated with variable growth and differentiation cell programs	• high sensitivity in differentiation of melanoma from nonmelanocytic tumours • controversial specificity in spindle cells melanoma • positive immunoexpression in neurothekeoma cells, histiocytes, and mast cells	• member of MiT family • melanocyte development and differentiation • Melan-A, Pmel, and tyrosinase transcription
HMB45	• recognized the *Silver* locus product Pmel17 located in pre-melanosomal vesicles	• positive immunoexpression for melanoma and junctional nevus cells • low sensitivity for metastatic melanoma • positive immunoexpression in clear cell sarcomas, PEComas, angiomyolipomas	• eumelanin polymerization
SOX10	• nuclei of melanocytes • nuclei of breast myoepithelial cells	• positive immunoexpression in melanoma, nevi, and focal positivity in desmoplastic melanoma • differentiates melanoma in situ from actinic keratosis with melanocytic hyperplasia (along with MITF1) • positive immunostaining of salivary and sweat glands, adenoid cystic carcinoma, atypical fibroxanthoma, granular cell tumour, dermatofibrosarcoma protuberans	• member of a family of 24 proteins involved in inflammation, cell transcription, differentiation, growth, cell cycle regulation, calcium homeostasis • transcription factor • specification of neural crest derivatives • melanocytes and Schwann cells maintenance
S-100	• melanocytes • Langerhans cells • chondrocytes • glial cells • Schwann cells	• high sensitivity and low specificity for melanoma	• inflammation • cell transcription • differentiation • growth • cell cycle regulation • calcium homeostasis

HMB45—Human Melanoma Black; IHC—immunohistochemistry; MART1—melanoma antigen recognized by T cells 1; MelanA—protein melan-A; MiT—microphthalmia transcription factor; MITF—microphtalmia-associated transcription factor; PEComa—perivascular epithelioid cell tumours; Pmel—premelanosomal protein; Pmel17— premelanosomal protein17; S-100—protein S100; SOX10—SRY-related HMG-box 10 protein.

3.3.3. THE TUMOR MICROENVIRONMENT IN MALIGNANT MELANOMA

3.3.3.1. Inflammatory microenvironment and melanoma's inflammasome interplay

The inflammation plays a crucial role in carcinogenesis considering its contribution as a source of cytokines and other tumor growth factors and its ability to eliminate transformed cells [Grivennikov et al., 2010].

Currently, the inflammatory microenvironment represents a key member in the regulation of different tumorigenesis stages, from early initiation to promotion and distant metastasis [Jang et al., 2021]. The inflammasome may have favourable roles in the innate immunity or can be abnormally activated in melanoma, as well as in other types of malignancies, with the overexpression of the correspondent effector molecules [Jang et al., 2021].

The inflammatory tumor microenvironment comprises the population of resident or infiltrating immune cells and inflammatory mediators near malignant cells, nowadays representing a key piece in carcinogenesis, from initiation to promotion and metastasis.

The inflammasomes are proteic complexes consisting of nucleotide oligomerization domain (NOD)-like receptors (NLRs), pro-caspase-1, and apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (CARD) domain—ASC, which are crucial for homeostasis maintenance. Inflammasomes are characteristic for different cell types, including antigen-presenting cells and T- and B-lymphocytes, along with cancer cells from the tumour microenvironment, with important roles in carcinogenesis in association with other concurrent factors [Schroder, Tschopp, 2010].

Inflammasome stimulation depends on DAMPs/PAMPs' (danger associated or pathogen associated molecular patterns) perception through cytoplasmic receptors (NLRP1, NLRP3) [Latz et al., 2013].

Various endogenous host proteins, such as CARD8, or different post-translational and transcriptional mechanisms regulate the inflammasome's activation [Latz et al., 2013].

Several studies have demonstrated the influence of single nucleotide polymorphism (SNP) of inflammasome genes in the development and progression of different cancer types [Machado et al., 2001; Liu et al., 2010; daSilva et al., 2016]. Although inflammasome constituents are widely expressed in immune and nonimmune cells, their encoding genes' expression is not always related to the inflammasome formation or activation [Pandey et al., 2021].

The characteristics of inflammasome biology are highlighted by the study of different cells, mouse bone marrow-derived macrophages (BMDMs) being the most investigated cellular type [Man, 2018; Pandey et al., 2021]. Other cells include mice bone marrow-derived intestinal epithelial cells and neutrophils, human airway epithelial cells, neutrophils, platelets, and peripheral blood mononuclear cells (PBMCs), as well as humans' and rodents' CD4+ and CD8+ T cells [Pandey et al., 2021]. Nevertheless, inflammasome type is different among cells and host species. Accordingly, the stimulation of these inflammasomes will induce distinct biological outcomes [Pandey et al., 2021].

The formation and activity of inflammasomes are closely dependent on cell organelles, leading to inflammation and cell death [Vanaja et al., 2015]. Several organelles can facilitate inflammasome assembly. Thus, the Golgi complex contributes to NLRP3 enrolment and activation, mitochondria are responsible for NLRP3 recruitment and inflammasome activation, endoplasmic reticulum can promote NLRP3 oligomerization with ASC and NLRP3 signalling regulation [Pandey et al., 2021]. AIM2 and interferon-inducible protein 16 (IFI16) activation are taking place in the nucleus, being followed by their translocation in the cytoplasm, forming a perinuclear inflammasome complex. Opposite to the inflammasome action, which can induce cell death, stress granules are responsible for cell survival and inhibit NLRP3 inflammasome formation [Pandey et al., 2021]. Ribosomes preserve cellular translation and generate inflammasomes and cytoskeleton components, especially the microtubule-organizing centre (MTOC), which contributes to NLRP3 and Pyrin formation, inflammasome translocation, and stability maintenance [Pandey et al., 2021]. This "headquarter" activates numerous apoptotic and inflammatory caspases, cytokine substrates, the plasma membrane rupture protein ninjurin-1 (NINJ1), and the pore-forming protein gasdermin D (GSDMD), followed by inflammation, cellular destruction, and pyroptosis [Kayagaki et al., 2015; Shi et al., 2015; Pandey et al., 2021; Kayagaki et al., 2021].

The innate immune system uses a group of pattern-recognition receptors (PRRs) as inflammasome components, which are expressed in different cell types involved in defence processes, such as dendritic cells, neutrophils, epithelial cells, macrophages, and monocytes [Schroder, Tschopp, 2010]. Moreover, PRRs include the nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), and the absent in melanoma 2 (AIM)-like receptor (ALRs) [Cantono, Guo, 2017]. The activated inflammasome sensor (NLRP1, NLRP3, NLRP6, NLRP9b, AIM2, caspase-11, or Pyrin) establishes the identity of the inflammasome complex [Pandey et al., 2021].

The protein absent in melanoma 2 (AIM2) is a member of the PYHIN family, with one pyrin (PYD) domain situated at the N-terminus and one or two hematopoietic, interferon inducible, and nuclear (HIN) domains located at the C-terminus. The gene encoding this inflammasome sensor was first identified as a tumour suppressor gene in human melanoma cell lines [Wang et al., 2020].

ASC recruits pro-caspase-1, which is converted into catalytically active caspase-1 [Cantono, Guo, 2017] and bioactive subunits p20 and p10, which will generate the bioactive forms of interleukins IL-1β, and IL-18 by proteolytic cleavage of pro-IL-1β and pro-IL-18 [Cantono, Guo, 2017].

Activation of the inflammasomes will subsequently lead to pro-inflammatory cytokine release by adjacent cells and tissues. Persistent inflammation will generate a chronic inflammatory status, which contributes to the pathogenesis of variable diseases, including cancer [Guo et al., 2015]. The complex inflammasome's interactions between different cytokines, endothelial, and tumour cells are contributing to angiogenesis and carcinogenesis, along with invasion and metastasis, in melanoma.

Melanoma involves upregulation of pro-inflammatory cytokines, such as interleukins 6 and 8 (IL-6, IL-8), C-C chemokine ligand 5 (CCL5), and IL-1β [Cantono, Guo, 2017], along with vascular endothelial growth factor (VEGF) [Yang et al., 2009]. In this regard, melanoma-derived IL-1β works as a stimulator of angiogenesis, tumor growth, invasion, and metastasis [Burrows et al., 1991; Vidal-Vanaclocha et al., 1994; Vidal-Vanaclocha et al., 1996; Song et al., 2003; Voronov et al., 2003; Okamoto et al., 2010; Dunn et al., 2012], the influence of the inflammasomes depending on the tumor cell types [Kantono, Guo, 2017]. The results of a study performed in a murine experimental model have demonstrated the dual functions of IL-1 and inflammasomes in inflammation-induced skin tumorigenesis [Drexler et al., 2012]. Moreover, norepinephrine (NE) is able to upregulate IL-6, IL-8, and VEGF in C8161 melanoma cell line, exhibiting an autocrine stimulation along with chemotactic and proangiogenic effects, its value being increased in advanced stage melanomas [Yang et al., 2009].

Other works have demonstrated that ASC is an inhibitor of carcinogenesis by NFκB transcriptional activity and IκB kinase α/β phosphorylation suppression in primary melanoma, while up-regulated ASC is stimulating the inflammasome, via IL-1β secretion and NF-κB activity, in metastatic melanoma [Zhai et al., 2017; Möller et al., 2020]. Moreover, the knockdown of NLR family pyrin domain containing 1 (NLRP1) reduces their tumor-promoter properties, both in vivo and in vitro [Zhai et al., 2017; Möller et al., 2020; Tengesdal et al., 2021].

Numerous studies have demonstrated that NLRP3-inflammasome dysregulation can activate the inflammasome-dependent IL-1β expression in human sporadic metastatic melanoma cells [Okamoto et al., 2010; Ahmad et al., 2013; Liu et al., 2013]. Furthermore, melanoma tumor growth is linked to the ATP-regulated K+ channel P2 × 7 activity associated with NLRP3-inflammasome stimulation [Gross et al., 2011; Latz et al., 2013; Makarenkova, Shestopalov, 2014].

Another study has reported that modified gain-of-function variants of inflammasome genes NLRP1 and NLRP3 could increase patients' risk for developing a sporadic malignant

melanoma [Verma et al., 2012; daSilva et al., 2016]. These findings highlight that the dysregulation of inflammasome activation, with subsequent IL-1β and IL-18 production, is crucial in tumorigenesis, the inflammasome molecules representing potential valuable prognostic melanoma biomarkers [daSilva et al., 2016].

Other studies on SNPs in melanoma genes demonstrated that various cytokines (tumor necrosis factor alfa (TNFa), IL-6, IL-10, interferon (IFN-c), and transforming growth factor-beta (TGF-b1)) are involved in melanoma progression and immune escape [Howell et al., 2003; Nikolova et al., 2007]. Some of the cytokines produced by human melanoma cells (IL-6, IL-8, C-C Motif Chemokine Ligand 5 (CCL5) (RANTES), Chemokine (C-X-C motif) ligand 13 (CXCL1–3) (MGSA-GROa-c), and monocyte chemotactic protein-1 (MCP1/CCL2) are associated with tumour invasiveness and aggressiveness [Raman et al., 2007]. Cytokines activity is stimulated by activated IL-1β [Dinarello, 2009; Dunn et al., 2012]. Biologically active melanoma-derived IL-1β has a wide range of actions in melanoma tumorigenesis, exhibiting paracrine and autocrine-like activity, increasing IL-1 synthesis in melanoma cells, contributing to macrophages recruitment and to in vitro angiogenesis [Okamoto et al., 2010]. It is considered that IL-1β has various effects on different cells of the tumor microenvironment, maintaining survival and proliferation of melanoma cells, immune suppressor cells, and macrophages while promoting invasion and metastasis [Allavena et al., 2008; Ostrand-Rosenberg, Sinha, 2009; Netea et al., 2009]. Moreover, IL-1β secretion becomes autonomous, as melanoma is progressing [Okamoto et al., 2010; Dunn et al., 2012].

Hedgehog (Hh) signalling plays an important role in melanoma pathogenesis, its activity being blocked by wogonin, an active component of flavonoids, in HT144 melanoma cells [Pietrobono et al., 2018]. It is accepted that wogonin has various inhibitory effects in different melanoma cells, including on invasion and migration of B16F10 cells, melanin synthesis in A375 melanoma cells, or the proliferation and tumor growth of HT144 melanoma cells [Zhao et al., 2014; Chen et al., 2017].

The anti-inflammatory effect of wogonin in HT144 melanoma is supported by the following activities: (i) pro-inflammatory factors decrease, (ii) anti-inflammatory factors increase, and (iii) inflammatory cytokines expression increase [Li et al., 2021]. Additionally, the anti-tumour effects are performed by: (i) glucose consumption decrease; (ii) production of ATP and lactic acid decrease; (iii) kinases' activities, such as phosphofructokinase (PFK), hexokinase (HK), and pyruvate kinase (PK) inhibition; and (iv) expression of glucose cotransporter-1 (GLUT1), monocarboxylate transporter 1 (MCT-1), and MCT4 inhibition [Li et al., 2021].

3.3.3.2. Melanoma's microenvironment components

The tumour microenvironment (TME) represents a complex biosystem with a great impact on tumour progression, which depends on the spatiotemporal interrelations between malignant and non-malignant cells [Hanahan, Weinberg, 2011; Shelton et al., 2021], its immune heterogeneity being significant for the prognosis of different types of cancers [Thorsson et al., 2018; Cesano, Warren, 2018].

The tumour microenvironment (TME) contains numerous immune cells, such as a variable amount of T lymphocytes, as well as B lymphocytes, dendritic cells (DCs), natural killer (NK) cells, M1 and M2 type macrophages, mast cells, and myeloid-derived suppressor cells (MDSCs). During the first stages of carcinogenesis, immune cells are involved in apoptosis, anti-tumour cytokines production, and cytotoxic reactions. Thus, NK cells engage antigen-presenting cells (APCs) through cytokine secretion, while DCs, macrophages, and neutrophils are involved in phagocytosis of dead melanoma cells and tumour antigens presentation, activating T cells immune responses [Simiczyjew et al., 2020] (Figure 3.1).

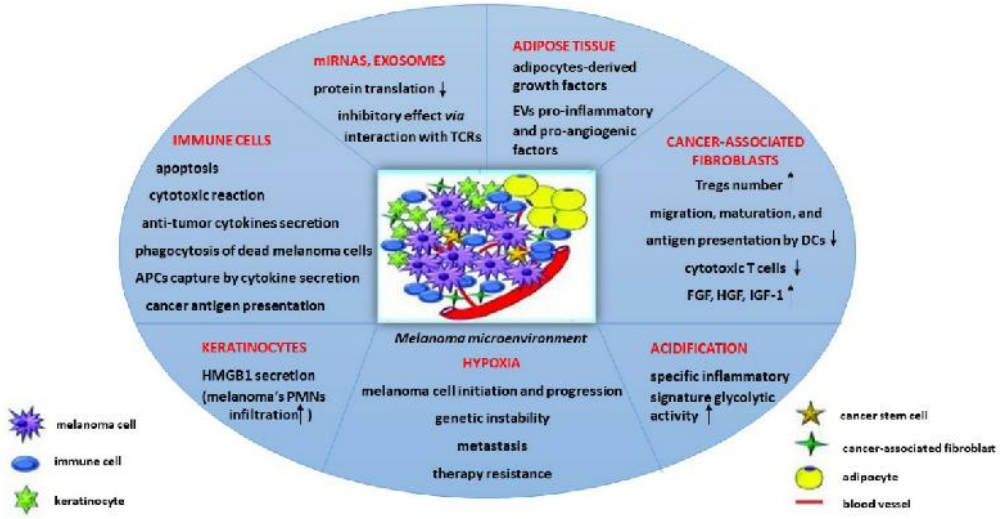


Fig. 3.1. Cellular and extracellular components of melanoma's microenvironment. The tumor microenvironment (TME) contains numerous cellular and extracellular components, forming together a complex, which supports melanoma development. APCs—antigen-presenting cells; CAFs—cancer- associated fibroblasts; DCs—dendritic cells; EVs—extracellular vesicles; HMGB1-high mobility group box 1 protein; MDSCs—myeloid-derived suppressor cells; miRNAs—microRNAs; PMNs— neutrophils; TCRs—T cells receptors; Tregs—regulatory T cells; ↑—increased level; ↓—decreased level.

T lymphocytes

The activity of the T lymphocytes' main subtypes is mandatory for melanoma remission. Accordingly, the functions of cytotoxic (effector), helper, and regulatory cells are: (i) CD8+ T effector lymphocytes (Teff) recognize antigens via major histocompatibility complex class I (MHC I) molecules, inducing cytotoxicity in melanoma cells and (ii) CD4+ T helper (Th) lymphocytes bind to APCs through MHC II molecules and provide different immune cell types, under the tumour cytokines influence [Huang et al., 2016; Durgeau et al., 2018; Marzagalli et al., 2019].

CD8+ T lymphocytes' infiltration in metastatic melanoma may be stimulated by the administration of B1 receptor agonist des-Arg9-bradykinin (DABK), considering the involvement of stromal bradykinin signalling and melanoma cells bradykinin receptors in the tumor microenvironment [Maria et al., 2016; Maria et al., 2019].

Melanoma cells have great plasticity, allowing the immune escape due to reduced antigen expression, MHC molecules' decreased level, as well as aberrations in their processing system [Marzagalli et al., 2019]. As T lymphocytes express programmed cell death protein (PD-1) checkpoint receptor, tumor cells are blocking T cell activity via increased production of ligand of PD-1 receptor (PD-L1), leading to an interaction between PD-1 and PD-L1, thus producing TILs apoptosis, stimulating the differentiation of CD4+ into regulatory T lymphocytes (Tregs), and inhibiting the immune system response for self-tolerance maintenance [Ahmadzadeh et al., 2009; Marconcini et al., 2018; Li et al., 2019]. It has been demonstrated that tumor Treg lymphocytes are correlated with melanoma growth and progression, their recruitment being performed by cancer cells through IL-10, IL-35, and tumor growth factor β (TGF-β) production in order to escape immunity [Marconcini et al., 2018; Simiczyjew et al., 2020]. Additionally, studies with experimental models shown that β3-adrenergic receptors expressed in melanoma microenvironment mediate Tregs' and myeloid-derived suppressor cells' activity, being involved in immune tolerance [Calvani et al., 2019].

B lymphocytes

Different studies have provided controversial results regarding the activity of B lymphocytes in melanoma TME. Accordingly, some authors consider that a high density in B cells is characteristic for non-metastatic melanoma, being associated with a better prognosis, while others found that melanoma cells produce fibroblast growth factor-2 (FGF2) which stimulates B cells to produce insulin-like growth factor-1 (IGF-1), exhibiting a potential resistance to B-Raf proto-oncogene, serine/threonine kinase (BRAF) and mitogen-activated protein kinase (MEK) inhibitors [Ladányi et al., 2011; Somasundaram et al., 2017]. Another study has emphasized that circulating B lymphocytes produce tumour necrosis factor α (TNF-α) and/or IL-6, these being associated with tumour unresponsiveness and poor survival of melanoma patients who underwent anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) antibody therapy [de Jonge et al., 2021]. The negative correlation between TNF-α expression and immune checkpoint blockade response suggests the role of B cells in tumor growth via inflammatory cytokines production [de Jonge et al., 2021].

Tumor-associated macrophages

Tumor-associated macrophages (TAMs), M1 and M2 types, could represent important prognostic markers due to their role in tumor cell migration, angiogenesis, and extracellular matrix degradation. M1 macrophages are found in low number in intratumoural infiltrate and have anti-tumoural effects, being activated by Th1 cells and pro-inflammatory factors (granulocyte-macrophage colony stimulating factor—GM-CSF, lipopolysaccharides, and IFN-γ) [Falleni et al., 2017]. M2 macrophages are involved in tumor progression and invasion, as they are mainly identified in early inflammatory infiltrate, being stimulated by Th2 cells and anti-inflammatory stimuli (IL-4, IL-10, IL-13, or monocyte colony-stimulating factor—MCSF) [Zhang et al., 2018]. Moreover, M2 macrophages can downregulate M1-mediated functions [Chen et al., 2011].

TAMs possess β3-adrenergic receptors and inducible nitric oxide synthase (NOS2, iNOS) and, therefore, NE and nitric oxide (NO) may modulate their activity, contributing to an increased tumor cells' growth and invasion [Filippi et al., 2020]. Moreover, TAMs may produce adrenomedullin, a vasodilator and stimulator of angiogenesis, involved in TAMs polarization toward M2 type and in melanoma progression [Simiczyjew et al., 2020].

Cancer stem cells

Cancer stem cells (CSCs) are related to TME and may recruit TAMs for tumor growth [Harlin et al., 2009]. In this regard, the involvement of CD34-melanoma tumour initiating cells (TICs) in chemoresistance and cancer progression promotion has been demonstrated, through M2 macrophages interaction, along with TGF-β and arginase pathway [Harlin et al., 2009].

Melanoma CSCs have self-renewal, indefinite proliferation capacities, high tumourigenicity, embryonic-like characteristics, ability to differentiation, and are involved in angiogenesis along with epithelial-mesenchymal transition (EMT) and metastasis [Frank et al., 2011; Kumar et al., 2017]. Furthermore, melanoma CSCs are involved in tumour microenvironment modulation by their expression of different miRNAs, along with their capacities of immune escape mechanisms and recurrences due to their limited response to conventional chemotherapy or radiotherapy [Zhou et al., 2014; Fomeshi et al., 2015; Skvortsov et al., 2015; Kumar et al., 2016; Hendrix et al., 2017].

Melanoma CSCs exhibit a weak immunogenicity and, in addition, show an immunosuppressive effect in the host organism [Kumar et al., 2017].

Myeloid-derived suppressor cells

The expansion and migration of MDSCs, which are the precursors of macrophages,

granulocytes, and DCs, are influenced by C-C chemokine receptor type 5 (CCR5) ligands (CCL3, CCL4, and CCL5) in melanoma [Harlin et al., 2009]. CD141 DCs from the melanoma immune microenvironment are activating CD8+ T lymphocytes, by CCR7 receptor involvement [Roberts et al., 2016].

Numerous evidence supports that the loss of CCR7 receptor promotes tumour growth, while its increased level is correlated with a better outcome [Roberts et al., 2016]. Furthermore, a reduced DCs number is associated to metastatic melanoma, while their increased amount is suggestive for lack of metastases or low recurrence risk [Roberts et al., 2016].

Neutrophils

Neutrophils of the tumour inflammatory infiltrate increase during melanoma progression, their accumulation depending on CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 molecules, which are stimulated by UV radiations [Masucci et al., 2019]. In an analogous manner to macrophages, neutrophils have also two subtypes: N1, which represent the dominant type of the early melanoma microenvironment, exhibiting anti-tumour activity, and N2 type, occurring in the advanced stages, with immunosuppressive effects [Masucci et al., 2019].

NK cells

The immune escape of melanoma cells is also produced through reduction in the expression of the main NK receptors (NKp30, NKp44, and NKG2D), which damage the mediated cytolytic anti-cancer activity of NK cells [Pietra et al., 2012].

Mast cells

Mast cells are involved in the development of melanoma, considering their ability to react to substance P neurogenic inflammation [Grimbaldeston et al., 2002; Vukman et al., 2017]. Consequently, they release different cytokines, proteases, growth factors, biological amines, such as histamine, which reduces the antitumoural defence mechanisms, chemokines, neuropeptides, variable enzymes, and angiogenic factors, such as heparin, VEGF, TGF-β, and IL-8, the latter being demonstrated as a growth factor in different melanoma cell lines [Vukman et al., 2017]. Moreover, their products seem to increase the immunosuppression resulting from UV-B exposure, using a complex mechanism of mast cells stimulation to release their products involving calcitonin gene-related peptide (CGRP), substance P (SP), and keratinocyte-produced nerve factor [Grimbaldeston et al., 2002].

TME includes also different extracellular elements or other cells of the tumor niche, which may contribute to the specific immune response, such as: fibroblasts, miRNAs or exosomes, acidification, keratinocytes, and adipose tissue (Figure 3.1).

Cancer associated fibroblasts

Peritumoural fibroblasts can be converted into cancer associated fibroblasts (CAFs), exhibiting analogous properties to myofibroblasts [Papaccio et al., 2021]. During melanoma progression, CAFs may represent an important amount within the tumor cells' population, displaying variable functions such as immunosuppression, due to TGF-β activity, which include inhibition of migration, maturation, and antigen presentation by DCs, increase in Tregs number, and reduction in the expression of perforin, granzymes, Fas ligand, and IFN-γ in cytotoxic T cells [Papaccio et al., 2021]. Tumor cells recruit fibroblasts under β3-adrenergic stimulation by NE [Filippi et al., 2020]. Moreover, NE stimulates fibroblasts metaplasia into myofibroblasts, which provide increased tumor cells motility and increased neoangiogenesis [Kharaishvili et al., 2014] along with the release of protumorigenic cytokines, such as FGF-2, IL-6, IL-8, and VEGF [Moretti et al., 2013; Kharaishvili et al., 2014]. CAFs are involved in

melanoma progression, metastasis, and drug resistance because of cell–cell interaction and secretion of extracellular matrix components, growth factors, and cytokines [Zhou et al., 2015; Shelton et al., 2021].

Cancer associated fibroblasts affect cellular growth and survival rates in melanoma [Gärtner et al., 1992; Otsuka et al., 1998]. The fibroblasts influence is manifested in early stage melanoma, and ussualy not in metastatic cells [Otsuka et al., 1998].

Some CAFs stimulate growth factors, like fibroblast growth factor (FGF), hepatic growth factor HGF) [Otsuka et al., 1998], and insulin growth factor-1 (IGF-1), promoting the growth of melanoma cells in early stages, through the activation of beta-catenin and MAPK (mitogen-activated protein kinase) [Satyamoorthy et al., 2001], which also stimulates epithelial growth factor (EGF) and vascular endothelial growth factor (VEGF), both potential mitogens for the melanoma cells growth and proliferation [Naves et al., 2017].

CAFs also synthesise hyaluronic acid (HA), one of the most important component of the extracellular matrix (ECM), which promotes angiogenesis, tumor growth, and metastasis [Willenberg et al., 2012]. Moreover, HA (HAS1 and HAS2) can activate melanoma synthesis of platelet derived growth factor receptor (PDGF)-AA (PDGF)-CC and PDGF-AA from dermis fibroblasts [Pasonen-Seppanen et al., 2012].

ADAM-9, a member of proteases family located at the tumour-stromal border and synthesized by stromal fibroblasts, activates melanoma cell proliferation and apoptosis, without knowing exactly whether it has stimulatory or inhibitory tumor growth effects [Abety et al., 2012; Naves et al., 2017].

Exosomes. Extracellular vesicles

Exosomes, a subtype of extracellular vesicles (EVs), are involved in tumor microenvironment activity and carcinogenesis, mediating the interrelation between cancer cells and CAFs [Shelton et al., 2021]. Several studies highlight the capacity of normal fibroblasts, CAFs, and cancer cells to secrete miRNA exosomes, providing a characteristic intercellular communication within the TME [Lener et al., 2015; Yamaguchi, Sakai, 2015].

The “tumour niche” intercellular communication carried out by extracellular vesicles (EVs) which are released by tumor cells and adipocytes has been the aim of recent studies [Hood, 2019; Mannavola et al., 2019]. Melanoma cells secrete EVs, which induce cancer progression by downregulation of skin adipocytes activity. These tumour EVs contain miRNAs, including miR-214-3p, which support the formation of a more favourable microenvironment by upregulation of lipogenesis genes, such as fatty acid-binding protein 4 (FABP4), adiponectin, and peroxisome proliferator-activated receptor G (PPARG) [Nieman et al., 2013; Xi et al., 2019].

Additionally, adipocytes are able to differentiate to a fibroblast-like phenotype through Wnt/β-catenin pathway activation and high expression of fibroblast specific markers, such as collagen and α-smooth muscle actin (α-SMA) [Nieman et al., 2013; Xi et al., 2019]. Moreover, melanoma-derived EVs induce EMT and tumour progression through let-7i family miR paracrine or autocrine signalling [Xiao et al., 2016]. In the same direction, tumour-derived EVs have been shown to be involved in the induction of apoptosis of cytotoxic T-cells, induction of M2 polarization of macrophages, and inhibition of cytotoxicity of NK cells in tumour microenvironment [Weidle et al., 2017].

All these melanoma-derived EVs actions are supported by the “activity” of skin adipocytes. Thus, adipocytes also release EVs internalized by melanoma cells. They contain nucleic acids (miRNA, mRNA, and other non-coding RNAs), lipids, and proteins involved in fatty acid oxidation (FAO), which promote tumour progression through a metabolic reprogramming [Clement et al., 2020] (Figure 3.2).

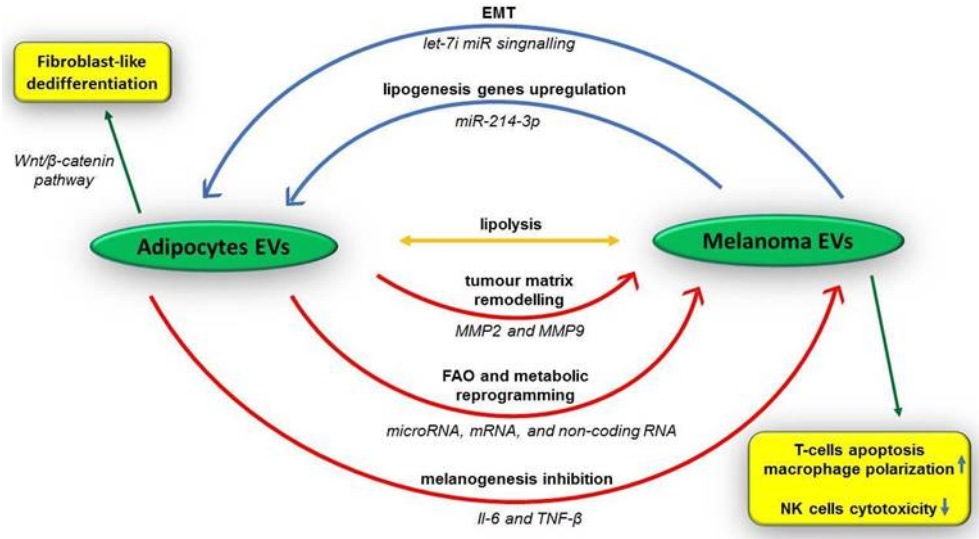


Fig. 3.2. Adipocytes and melanoma cells EVs "dialogue" in tumour microenvironment. Adipocytes and melanoma cells secrete EVs, which promote tumour progression and metastasis by tumour matrix remodelling, melanogenesis inhibition, fatty acid oxidation (FAO), and metabolic reprogramming into melanoma cells, along with epithelial-mesenchymal transition (EMT) induction, lipolysis, and li- pogenesis genes upregulation in fat cells. EVs—extracellular vesicles; EMT—epithelial-mesenchymal transition; FAO—fatty acid oxidation; IL-6—interleukin 6; miRNA—microRNA; MMP—matrix met- alloproteinase; NK cells—natural killer cells; T-cells—T lymphocytes; TNF-β—tumour necrosis factor β; ↑—increased level; ↓—decreased level

MiRNAs

MiRNAs, small, non-coding RNAs involved in protein translation attenuation or inhibition, can regulate the melanoma immune microenvironment [Motti et al., 2020]. Melanoma cells secrete exosomes, which also provide membrane-bound ligands such as PD-L1, with an inhibitory effect of the anti-tumor response via interaction with the T cells receptors [Motti et al., 2020].

Acidification

Another extracellular factor which mediates the immune response to cancer cells is acidification, the characteristic lower melanoma pH (6.0–7.0) providing an enhanced glycolytic activity and a specific inflammatory signature [Boussadia et al., 2018]. Most of the published data have revealed the immunosuppressive role of acidosis, which develops a "migratory" phenotype of melanoma cells [Bohn et al., 2018; Erra Díaz et al., 2018; Böhme, Bosserhoff, 2020].

The lower melanoma pH is responsible for decreased cytolytic activity of CD8+ T cells and increased secretion of IL-1β by monocytes and TAMs [Erra Díaz et al., 2018], as well as a functional orientation of TAMs toward the M2 type.

Hypoxia

Without sufficient oxygen supply, the dermal environment becomes hypoxic, promoting carcinogenesis [Hockel et al., 1996]. The hypoxic tumors have worse prognosis, because of their resistance to chemotherapy and radiotherapy [Harrison et al., 2004].

Melanoma initiation, progression, genetic instability, metastasis, as well as therapy resistance are influenced by tumor hypoxic environment and tumor-stromal cells. Moreover, hypoxia decreases drug-induced apoptosis, favouring hypoxia-tolerant tumor cell clones

growth [Schnitzer et al., 2006; Brahimi-Horn et al., 2007].

When the tumor diameter is more than 1mm, hypoxia-inducible factors (HIFs) are found in a solid tumor. In normal skin, the melanocytes from the dermal-epidermal junction are hypoxic, representing an important factor for melanocytes transformation. HIF-1α is found in melanocytes nuclei, suggesting that HIF-1α is triggered in melanocytes [Buscà et al., 2006].

Although hypoxic in normal skin, melanocytes are susceptible to oncogenic transformation, if HIF-1α stabilizes in the hypoxic microenvironment [Naves et al., 2017].

Adipose tissue

An important component of the cancer microenvironment involved in the progression of melanoma is the adipose tissue, comprising the hypodermis [Clement et al., 2017]. This area of fat tissue is mainly composed of white adipocytes, associated with other types of cells such as endothelial cells, pericytes, monocytes, macrophages, and stem cells [Booth et al., 2015]. Current knowledge allows us to consider adipose tissue not only as a lipid storage area but also as an inflammatory and endocrine organ [Mazurkiewicz et al., 2021]. Thus, adipose cells are the source of growth factors such as fibroblast growth factor-21 (FGF-21), hepatocyte growth factor (HGF), IGF-1, VEGF, and endocan, along with insulin-like growth factor-binding protein (IGFBP), leptin, retinol-binding protein 4 (RBP-4), resistin, leukaemia inhibitory factor, IL-6, IL-11, TNF-α, plasminogen activator inhibitor-1 (PAI-1), and TIMP-1 [Coelho et al., 2016; Robaldo de Lope et al., 2018]. Melanoma cells express surface membrane receptors for these adipocytes-derived factors, which support the tumour cells' proliferation, metastasis, and drug resistance via MAPK, PI3K/AKT, and JAK/STAT pathways [Malvi et al., 2015].

Recent reports show a "bidirectional communication" between melanoma cells and adipocytes, especially in obese patients, consisting of the secretion of high amounts of pro-inflammatory factors such as IL-6, IL-11, TNF-α, monocyte chemoattractant protein (MCP)-1/CCL2, and PAI-1 by the excessive fat [Ouchi et al., 2011; Smith et al., 2020]. Furthermore, the inflammatory profile of subcutaneous adipose tissue is associated with an increased release of leptin and resistin, supporting the progression of tumour cells and increasing the risk of lymph node metastasis [Oba et al., 2016].

In addition, adiponectin, with characteristic low levels in obese patients, has the opposite effect, analogous to leptin and resistin in melanoma [Katira, Tan, 2016].

Adiponectin induces apoptosis and inhibits cancer cells growth by activation of the AMP activated protein kinase (AMPK) signalling pathway [Katira, Tan, 2016].

These data are also supported by other studies on murine melanoma models which demonstrate a positive correlation between melanoma progression and obesity [Pandey et al., 2012; Malvi et al., 2015; Pereira et al., 2021].

In this respect, excessive subcutaneous white adipose tissue, together with enhanced secretion of pro-inflammatory factors, lead to the progression of melanoma by supporting tumour neoangiogenesis, following the release of pro-angiogenic factors, such as endocan, hepatocyte growth factor (HGF), and VEGF, added to an altered energy metabolism [Coleho et al., 2016; Clement et al., 2017]. In addition, other experimental studies revealed that adipocytes co-cultured with melanoma cells induce the secretion of chemoattractant factors (CXCL1, CXCL2, and CXCL5) added to a local immune cell recruitment, especially of M2 macrophages, in the "tumor niche" [Gilbert et al., 2013; Chen et al., 2016; Robado de Lope et al., 2018].

Analogous results have been reported by another research team, leading to the conclusion that skin adipocytes are involved in melanoma cell immune escape, by high expression of PD-L1, which interact with PD-1 molecule on the T lymphocytes membrane [Wu et al., 2020].

The adipose tissue β3-adrenergic receptor also influences the anti-tumor response of immune cells, its upregulation in the melanoma microenvironment resulting in tumor growth stimulation [Calvani et al., 2015].

Keratinocytes

Keratinocytes can also contribute to the immune escape, influencing the melanoma's immunosuppressive environment. Usually, the UV-absorbing melanin from keratinocytes protects against melanocytes mutations induced by prolonged radiation exposure, although the UV radiations can also stimulate cancer progression through a different pathway [Simiczyjew et al., 2020].

Recent data have demonstrated that keratinocytes secrete a high mobility group box 1 (HMGB1) protein, which promotes neutrophils infiltration into the melanoma microenvironment, being responsible for melanoma plasticity [Simiczyjew et al., 2020].

Recent data have demonstrated a correlation between keratinocytes and corticotropin releasing hormone-proopiomelanocortin (CRH-POMC) axis in about 80% of melanomas, along with adrenocorticotropic hormone (ACTH) production in about 70% of melanomas, with α-melanocyte-stimulating hormone (α-MSH) release in over 50% of melanomas [Kim et al., 2006] and with the functional cell-specific MSH receptor or melanocortin 1 receptor (MC1R) [Abdel-Malek et al., 2008].

CRH stimulates melanoma cells invasion via extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway [Yang et al., 2007]. Supplementary, desmoglein 1 is involved in the signalling between melanocytes and keratinocytes, with cytokines and POMC production, leading to a high level of melanin and pagetoid melanoma cells spread [Arnette et al., 2020]. However, POMC overexpression reduces the melanoma growth by apoptosis and autophagy via complex α-MSH-HIF-1 α/BCL2 and adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) signalling pathways [Wu et al., 2018].

Keratinocytes are stimulated by calcitonin gene-related peptide (CGRP) resulting in stimulation of melanin production and melanocytes trophicity [Zhou et al., 2015]. However, CGRP may induce melanocyte apoptosis via increased Bax/Bcl-2 ratio, while substance P (SP) association with CGRP is inhibiting the process of melanogenesis [Zhou et al., 2015].

Keratinocytes are expressing enkephalins, or opioid receptors (ORs), belonging to G protein-coupled receptors [Valentino, Volkow, 2018; Azzam et al., 2019], while low levels of enkephalin and proenkephalin (PENK) have been detected in melanomas [Slominski et al., 2011]. Moreover, methionine (met)-enkephalin (MENK) has shown melanoma growth inhibition via apoptosis associated with opioid growth factor receptors (OGFrs) increased expression in animal models [Wang et al., 2017].

Furthermore, it seems that the addictive mechanism of UV exposure is mediated by β-endorphin production in keratinocytes, added to its role in tumor cell proliferation and immune reactions inhibition, by decreasing the amount of tumor lymphocytes [Fell et al., 2014].

The inflammatory phenotype of the uveal melanoma, the most common primary ocular cancer in adults, is associated with a poor outcome, correlated to a greater number of inflammatory cells populating mainly epithelioid-cell-type tumors, characterized by chromosome 3 loss [Coupland et al., 2020], while CD8+ T cells and macrophages represent the main population in its inflammatory milieu [Lei et al., 2021].

Among the four molecular subsets of uveal melanoma (A, B, C, D), identified in recent studies according to their immunological features and gene expression profiles [Robertson et al., 2017; Jager et al., 2018], only the subset D shows a characteristic inflammatory phenotype, with excessive infiltration of lymphocytes and macrophages [Bronkhorst, Jager, 2013; Jager et al., 2018; Souri et al., 2019]. Moreover, variant D has an increased metastatic potential and several specific genetic aberrations (monosomy 3,

chromosome 8q gain, and BAP1 loss), which seem to be correlated with its specific inflammatory phenotype [Bronkhorst, Jager, 2013; Gezgin et al., 2017]. Recent data regarding the specific immunological and genetic profile of uveal melanoma TME provide a gene-based prognostic signature, with possible impact on prognosis and on targeted therapy perspectives related to metastasis prevention [Lei et al., 2021].

Another interesting study has assessed the crosstalk between cultured uveal melanoma cells and hepatic stellate cells, demonstrating that metastatic melanoma cells are more sensitive to the paracrine signalling of stellate cells than their non-metastatic category, this interrelation involving profibrogenic interleukins [Babchia et al., 2019]. Thus, metastatic melanoma cells are able to regulate hepatic stellate cells activity, promoting their growth and survival [Babchia et al., 2019].

Tumor progression needs an early and persistent inflammatory response, as cancer cells can modulate the functions of the surrounding cells to favour their growth, invasion, metastasis, and survival [Bronkhorst, Jager, 2013].

3.3.4. MICROBIOTA ROLE IN MALIGNANT MELANOMA

During the last decade, the gut and oral cavity microbiota came to represent a key factor of tumour development by its immunomodulatory function [Mitsuhashi, Okuma, 2018; McQuade et al., 2020]. Moreover, recent data have demonstrated that gut microbiota, added to intratumour bacteria, may modulate the response to immunotherapy in many cancers, including melanoma, and modulate its toxic effects [Panebianco et al., 2018; Ribas, Wolchok, 2018].

The growth of beneficial bacteria is enhanced by fibres or non-digestible compounds, or prebiotics, while healthy microbial species or probiotics are represented by Lactobacilli, Bifidobacteria, Saccharomyces yeasts, along with Enterococcus, Bacillus, and Streptococcus [Mego et al., 2013; Zitvogel et al., 2015]. The probiotics associated to prebiotics, which selectively stimulate the growth of probiotics, or the synbiotics, lead to a synergistic effect, while the use of nonviable microbial metabolites, or postbiotics, such as acetate, propionate, and butyrate (short-chain fatty acids) may mimic the effects of probiotics [Patel et al., 2013].

According to experimental studies, Staphylococcus aureus is stimulating Foxp3+Tregs, while Enterococcus, Alistipes shaii, and Lactobacilus are involved in Th17 and Th1 differentiation and in cytokines production [Iida et al., 2013; Mitsuhashi, Okuma, 2018]. The unbalanced microbiota, or dysbiosis, induced by antibiotics and immune checkpoint inhibitors, such as anti-CTLA-4 antibody, may led to the development of an immune-compromised tumour microenvironment [Mitsuhashi, Okuma, 2018], while Bacteroides fragilis, Bacteroides thetaiotaomicron, and Bifidobacterium improve the response to immunotherapy in mice models [Sivan et al., 2015; Vétizou et al., 2015; Mitsuhashi, Okuma, 2018]. It has been also demonstrated that anti-PD-1 efficiency is higher in patients who had a microbiota rich in Enterococcus faecium, Bifidobacterium longum, Collisella aerofaciens, and Ruminococcaceae [Matson et al., 2018; Wong et al., 2021]. Anti-CTLA-4 immunotherapy results in a dominance of selected Bacteroides species [Vétizou et al., 2015]. The comprehensive analysis of commensal microbiota is valuable for detecting novel biomarkers or therapeutic targets in tumour patients treated with immune checkpoint inhibitors [Mitsuhashi, Okuma, 2018]. The abundance of gut Ruminococcaceae bacteria, along with Akkermansia muciniphila administration, contributes to the clinical response to anti-PD-1 treatment in melanoma patients [Mitsuhashi, Okuma, 2018; Gopalakrishnan et al., 2018].

Additionally, it has been demonstrated that patients containing Bacteroidaceae, Barnesiellaceae, and Rikenellaceae in their microbiota do not show colitis induced by anti-CTLA-4 therapy [Dubin et al., 2016].

Furthermore, microbiota may influence immunotherapy response and toxicity, as demonstrated by the intratumour administration of CpG oligodeoxynucleotides, which mimic bacterial DNA administrated in melanoma experimental mice models, in association with an antibody against IL-10 receptor, which increase TNF production and CD8 T cells stimulation, resulting in tumour growth inhibition [Iida et al., 2013].

Based on mice experiments observations [Sivan et al., 2015], faecal microbiota transfer (FMT), or the transfer of a donor entire microbial ecosystem are currently tested in clinical trials, in the immunotherapy context [Sivan et al., 2015]. This is recommended mainly to patients who are refractory to anti-PD-1 treatment, with a possible pretreatment antibiotic ablation of their own microbiota [Freitag et al., 2019]. Further studies would be necessary to evaluate the clinical utility of salivary or faecal microbiomes in patients which have an anti-PD-1 therapy [Mitsuhashi, Okuma, 2018].

3.3.5. CURRENT MELANOMA THERAPEUTIC APPROACHES. THERAPEUTIC STRATEGIES ASSOCIATED TO MELANOMA IMMUNE MICROENVIRONMENT

The current therapeutic approach to melanoma depends on its location, stage, its genetic biology, or other factors. Thus, in addition to surgical therapy, immunotherapy and targeted therapy represent important recommendations as adjuvant medical therapy for advanced stages, as well as chemotherapy and radiation therapy.

A variable and complex network of interactions is characteristic for the melanoma microenvironment. The specific cells of the tumour milieu contribute to the plasticity and heterogeneity of melanoma, as they represent factors, which are influencing the tumour immune escape and therapy resistance [Simiczyjew et al., 2020]. The different types of tumour cells, such as immune cells, CAFs, adipocytes, and keratinocytes, can communicate with each other or with extracellular matrix molecules, thus getting involved in the immune escape and affecting the immunosuppressive environment of the melanoma and, thus, the treatment efficiency [Simiczyjew et al., 2020]. The interaction between cancer cells and surrounding elements creates new therapeutic protocol opportunities [Bronkhorst, Jager, 2013]. In this direction, a therapy based on antibodies directed against adrenomedullin or an antagonist of its receptor has proven its efficiency both in vitro and in vivo [Chen et al., 2011; Simiczyjew et al., 2020].

It was revealed that CAFs and TAMs may contribute to therapy tolerance through a cytokine-signalling network that includes fibroblast-derived CXCR2 ligands and macrophage-derived IL-1β, inflammatory niches' signalling being amplified by MAPK inhibitors, providing early drug tolerance toward BRAF and MEK inhibitors during treatment [Young et al., 2017].

Furthermore, melanoma aggressiveness may be correlated to peritumoural mast cells density and their population may be targeted in new therapeutic approaches [Lichterman, Reddy, 2021].

The modern melanoma therapy directed against immune cells is targeted against PD-1 ad CTLA-4. The best-known anti-PD-1 antibodies are nivolumab and pembrolizumab, used in the therapy of metastatic melanoma and advanced melanoma treatment, respectively.

Nivolumab has shown superior therapeutic effects, improving the median progressionfree survival, compared to ipilimumab, which blocks CTLA-4 inhibitory signalling pathway [Simiczyjew et al., 2020].

Considering that BRAF and MEK inhibitors can lead to changes in TME immunogenicity, therapeutic strategies consist of a combination between anti-MEK/BRAF drugs and immune checkpoint inhibitors [Simiczyjew et al., 2020]. This approach is based on the observation that BRAF-mutated melanoma cells have a low T cell infiltration and a high

level of IL-6, IL10, and VEGF, which increase the number of Tregs or myeloid-derived suppressor cells within TME [Simiczyjew et al., 2020]. Clinical investigations which have shown promising results involved the following combinations: trametinib (MEKi), dabrafenib (BRAFi), and murine anti-PD-1 antibody or cobimetinib (MEKi), vemurafenib (BRAFi), and atezolizumab (anti-PD-L1 antibody) [Sullivan et al., 2019; Gutzmer et al., 2020]. Moreover, the melanoma survival was successfully improved by using combination targeted therapies for checkpoint inhibitors and MEK-ERK pathway [Lee et al., 2020].

Other immune checkpoint proteins have been studied as potential markers for new therapy strategies, such as lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulinmucin domain-containing molecule 3 (TIM-3), and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT), all of them being valuable targets for immunotherapy [Anderson et al., 2016; Simiczyjew et al., 2020].

Glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR) represents another promising molecule for immune checkpoint therapy. GITR alteration has been shown to induce inhibition of T cell-mediated cancer cell apoptosis [Knee et al., 2016; Guo et al., 2019]. Moreover, GITR is correlated with the activity of E3 ubiquitin protein ligase neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4), often involved in metastatic melanoma [Knee et al., 2016; Guo et al., 2019]. In this regard, the anti-melanoma immune response could be increased by inhibiting the activity of this enzyme [Knee et al., 2016; Guo et al., 2019].

It was demonstrated that human and mouse melanoma cells metastasis was inhibited in vitro by thymoquinone, a bioactive phytocompound, by decreasing the NLRP3 inflammasome expression, accompanied by a reduction in caspase-1 proteolytic cleavage, which led to IL-1β and IL-18 inhibition, as well as NF-κB activity suppression [Ahmad et al., 2013]. Moreover, the incomplete inactivation of NLRP3 inflammasome was due to reactive oxygen species (ROS) inhibition by thymoquinone. These results promote thymoquinone as a potential agent for both adjuvant immunotherapy and metastatic melanoma prevention [Ahmad et al., 2013].

Brain metastases of cutaneous melanoma, including acral type, react to both targeted and immune therapies, responding with targeted therapy resistance mediated by extrinsic factors. The mechanism involved is phospho-inositide 3-kinase (PI3K) pathway activation, which is a main target for brain metastases in melanoma [Lee et al., 2020].

Immune therapy's escape of the succeeding checkpoint blockade was related to specific TME changes, represented by a lower infiltration of effector T cells, a higher number of alternatively activated macrophages, and a characteristic gene expression profile [Lee et al., 2020]. Numerous therapeutic strategies in melanoma have successfully targeted different tumourigenic mechanisms and pathways, taking into account the inflammatory microenvironment variability, such as the involvement of dendritic cells, as APCs and cytotoxic T-cell stimulators via CCR7 expression, T cell infiltration, alteration of immunosuppressive signalling pathways, neoantigen specific T cell response obtained by vaccination (mutant epitopes, mRNA, and dendritic cells) [Gubin et al., 2014; Boudewijns et al., 2016; Sahin et al., 2017], antitumour immune response with oncolytic viruses [Ledford, 2015], and antitumour activity of cytokines (IL-2 pathway and granulocyte-macrophage colonystimulating factor—GM-CSF)) [Everly, Lonial, 2005; Rosenberg, 2014; Diab et al., 2020].

Due to heterogeneity of the melanoma immune microenvironment and weak therapeutic response to different monotherapies, attempts have been made in recent years to combine therapeutic variants in order to improve the therapeutic response, mainly with immune checkpoint inhibitors [Pardoll, 2012; Haanen, Robert, 2015; Wilgenhof et al., 2016;

Ott et al., 2017]. The promising results of combination therapy in melanoma could address the complexity of the inflammatory microenvironment and tumour heterogeneity by aiming for a therapeutic precision that exceeds the immune resistance or side effects encountered in current therapies [Tang et al., 2021].

Numerous studies have assessed the therapeutic efficiency of c-KIT inhibitors, but the results have limited value, as most patients have eventually manifested tumour progression, possibly due to numerous cases of melanoma with central nervous system metastases, characterized by a limited drug penetration capacity [Wolf Horrell et al., 2016]. Thus, current strategies are exploring combination therapy, such as an association of c-KIT inhibitors with those targeting its downstream pathways or with the immunological checkpoint blockade [Pham et al., 2020].

The concept that frequent ARID2 mutations are influencing the immune checkpoint inhibitors, being correlated to increased CD8+ T cells, supports the role of ARID2 as a potential biomarker of therapeutic efficiency of immune checkpoint inhibitors in patients with melanoma [Fukumoto et al., 2021].

Moreover, SETD2 loss provides a potential role of this marker in melanoma therapy assessment [Fahey, Davis, 2017].

Supplementing the surgery, radiotherapy, and immunotherapies (systemic therapy), targeted therapy is efficient, such as anti-BRAF (vemurafenib, dabrafenib, and encorafenib) and anti-c-kit (imatinib, nilotinib, and regorafenib), while anti-NRAS therapy (lonafarnib and tipifarnib) has failed in clinical trials due to NRAS activation via alternative posttranslational alterations [Dummer et al., 2018; Yang et al., 2020].

Targeting MAPK proteins and their regulatory components may contribute to inhibit melanoma cell genesis, thereby using them as potential therapeutic tools. Tipifarnib is targeting RAS, while Sorafenib is specifically targeting RAF. Pharmacological agents used in clinical trials, such as AZD6244, U0126, PD0325901, CI-1040, XL518, AZD8330, ARRY-162, and ARRY-300 are selective inhibitors of MEK [Montagut, Settleman, 2009], while AZ628 inhibits this pathway at the ERK level. The addition of an anti-RAS, RAF, MEK, ERK agent, MEK inhibition (MEKi) has shown considerable effects and ability to inhibit growth and induce melanoma cell death, especially in the BRAF-mutant metastatic melanoma [Lee et al., 2020]. Thus, all these pharmacological agents remain promising therapeutic targets in the MAPK pathway.

3.3.6. FINAL REMARKS

The melanoma microenvironment, consisting of a cell complex which comprises a population of resident or infiltrating immune cells, inflammatory mediators, adipose cells, and adipocytes-derived factors supports tumour cell proliferation, drug resistance, and metastasis.

Thus, an insight into its distinct features and their interplay may reveal new pathways and molecules which prove useful in an innovative therapeutic approach.

Recent progress in immunomodulatory therapy and in manipulation of melanoma cells–adipocytes interactions has been added to the current arsenal against melanoma, and considerable efforts are made to identify suitable biomarkers for early diagnosis, staging, differential diagnosis, prognosis, and tailored therapy.

The deciphering of the complex tumour microenvironment which governs the molecular mechanisms involved in the melanoma progression is opening new therapeutic targets in these patients, especially from the perspective of FAO inhibitors and/or molecules use to prevent the release of EVs in the “tumour niche”.

The gut and oral cavity microbiota has been recently considered a key factor of tumour development by its immunomodulatory function, and current research is aimed at

modifying patients' microbiota as an adjuvant therapy in melanoma.

Considering the complexity of the interplay between melanoma cells and their microenvironment, along with tumour heterogeneity, combination therapy is now being developed in an attempt to overcome the immune resistance and the side effects of current therapies, opening new perspectives for a better management and an improved prognosis for patients.

SECTION III.
FUTURE PROJECTS IN ACADEMIC, PROFESSIONAL, AND SCIENTIFIC FIELDS

Medicine and education are two professions that combine harmoniously, through similar qualities that a person who practices them must fulfill, such as: availability and ability to understand the human personality, empathy, desire for knowledge and improvement, time efficiency, patience, efficient communication, respect for oneself and others, adaptability, meticulousness, human ethics, and fairness. Just as medicine consists in a partnership between physician and patient, so education represents a partnership between teacher and student, these connections being based primarily on individual responsibility and mutual respect.

Medical education can be considered an art which is created to serve the future community.

It can be assumed that the continuous self-improvement in both fields, as well as the permanent assurance and control of the quality of medical education represent the top of the mastery achievement in both fields.

III.1. FUTURE PROJECTS REGARDING ACADEMIC AND PROFESSIONAL ACTIVITY

The university career development involves the determination of objectives that are established according to: personal priorities, final goal, personal potential related to the socio-professional environment, as well as the profession particularities. In this regard, I have set myself two major goals:

• A permanent updating of the personal professional knowledge and competences according to the international didactic and scientific standards, to which the entire academic activity of the University is related;

• The constant involvement in the complex life of the University, aiming to increase the national and international recognition, with an impact on the personal prestige and of the academic community to which I belong.

ACADEMIC ACTIVITY

As a teacher at the Histology Discipline and a pathologist, I believe that updating information and correlating it with specific fundamental and clinical sciences is a priority that we must consider in the process of morphological training of students. Although Histology is considered a relatively static science, at present it becomes the link of new discoveries in the top fields of scientific research, represented by Molecular Biology, Immunology and Genetics, thus acquiring a dynamic character, in a continuous development. From a methodological point of view, this update implies an adaptation to the level of knowledge of each trained category (undergraduate studies, postgraduate studies: residents, specialists), appropriately adjusting the volume and complexity of information, connections with other medical fields and practical applicability of theoretical notions.

An important goal consists in the training of young teachers. In this regard, the development of the Histology Discipline involves a careful selection of the new generation of university assistants, the most appropriate profile being that of a Pathology resident or specialist, PhD student, who has willingness and reliable professional qualities.

The main future directions for the academic activity include:

1. Student training. One of the objectives is the introduction of innovative virtual microscopy teaching methods for medical students, who will look with more interes at computer-assisted microscopic morphology. It will create the possibility of dynamic teacher-student interaction, as well as instant verification by MCQ (multiple choice questions) type tests of the acquired knowledge. Educational computer technology is a valuable support for both teachers and students, contributing, overall, to increasing the quality of the educational process.

2. Pathology residents training. The involvement in the Pathology Residency Program as a coordinator represents a major responsibility. In this sense, I intend to facilitate the residents the access to the latest theoretical information, and to offer the expertise of a quality practical training within the modules of Histology and Cytology, focusing on placental and fetal pathology, breast and gynecological pathology, cervico-vaginal cytology. The virtual microscopic morphology can also play an important role in the residents training. I believe that, by using the technical facilities of this technology, the residents will have the opportunity to acquire solid skills in the practice of pathology diagnosis.

3. Postgraduate courses. Continuing medical education is a perpetual educational program, aiming to develop a dynamic offer of postgraduate courses for the pathologists and other related specialties (Oncology, Surgery, Gynecology, Dermatology, and Forensic Medicine). These courses intend to reflect the theoretical and practical medical experience.

MEDICAL ACTIVITY

Since 2002 I have been carrying out my integrated medical activity at the Pathology Laboratory of the “Elena Doamna” Clinical Hospital of Obstetrics and Gynecology Iasi. The histopathologist activity allows me to coordinate both theoretically and practically the Pathology residents or those enrolled in other related specialties with Pathology module in their curricula, as well as the development of professional collaborations with other pathologists or specialists.

Due to the increasingly varied and complex cases which I face in the Pathology Laboratory where I work, I intend to improve the modern infrastructure of the laboratory with equipment that will create the opportunity to perform immunohistochemical examinations complementary to routine diagnosis.

Moreover, I intend to improve the quality standards by introducing also a modern equipment for digital microscopy and telepathology, with the possibility of creating a data base with virtual images, as well as the virtual transmission of microscopic images in order to create the opportunity of remote second-opinion consultation, which will provide sometimes a faster diagnosis and also the possibility of specialists inter-consultations, improving the histopathological diagnosis, with a major positive impact on patient therapeutic management and on academic and research activity.

III.2. FUTURE PROJECTS REGARDING THE SCIENTIFIC ACTIVITY

Future research projects will focus largely on the continuation and development of research in the fields of HPV-associated and HPV-independent cervical neoplastic pathology, as well as endometrial, and placental pathology, through studies aimed at identifying new potential molecular markers with prognostic and pathogenic impact.

In this regard, the most important objectives for conducting my research activity will follow collaboration with multidisciplinary teams from Romania and abroad, writing new articles with valuable results that can be published in high impact journals, permanent information in the subspecialties of interest by participating in intensive training courses as well as national and international scientific events.

Among the main topics of my future research projects, the following subjects are included:

III.2.1. DISTINCT MOLECULAR PROFILES OF HPV-ASSOCIATED AND HPV-INDEPENDENT CERVICAL CARCINOMA AND INTRAEPITHELIAL PRECURSOR LESIONS

A topic of interest in gynecological pathology is cervical cancer, due to the incidence, morbidity and mortality characteristic of cervical neoplasms, among female genital tumors, subject that acquires special values for both the field of pathology and gynecology.The involvement of HPV in uterine cervix carcinogenesis is well characterized [Arbyn et al., 2020; Fernandes et al., 2021]. HR-HPVs represent the most important factors involved in the pathogenesis of intraepithelial precursor lesions and cancer of uterine cervix, which make them a reliable biomarker of the prognosis assessment of high-grade and low-grade squamous intraepithelial lesions (HSILs and LSILs). Moreover, as the p16INK4a protein overexpression is strongly correlated with the presence of HR-HPV, immunohistochemical interpretation of p16INK4a expression represents the proper method in the diagnosis of cervical cancer [Farzanehpour et al., 2020]. Nevertheless, HPV-independent cervical cancers still have an unclear mechanism [Liu et al., 2011]. According to numerous studies, HPV-independent and HPV-associated are characterized by differences in various genes expression [Cancer Genome Atlas Research Network, 2017; Park et al., 2017; Liu et al., 2021], such as the proliferation markers Ki67 [Kedzia et al., 2002] and PCNA [Shikano et al., 1993], tumor suppressor proteins p16 [Houghton et al.,2010; Park et al., 2011; Omori et al., 2015; Nicolas et al., 2019], p53 [Brewer et al., 1996; Kedzia et al., 2002; Park et al., 2011; Omori et al., 2015; Nicolas et al., 2019], p21, p14, and p27 [Omori et al., 2015], and the protooncogenes c-myc [Brewer et al., 1996], c-Erb2 [Kedzia et al., 2002], and EGFR [Kedzia et al., 2002]. While, HPV-associated neoplasia present an overexpression of p14, p16, and p27 [Houghton et al., 2010; Omori et al., 2015; Fernandes et al., 2021], HPV-independent tumors possess a decreased proliferative activity [Shikano et al., 1993], p53 nuclear immunoexpression [Brewer et al., 1996; Kedzia et al., 2002; Park et al., 2011; Omori et al., 2015]. This pattern of HPV-negative tumors, with increased immunostaining of p53 abnormal [Nicolas et al., 2019], indicates a specific mutational phenotype correlated with the tumor deregulation, high growth capacity, and metastasis [Fernandes et al., 2021].

In this context, my aim is to attempt an immunohistochemical characterization of these two categories of uterine cervix neoplasias, HPV-associated and HPV-independent, to confirm or find new similarities and differences between the profiles of the two entities, with an impact on the diagnostic and prognostic management of patients.

III.2.2. ENDOMETRIAL CARCINOMA –NEW MOLECULAR PROFILES RELATED TO THE NEW WHO CATEGORIES. CORRELATION BETWEEN ACTUAL GENE EXPRESSION PROFILE AND IMMUNOHISTOCHEMICAL PARAMETERS

Endometrial cancer is one of the most common gynecological malignancies. Molecular studies have contributed to the understanding of the genetic events involved in the development and progression of endometrial carcinoma. DNA mutations can alter the genes involved in controlling cell growth, their disruption promoting carcinogenesis. In this regard,

gene therapy is one of the major targets of current and future research, including in endometrial carcinomas, which will be able to correct DNA defects.

There are many mutated genes involved in endometrial carcinogenesis, which interfere with the cell cycle, apoptosis, characterizing the molecular pathogenesis of one of the two major types of endometrial carcinomas, endometrioid or serous. The immunohistochemical (IHC) and molecular profiles differ among these two types of carcinomas, as demonstrated: (i) ER positive, along with variable mutations of tumor protein p53, β-catenin, ARID1A, PTEN, PIK3CA, and KRAS genes, in type 1; (ii) p53 and p16 positive, along with variable mutations of PIK3CA, FBXW7, and PPP2R1A genes in type 2 [Hedrick et al., 2011; Goebel et al., 2017; Bilyk et al., 2017].

The main WHO 2020 change related to endometrial carcinoma is the new molecular classification according to The Cancer Genome Atlas (TCGA), which comprises four molecular subtypes - POLEmut, MMRd, p53abn, and NSMP, identified by genomic architecture (ultramutate, hypermutated, high copy number, low copy number) [Kandoth et al., 2013; WHO, 2020; McCluggage et al., 2022].

This new molecular subclassification of endometrial carcinoma is more consistent than the histotype diagnosis, and can be certified on the biopsy specimen at the initial diagnosis, providing additional data for the therapeutic and prognostic management. It was found that molecular groups are heterogeneous compared to histotypes, p53abn endometrial carcinomas presenting the widest variation [McCluggage et al., 2022].

From this perspective, my future research purpose is to complete the immunohistochemical characterization of the new molecular categories of endometrial carcinoma, using known or new markers to correlate the classic histological types with the new four classes of endometrial carcinoma. This will be able to provide new diagnostic, prognostic and therapeutic approaches.

III.2.3. MULTIFACETED PLACENTA. IMMUNOHISTOCHEMICAL ASSESSMENT OF PARTIAL AND COMPLETE HYDATIDIFORM MOLES

Molar pregnancy is considered a category of gestational trophoblastic disease (GTD) included in precancerous lesions [Kubelka-Sabit et al., 2017]. Hydatidiform mole represents the most common disease among gestational trophoblastic diseases, its main etiological factors including maternal age, ethnicity and genetical abnormalities. However, the etiology of gestational trophoblastic tumors following a normal pregnancy is still unrevealed [Hui et al., 2017].

Histopathological diagnosis is considered the essential method in the management of abortion from the first trimester, the product of conception classification in molar or nonmolar pregnancy being required [Howat et al., 1993]. Nevertheless, there is not always a diagnostic certainty, most specialists diagnosing “suspicion of molar pregnancy” in complex cases with atypical villous morphology [Colgan et al., 2017]. Thus, given the limited morphological assessment and the need for an accurate diagnosis of molar pregnancies, specialists recommend the use of auxiliary techniques for refining the diagnosis of hydatidiform moles [Hui et al., 2017]. Studies have shown the importance of immunohistochemistry through the p57 marker and DNA genotyping in improving the diagnosis of this entity [Bifulco et al., 2008], these techniques describing the particular genetic aspects of molar and non-molar specimens in order to obtain a more accurate diagnosis [Ronnett et al., 2019]. One study underlined that p57 represents the most useful marker for the diagnosis of a complete hydatidiform mole, being absent in villous stromal cells and cytotrophoblast from these cases, [Kubelka-Sabit et al., 2017]. Although the role of p63 and Ki67 in the diagnostic of GTD is not fully elucidated, it was demonstrated in the same study that the concomitant assessment of

p63, and Ki67 can considerably complete the diagnosis of a molar pregnancy [Kubelka-Sabit et al., 2017. Other studies assess the different immunoexpression pattern of cyclin E, p63, Ki67, and CD34 in complete and partial hydatidiform moles [Lisman et al., 2005; Hussein et al., 2010; Kar et al., 2019].

From this perspective, the aim of my future research is to assess and to find a proper antibodies panel for a comprehensive characterization of hydatidiform moles and a facilitation of the differential diagnosis, solving the frequent diagnostic dilemmas.

III.3. FINAL REMARKS

My professional interest extends beyond the mentioned scientific projects – this fact being justified by the permanent diagnostic challenge due to the variability of the histopathological forms of the pathology I confront in my daily medical practice.

The opportunity to coordinate PhD theses will contribute to fulfill my present and future research projects, hoping to succeed in promoting and honoring the "Grigore T. Popa" University of Medicine and Pharmacy I belong, with valuable publications.

SECTION IV.
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