UNIVERSITY OF MEDICINE AND PHARMACY
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THE IMPACT OF ENDOSONOGRAPHY IN THE EARLY DIAGNOSIS AND PROGNOSIS OF PANCREATIC TUMORS

PhD THESIS

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INTRODUCTION

Pancreatic adenocarcinoma represents a major health problem due to its aggressive pattern and difficulty in early diagnosis. Pancreatic adenocarcinoma (PDAC) is the most common form of pancreatic cancer and represents the subject of our research.

The diagnosis of PDAC is predominantly made through imaging scans and endoscopy (ultrasound endoscopy in particular), as the clinical examination and laboratory investigations have low specificity at this moment. The treatment is done in a multidisciplinary team, but since most patients come to the hospital in advanced forms, the treatment is predominantly palliative.

Endosonography has become essential in the diagnosis and treatment of pancreatic diseases. It provides additional information with the help of integrated modules: elastography, color Doppler module, use of contrast agent for better characterization and biopsy in particular. The ability to harvest tumor tissue by minimally invasive technique (fine needle aspiration or fine needle biopsy) is crucial both for PDAC diagnosis and prognosis, but also for research purpose, offering the possibility of molecular testing of tissue samples.

More and more patients with this pathology have been presenting to our clinic, with an increase in incidence both nationally and globally. Through this study, I want to take a first step in addressing this issue by looking for early diagnostic and prognostic markers in PDAC by using ultrasound endoscopy. Since CA 19-9 is only a viable biomarker in selected cases, our research focused on tissue miRNA expression in PDAC and identifying other diagnostic and prognostic parameters in PDAC.

Accordingly, PDAC remains one of the hot topics among specialized congresses. This aspect is also highlighted in the case of the specialized literature, as there are more and more studies on this subject. Research in this field is growing exponentially, being facilitated by technological innovations. The national research in this field is not yet extensive rare studies having appeared on new biomarkers in PDAC.

The research results were the starting point for 2 original ISI articles published in international journals: Cells (ISSN: 2073-4409, IF: 6,600) and Endoscopy (ISSN: 1438-8812, IF: 10,093).

I. GENERAL PART

Pancreatic cancer is one of the deadliest forms of cancer, due to the late presentation to the doctor in stages that no longer present an indication for curative treatment, and because of the lack of early diagnostic methods. Even though there are many developments in pancreatic cancer through the invention of immunotherapy and improvements in pancreatic surgery, 5-year survival is less than 10% (1).

Chapter 1 describes the epidemiological data, etiopathogenesis and risk factors, and diagnostic methods of pancreatic adenocarcinoma. The risk of pancreatic cancer is directly increased with age, rarely being diagnosed before the age of 55 years old. The peak incidence is between 65 and 69 for men and 75 and 79 for women. Pancreatic cancer is more commonly encountered amongst developed countries, most likely due to increased accessibility of diagnostic and treatment methods (2; 3). The 5-year survival for metastatic disease is 2.9%, 12.4% for regional disease and 37.4% for localized disease (2). Nevertheless 80% of newly diagnosed patients have metastatic disease, and of those operable almost 80% will have local or distant metastases (1; 3).

Preventive medicine tackles the risk factors involved in the etiopathogenesis of PDAC. Smoking, alcohol consumption, obesity, diet that is rich in red meat, exposure to toxic substances are risk factors that can be modified, crucial since these can be removed, decreasing the overall risk of PDAC. Older age, male gender, African-American ethnicity, personal history of chronic infections or chronic pancreatitis, presence of diabetes mellitus, blood group B, family history of PDAC are risk factors that cannot be modified but may alert the physician to the associated risk of pancreatic cancer. The risk of PDAC is highest among patients with blood group B, whilst patients with blood groups A and AB have intermediate risk (4).

Most cases of PDAC are sporadic, only 10-15% having a genetic cause (3; 5). There are two categories of hereditary risk in PDAC: syndromes with a well-defined mutation and familial pancreatic cancer. The first category includes: hereditary breast and ovarian cancer syndrome (BRCA1, BRCA2, PALB2 mutation), Lynch II syndrome (MLH1, MSH2, MSH6, PMS2 mutation), Peutz-Jeghers syndrome (STK11 mutation), familial atypical multiple melanoma syndrome (CDKN2A mutation), hereditary pancreatitis (SPINK1, CFTR mutation). Ataxia-teleangiectasia, Li-Fraumeni syndrome, familial adenomatous polyposis, hereditary non-polyposis colon cancer, Fanconi anemia are rarely incriminated in the etiology of PDAC (3; 5; 6; 7). Intrafamilial cluster formation

are encountered in familial pancreatic cancer, without being able to identify a genetic susceptibility syndrome. These are more than frequent young patients (mean age 68 years) (8).

PDAC develops from glandular exocrine elements (ductal and acinar cells). Macroscopically it presents as a hard, imprecisely delineated lesion. 60-70% are located head of the pancreas, 5-10% in the body, 10-15% in the tail. In advanced forms metastases may occur in (in order of the frequency): lymph nodes, hepatic, peritoneal, lung, pleural, bone (3). Microscopically, PDAC can be classified according to the degree of differentiation into: well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3). One of the microscopic signatures of pancreatic adenocarcinoma is the presence of desmoplastic stroma (5). PDAC may also develop from premalignant lesions secondary to genetic changes: cystic mucinous neoplasm, tubulopapillary intraductal neoplasm, oncocytic intraductal neoplasm, mucinous papillary intraductal neoplasm (the most frequently incriminated) (6; 9). Pancreatic tumorigenesis is due to a combination of irregular events, therefore the effects of multiple intracellular genetic mutations are combined with the existence of abnormal cellular interactions and the morphological anomalies of pancreatic tissue. The role of pancreatic stellate cells has gained interest in PDAC, as they are activated as early as the PanIN stage (10). In the early phases tumor cells evade recognition, a process termed tumor immunoediting by loss of surface antigens as a consequence of tumor genetic instability and continued cell division. Neoangiogenesis occurs secondary to angioblast proliferation under the influence of VEGF and FGF, resulting in the appearance of immature endothelial cells. Most frequently encountered mutations in PDAC include the following genes KRAS, CDKN2A, TP53, SMAD3, MLH1, MSH2, MSH6 (5; 10; 11).

New biomarkers such as microRNAs circulating (serum or plasma, pancreatic fluid, saliva, faecal) or tissue derived, circulating DNA, non-coding RNA have been discovered in an attempt to determine new methods of early diagnosis. MicroRNAs are short, non-coding fragments of RNA that regulate the expression of other genes and are involved in the post-transcriptional regulation of gene expression. miRNAs can behave as oncomir when they stimulate proto-oncogenes and/ or suppressors by inhibiting oncogenes (13).

The clinical diagnosis in PDAC does not provide much information as it is non-specific, but in early forms the following symptoms predominate: abdominal pain, meteorism, flatulence, diarrhea, vomiting, general malaise (3; 14). Jaundice,

hepatomegaly, palpable right upper quadrant mass, cachexia, ascites, Courvoisier sign are the most common signs in PDAC, though in advanced disease (6; 15). The European Cooperative Oncology Group (ECOG) staged functional capacity grade as a major prognostic factor, higher values being associated with poor survival (5).

Diabetes mellitus and impaired fasting glucose are seen in 85% of patients at diagnosis, 55-85% of cases having this diagnosis within the first 2 years of diagnosis. CA 19-9 is the only useful biomarker in selected cases such as immediately after neoadjuvant treatment preoperatively, immediately postoperatively prior to adjuvant treatment and for postoperative surveillance. However, CA 19-9 has low specificity and can be increased non-specifically in non-malignant pathologies (16; 17). Rare studies demonstrate the usefulness of neutrophil/lymphocyte ratio (NLR), with values above 5 being associated with poor survival (18).

Imaging is the gold standard in the diagnosis of pancreatic adenocarcinoma, represented by multidetector computed tomography with pancreatic protocol or magnetic resonance imaging, though endosonography can bring further information. EUS has become essential in pancreatology (19). CT is useful in staging PDAC according to resectability criteria (6; 20). Contrast-enhanced MRI with pancreatic protocol may be useful in staging PDAC, particularly to characterize liver lesions that cannot be adequately depicted by CT examination, in case of suspicion of pancreatic tumors that cannot be visualized by CT or in case of allergy to the contrast agent, as it uses non-iodinated contrast agents (19). MRI-associated cholangiopancreatography (MRCP) can be used as a diagnostic method for jaundiced patients who cannot perform ERCP: gastric obstruction by tumor mass effect or with ERCP failure (6). PET-CT can be combined with CT with pancreatic protocol only in high-risk patients for detection of metastases, observing different functional activity of PDAC compared to benign masses. Benign masses do not uptake tracers except for inflammatory lesions in chronic pancreatitis (6; 16).

Endoscopic ultrasound is complementary to CT, with increased sensitivity and specificity particularly for tumors below 3 cm (3; 21). EUS is essential in the management of patients with pancreatic pathology and has opened new doors in the molecular study of PDAC. Tehniques that are associated with EUS and offer a gret advantage are: fine needle aspiration or fine needle biopsy, color Doppler evaluation, contrast enhanced EUS, elastography (22; 23). Retrograde cholangiopancreatography is rarely used

diagnostically, presenting therapeutic purpose in PDAC by stenting (18). The indication for exploratory laparoscopy is scarce. Surgical diagnosis is necessary only if imaging explorations raise suspicion of occult metastatic disease, in borderline tumors before neoadjuvant treatment or if there are clinical or paraclinical indicators that are not consistent with the imaging stage (large lymph nodes, excessive weight loss, significant abdominal pain, CA 19-9 above 100 U/mL) (19). Biopsy examination may be obtained by ultrasound or CT puncture, by endoscopic ultrasound (fine needle aspiration or fine needle biopsy puncture) or surgically. EUS biopsy is the preferred method. The Papanicolau Society of Cytopathology has established a standardized system for evaluation of pancreatic cytology, structured into 6 categories: 'nondiagnostic', 'negative', 'atypical', 'neoplastic', 'suspicious for malignancy' and 'positive for malignancy' (24).

The differential diagnosis of PDAC is made with chronic pancreatitis, autoimmune pancreatitis, pancreatic neuroendocrine tumors, primary pancreatic lymphoma, pancreatic intraepithelial neoplasia, secondary determinations from primary extrapancreatic tumors (6).

Current treatment modalities in pancreatic adenocarcinoma are presented in **Chapter 2**. Treatment of PDAC depends on the stage of the disease (Image 1). Surgery is the only curative treatment but reserved for patients with resectable or borderline resectable tumor (taken into consideration only after neoadjuvant treatment). Although there have been advances in medicine, no effective drug treatment has been found in PDAC. miRNA, molecular or gene mutations have been identified and in the future these can be drug-targeted, but studies are still needed to certify the data.

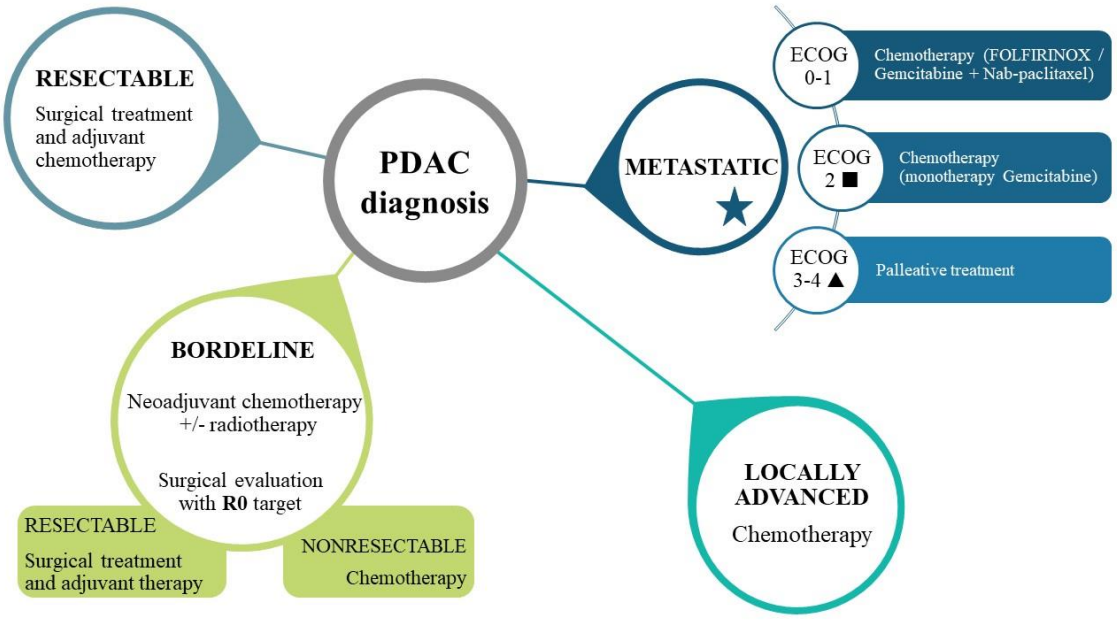

Image 1. Treatment in pancreatic cancer (25); ■ Performance status 2 and/or total bilirubin above 1.5x N; ▲ Performance status 3-4 or patient with comorbidities ★ Immunotherapy is recommended in patients with locally advanced or metastatic PDAC and MMR and MSI mutations (Pembrolizumab, NTRK mutations (Larotrektinib or Entrectinib), BRCA1 or BRCA2 mutations (Olaparib as second line) (26)

New directions in the management of pancreatic adenocarcinoma are presented in **Chapter 3**. New directions in PDAC treatment refer to personalized medicine, bringing to the forefront targeted treatments against affected genes, molecules, signaling pathways or even miRNAs. Targeted therapy taking into account miRNA profiling offers the advantage that a single type of microRNA can target multiple genes simultaneously.

The determination of genomic biomarkers has gained momentum through spectrophotometry and genomic sequencing. miRNAs can be used both in predicting the risk of malignant transformation of premalignant pancreatic lesions and also for diagnostic and prognostic role. miRNAs can predict the aggressive pattern of pancreatic cancer or even loss of sensitivity to chemotherapy or radiotherapy. Proteomic biomarkers are tumor-derived proteins that are derived from cell lines, tumor tissue, pancreatic juice and blood (6). Fluid biopsy is under evaluation as an early detection method for PDAC. Liquid biobsy determines circulating tumor cells, free circulating nucleic acids, microvesicles containing exosomes. Despite all its advantages, liquid biopsy has certain limitations: low sensitivity compared to conventional biopsies due to the rarity of

circulating tumor cells and circulating nucleic acids in patients' blood, the possibility of other extra-pancreatic sources, lack of consensus on sampling methodology (27).

II. PERSONAL CONTRIBUTION

The working hypothesis and general objectives are described in **Chapter 4**. The aim of the present research is to evaluate the diagnostic tools, markers or scores that are used in PDAC and to develop new diagnostic and prognostic markers. The personal contribution has been structured in two separate but interlinked studies.

In the first study we characterized the study group containing 43 patients diagnosed with PDAC after biopsy obtained by EUS method (from the initial cohort of 57 patients) by detailed evaluation of epidemiological, clinical, biological, imaging, endosonography and histological parameters (biopsies obtained by EUS-FNA). EUS offered us the possibility to analyze biopsy specimens, being a minimally invasive procedure essential in the management of patients with PDAC. We correlated these parameters with PDAC aggressiveness criteria: tumor size, existence of metastases, vascular invasion and survival.

In the second study we aimed to determine tissue miRNA expression in PDAC from samples obtained by EUS guided fine aspiration puncture from 41 patients, evaluated by RT-qPCR. In the first phase we aimed to identify the miRNA signature of pancreatic adenocarcinoma by comparing the miRNA profile in PDAC with the miRNA profile in non-malignant pathologies. For this we used 2 groups: the comparative group formed of samples from chronic pancreatitis tissue (taken by EUS-FNA, patients that belonged in the initial cohort) and the control group formed of samples of normal peritumoral pancreatic tissue (biobank of the Victor Babeș National Institute of Pathology). The original study group of 43 patients in whom miRNA profile analysis was performed was modified by the subsequent exclusion of 2 patients. This was done using SPSS statistical processing software, a program for the detection of values significantly different from the mean of the study group (outlier detection). Exclusion from the study group was based on PCA Analysis (Principal Component Analysis) which classifies patients according to total miRNA expression. The primary hypothesis is the correlation of tissue miRNA expression (echoendoscopically sampled from PDAC patients) with patients' clinical, biological and imaging characteristics, tumour aggressiveness, response to specific treatment and prognostic factors.

The general objectives of the study are:

1. To establish correlations between epidemiological, clinical, biological, endoscopic and histological parameters (biopsy samples obtained by EUS-FNA) and the aggressive nature of pancreatic adenocarcinoma;
2. Identification of the specific miRNA profile of pancreatic malignant tumour tissue, by comparison with normal peritumoral tissue, respectively with tissue from chronic pancreatitis;
3. To identify correlations between altered expression of an echoendoscopically sampled tumour miRNA panel (from patients with pancreatic adenocarcinoma) and patients' clinical, biological, imaging and endoscopic characteristics, tumour aggressiveness, response to specific treatment and survival;
4. To determine signaling pathways and target genes of the modified miRNAs showing altered expression in the study group by using bioinformatics (DIANA TarBase).

The research methodology is presented in **Chapter 5**. A prospective, observational, multicenter study was conducted including 57 patients with solid pancreatic tumor (diagnosed by imaging methods) from March 2019 to September 2021. Patient follow-up was conducted over 2 years and 6 months, consisting of periodic telephone visits at 1 month, 3 months, 6 months, 1 year, 2 years, and 2½ years (until the end of the study), respectively, collecting the following data: history and ECOG score.

As we are discussing a multicenter study, the contribution of several medical centers was facilitated by the good collaboration between experts in the field. The partner hospitals in the study are represented by: the Emergency Clinical Hospital "Prof Dr Agrippa Ionescu", the Central Military Emergency University Hospital "Dr Carol Davila" and the Emergency Clinical Hospital Bucharest, in collaboration with Victor Babeș National Institute of Pathology. The study was launched after obtaining confirmation from the Ethics Committees of the partner-hospitals and the confirmation from the heads of the Gastroenterology Departments. The multidisciplinary aspect of this research also consists of collaborations between different specialties: gastroenterology, pathology and molecular biology. The gastroenterology teams were responsible for the evaluation and diagnosis of patients: clinical, anamnestic, biological, imaging and EUS with biopsy sampling. Endoscopic examination was performed in the Digestive Endoscopy Laboratory of the partner hospitals, followed by histopathological evaluation in the

dedicated Pathology Laboratory. Evaluation of miRNA expression in the biopsy sample was performed in the Department of Histopathology and Imunohistochemistry of the Victor Babes National Institute of Pathology. The present observational, prospective study was registered in the clinicaltrials.gov database with registration number NCT04765410.

The study population consisted of patients who were diagnosed by imaging techniques with an unidentified or suspected solid pancreatic tumor mass (CT scan and/or nuclear magnetic resonance) presenting for ultrasound endoscopy guided fine aspiration (EUS FNA). Inclusion criteria for the study are as follows: presence of a solid pancreatic formation with unknown histopathological diagnosis; age over 18 years; existence of a signed informed consent. Non-inclusion criteria are as follows: presence of a pancreatic solid formation less than 10mm in diameter or with known histology or a pancreatic cystic mass without a solid component; coagulation disorders present (INR > 1. 5, APTT > 42 seconds, thrombocytopenia < 60000/mmc) or failure to stop antiaggregants or anticoagulants according to European Society of Digestive Endoscopy (ESGE) guidelines; evolving pregnancy; age below 18 years; refusal or failure to obtain informed consent; presence of vascular structures or dilated Wirsung duct that are interposed in the puncture needle path, between the needle entry site and the solid pancreatic lesion, which cannot be bypassed during the puncture and increases the risks of the puncture procedure (endoscopist's decision); presence of another malignancy in the same patient which may interfere with the determined biomarkers. Participation in other clinical trials is not a non-inclusion criterion.

The study protocol aimed at enrolling and evaluating the 57 patients in the study and performing the history and objective examination. After explaining the diagnosis, risks and benefits of the research protocol, the patient signed informed consent for participation in the study. Subsequently, biological samples (parameters relevant to the underlying disease) were collected and EUS-FNA was performed. At this time biopsy samples were collected: minimum 2 passes (one was directed to our study). It was preserved by immediate immersion after biopsy in RNA stabilizing solutions, kept refrigerated 24-48h. EUS-FNA biopsy specimens were analyzed by a pathologist (which were not directed to the study) for confirmation of the diagnosis (Image 2). The tissue samples that were kept in solution were kept at la -80^{o}C until RNA analysis. Certain steps

were followed to obtain the expression of a panel of 84 miRNAs with implications in tumor pathology.

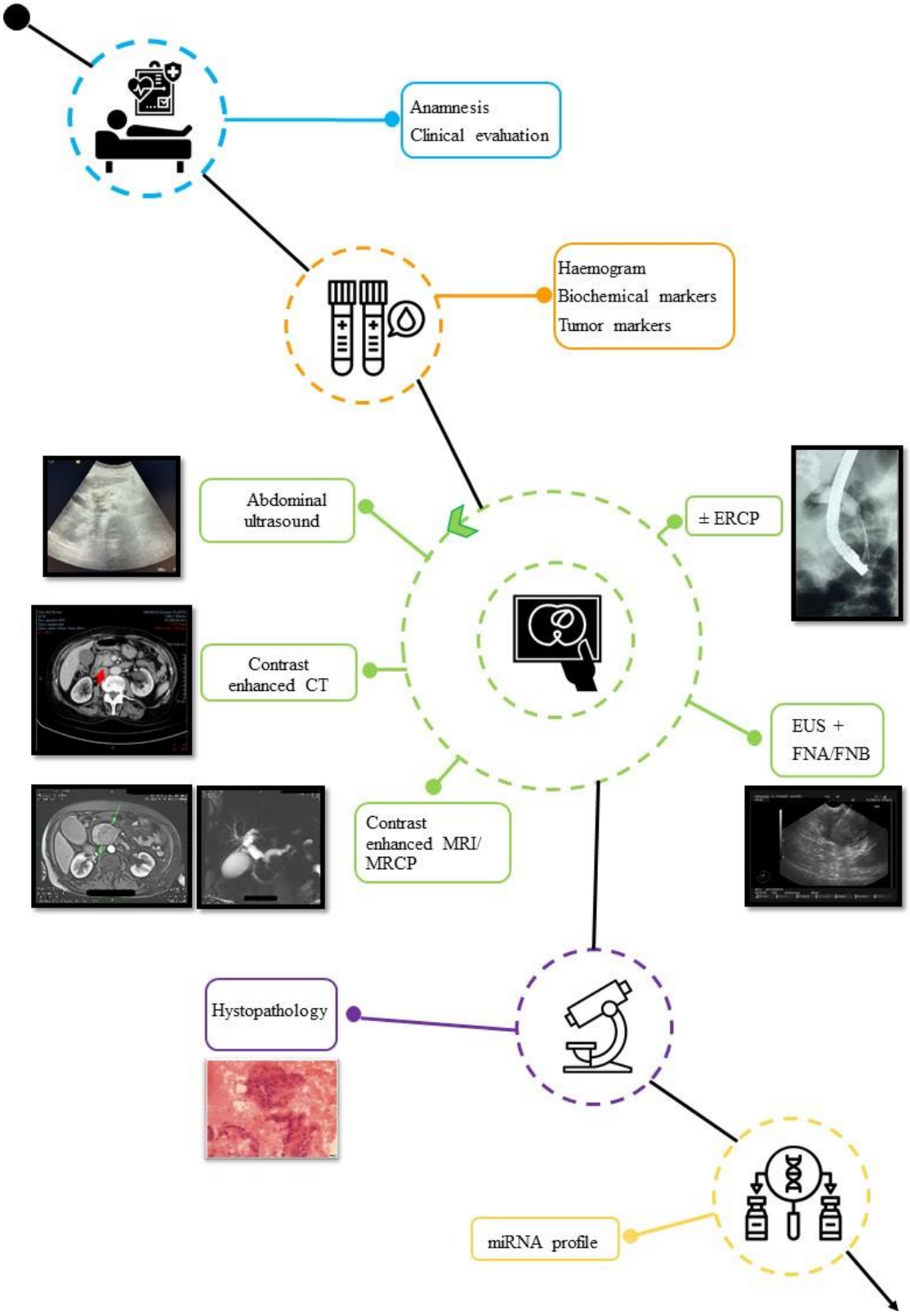

Image 2. Procedural steps followed by the patients in the study: after anamnesis and objective examination, biological samples were taken, then patients underwent imaging

and endoscopic investigations (EUS-FNA - minimum 2 passages, one being placed in a special tube containing RNA-later to be sent to the Victor Babeș Institute for miRNA profiling). The sample obtained endoscopically (not the one immersed in RNA-later) was sent to the pathology laboratory and analysed, thus confirming the diagnosis of pancreatic cancer. If the diagnosis was PDAC, the patient remained in the study cohort (images are taken from the in-house archive of the Bucharest Emergency Clinical Hospital and the Emergency Clinical Hospital "Prof Dr Agrippa Ionescu").

MicroRNA isolation was performed using the miRNeasy mini kit (Qiagen) and miRNA expression identification using the miRCURY LNA RT kit, miRCURY LNA SYBR Green PCR kit and miRCURY LNA miRNA Focus panel (YASH - 102Y) (Qiagen, GmbH). The isolation procedure is based on lysis of the sample with phenol and guanidine thiocyanate, purification of the sample by fixation of total RNA extracts in silica membrane and storage at -80^{o}C. A mandatory checkpoint in molecular biology experiments is checking of the quality of total RNA as well as the determination of the concentration, performed by UV spectrophotometry using the NanoDrop 2000 spectrophotometer (ThermoScientific). The microRNA expression study was carried out by PCR array method, by obtaining complementary DNA and reaction mixture, then amplification by real-time PCR and finally analysis of the results. The results obtained, expressed in TC (treshold cycle) values, were exported to an Excel document, followed by the actual analysis. If the TC value of the Blank control is ≤ 35, the amplification is non-specific and the results are not correct (repeat the reaction). For each plate, check the replicates for the 3 IPCs (interplate calibrator) and if the difference between them is $\leq$ 0.5, the results can be analysed. Fold change and fold regulation values were calculated to specify the intensity of underexpression or overexpression.

Statistical analysis was performed using Statistical Package for the Social Sciences SPSS software (version 28.0, SPSS Inc., Chicago, IL) and Microsoft Office Professional Plus 2016 Excel. SPSS, Excel and GraphPad Prism 8.4.3 were used for graphical representations. Using the Shapiro-Wilk test (<0.05) we observed that the distribution of the data in the second study was not normal, therefore non-parametric tests were used. For the analysis of categorical variables, we used the Chi-Square test, as in some cases the number of patients per category was relatively small. The Pearson/Spearman correlation coefficient was used to estimate correlations between continuous variables. Independent t-tests or Mann-Whitney test were used for analysis of continuous data,

expressed as mean ± standard deviation or mean standard error. The Kruskal-Wallis test was used to compare groups and to compare different parameters within the study group if there were at least 3 categories. Statistical significance was given by p-values <0.05 and fold regulation is significant if below -2 or above 2. To show the graphical differentiation of miRNA expression levels in the 3 groups we used heatmap using Prism GraphPad 8.4.3.

The results of epidemiological, clinical, biological, imaging and endoscopic correlations in PDAC patients have been elaborated in **Chapter 6**. Of the 57 patients enrolled in the study: 45 were confirmed with PDAC at histopathology examination, 5 patients were diagnosed with chronic pancreatitis (this being the comparative group in the second study); 1 patient was diagnosed with primary pancreatic lymphoma, 6 patients were not confirmed with PDAC at histopathology analysis. Of the 45 patients confirmed with PDAC, 2 patients were excluded as the biopsy sample was not sufficient to perform miRNA processing. This left 43 patients that formed the study group. Among them, epidemiological, clinical, biological, imaging, endoscopic and prognostic parameters were analyzed and possible correlations between these parameters and the aggressive features of the pancreatic tumor were identified.

Analyzing the study cohort, minimal preponderance of female patients was observed. Analyzing survival by sex in our cohort, we observed that female patients show higher survival than male patients (p=0.026), but the results may also be influenced by the higher prevalence of other risk factors for PDAC among males (smoking, alcohol).

The peak incidence of PDAC patients is in the 7^{th} -8^{th} decade of life, similar to the data in our study (20). There is a negative correlation between older age and poor survival in our research (*p=0.012*).

65.1% of patients come from urban areas, while only 34.95% of patients come from rural areas. This is most likely due to the increased accessibility of diagnostic and treatment methods for patients in urban areas.

Patients in the study group have modifiable risk factors encountered in our analysis: smoking, alcohol consumption and toxic exposure; respectively risk factors that cannot be modified: old age, presence of other comorbidities such as diabetes mellitus, hereditary history of PDAC.

Pain remains the main symptom encountered in 88% of patients, although non-specific in PDAC, followed by weight loss and jaundice which were also identified in an

increased proportion within the study group in 74% and 40% of patients respectively. Mean BMI was 22.02 ± 4.21, with the majority of patients being normal weight.

There were statistically significant differences in survival between ECOG stages: as ECOG stage increased, survival decreased (*p=0.001*).

Only 17% of patients had normal blood glucose values, while 32% had impaired fasting glucose, which is consistent with literature data. In our cohort 82.9% of patients have impaired fasting glucose or diabetes, similar to data obtained by Modi et al. who showed that 40% of patients with impaired fasting glucose and 85% of patients with PDAC at diagnosis have diabetes or impaired fasting glucose (3). CA 19-9 remains the most widely used biomarker at present, but with utility in selected cases. An interesting element analyzed in our cohort is the normal CA 19-9 values among 3 patients. We found no statistically significant differences between hemoglobin, NLR, albuminemie and survival.

60-70% of pancreatic adenocarcinomas are localized in the head of the pancreas according to literature data, which is confirmed in our study demonstrating cephalic localization of PDAC in 60.5% of cases (2). Patients with tumors localized in the head of the pancreas present earlier to the hospital because of early onset of symptoms secondary to tumor contact with coledocus. Thus, the statistically significant association of higher values of cholestatic enzymes (total and direct BR, GGT) and hepatic lysis enzymes (ALT, AST) with pancreatic cephalic location, is also justified. A surprising element is the association of cephalic tumor location with increased CA 19-9 values (*p=0.032*) since at diagnosis, tumors localized in the body or tail of the pancreas are larger in size and thus more frequently associated with higher CA 19-9 values.

90.69% of the patients in the study presented in inoperable forms, while no patient is in resectable form of the disease and only 4 patients are borderline resectable (9.31%). According to TNM staging two thirds of the study group are in stage IV disease.

Poor survival was associated with older age (Image 3), high ECOG status (Image 4a), presence of metastases (Image 4b) and large tumor size (Image 5), associations that were shown to be statistically significant ($p<0.05$).

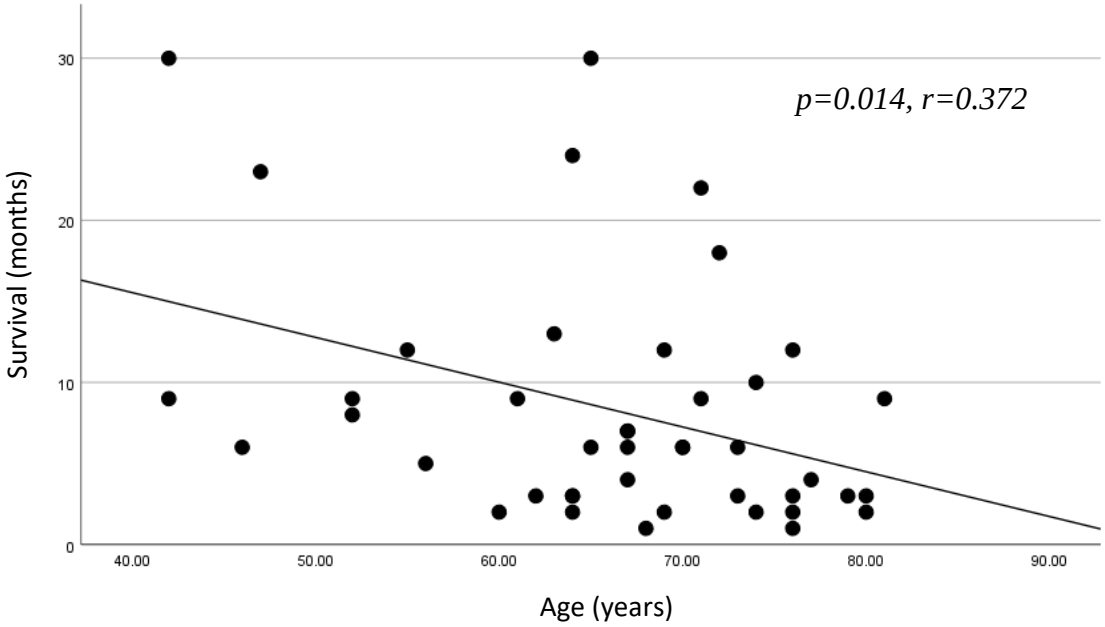

Image 3. Negative correlation between age and survival by Pearson test

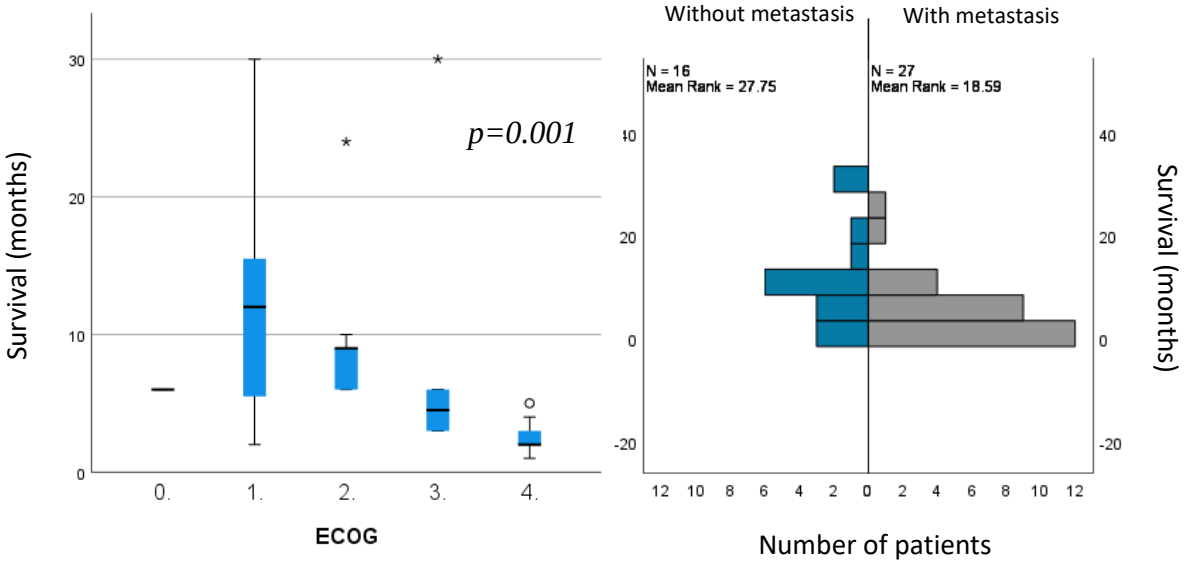

Image 4. a) Differences in survival according to ECOG category; b) Distribution of patients with and without metastases according to survival (statistically significant correlation p=0.02)

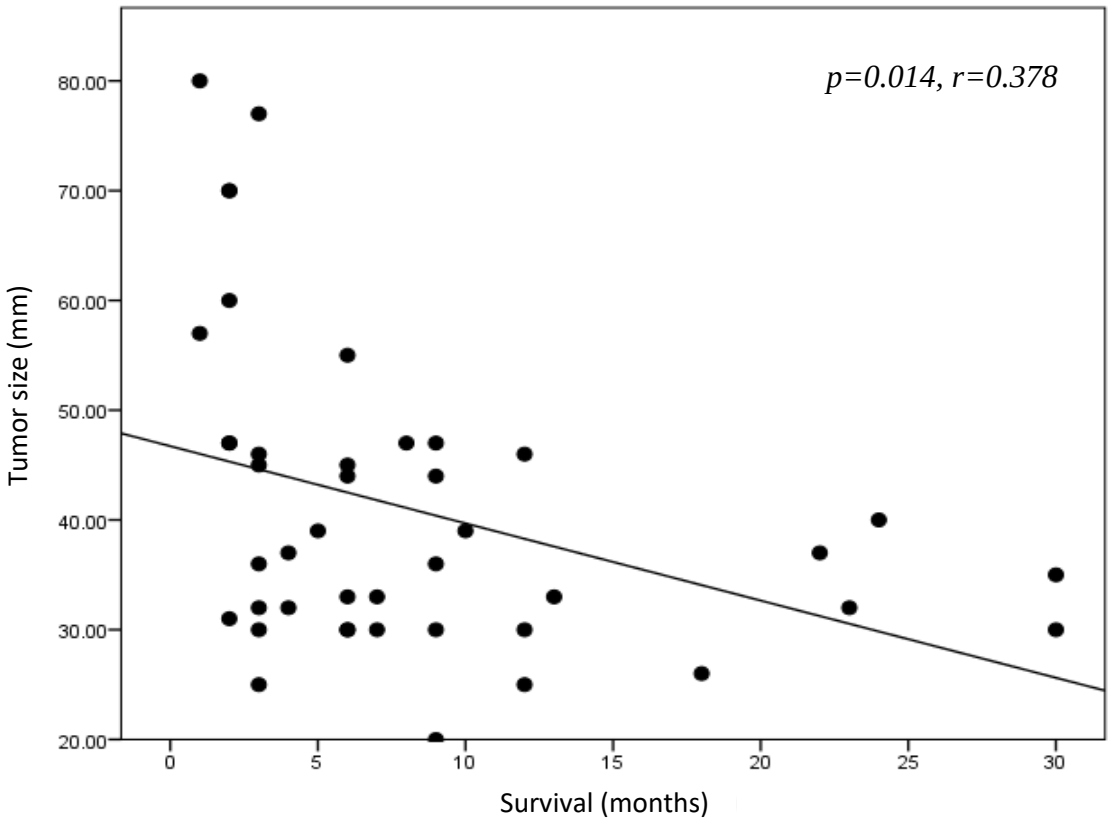

Image 5. Statistically significant Pearson correlation between survival (months) and tumor size (mm)

Tissue miRNA expression in PDAC was analyzed in **Chapter 7**. miRNA represents 1-4% of the human genome, controlling the expression of over 60% of human protein-coding genes. miRNA is involved in the proper functioning of cellular processes: growth, cell differentiation and apoptosis. miRNA are novel parameters obtained from biological products of the patient (blood, tissue, saliva, faeces, pancreatic juice). These are not only biomarkers in the diagnosis of PDAC, but also they can express the aggressive pattern of PDAC (the presence of metastases, vascular invasion, increased tumor size) (12). Evidence of miRNA expression was analyzed from biopsy samples taken by ultrasound endoscopy FNA/FNB. Starting from the cohort in the previous study of 43 patients, 2 patients were excluded after PCA analysis from SPSS statistical processing software "outlier detection". For the comparative analysis it was necessary to create 2 comparative groups: group 1 consisting of tissue samples from patients with chronic pancreatitis taken by fine needle aspiration EUS, called the *comparative group*, and group 2 consisting of normal peritumoral pancreatic tissue samples (from the biobank of the "Victor Babeș" Institute of Pathology) which we will name the *control group*. Patients in the comparative

group were part of the initial cohort of 57 patients, but following histological evaluation and subsequent imaging re-evaluation, the diagnosis of chronic pancreatitis was made.

Within the 3 groups 84 miRNAs involved in the development and progression of cancer were analyzed. Of these 84 miRNAs studied, 3 miRNAs (miR-149-3p, miR-202-3p, miR-206) were not detected in the studied tissues (Ct > 35) according to the protocol. The assay demonstrated that 75 miRNAs were differentially expressed between the 3 groups (Table 1 and Image 6).

Tabel 1. Different miRNA expression in pancreatic adenocarcinoma versus normal peritumoral pancreatic tissue versus chronic pancreatitis, respectively

		Study lot (PDAC) vs control lot (normal pancreatic tissue peri-tumoral), pair-wise analysis		Study lot (PDAC) vs comparative lot (chronic pancreatitis), pair-wise analysis	
miARN	Kruskal Wallis test	p-value	FR ■	p-value	FR ■
let_7a_5p	***0.004***	0.717		***0.004***	***-15.34***
let_7b_5p	***<0.001***	***0.033***	***-3.16184***	***0.004***	***-14.20***
let_7c_5p	***0.003***	0.311	-1.89073	***0.004***	***-9.59***
let_7d_5p	***0.007***	1		***0.005***	***-10.78***
miR_200a_3p	***<0.001***	***0.001***	***-8.9012***	0.228	-3.16
let_7g_5p	***0.021***	0.501		***0.032***	***-8.47***
let_7i_5p	***0.044***	1		***0.037***	***-2.75***
miR_1	***0.005***	***0.02***	***-8.68729***	0.118	-2.46
miR_100_5p	***0.002***	***0.012***	***-4.10719***	0.051	-1.44
let_7f_5p	***0.009***	0.57		***0.011***	***-9.08***
miR_101_3p	***0.005***	0.172	-1.34124	***0.013***	***-8.86***
miR_103a_3p	***0.004***	1		***0.003***	***-8.08***
miR_106a_5p	***0.004***	1		***0.012***	***-15.45***
miR_106b_5p	***0.011***	1		***0.011***	***-9.72***
miR_107	***0.012***	1		***0.01***	***-8.03***
miR_125b_5p	***<0.001***	***<0.001***	***-39.7994***	***0.03***	***-7.74***
miR_126_3p	***0.001***	***0.034***	***-3.22119***	***0.005***	***-26.97***
miR_130a_3p	***0.005***	0.153	-2.16855	***0.014***	***-21.96***
miR_132_3p	***<0.001***	***0.002***	***-18.433***	***0.006***	***-13.39***

miR_10b_5p	***<0.001***	***0.001***	***-14.3183***	***0.019***	***-6.65***
miR_133a_3p	***0.001***	***0.011***	***-5.46625***	***0.023***	***-4.39***
miR_141_3p	***<0.001***	***<0.001***	***-54.4185***	0.09	-4.99
miR_143_3p	***<0.001***	***0.002***	***-5.71145***	***0.025***	***-4.21***
miR_145_5p	***<0.001***	***0.001***	***-11.1326***	***0.02***	***-6.82***
miR_146a_5p	***0.001***	0.205	-3.99766	***0.002***	***-7.75***
miR_26a_5p	***<0.001***	***0.022***	***-4.18862***	***0.004***	***-15.15***
miR_150_5p	***0.001***	0.47	-5.73382	***0.001***	***-28.60***
miR_155_5p	***0.005***	1	-2.72754	***0.004***	***-22.55***
miR_15a_5p	0.085				
miR_15b_5p	***0.004***	0.11		***0.065***	***-6.68***
miR_149_3p					
miR_16_5p	***0.008***	1		***0.009***	***-20.00***
miR_17_5p	***0.005***	1		***0.007***	***-10.30***
miR_181a_5p	***<0.001***	***0.024***	***-4.92669***	***0.003***	***-14.70***
miR_181b_5p	***<0.001***	***0.012***	***-7.07596***	***0.004***	***-6.68***
miR_182_5p	***0.002***	0.108	-1.74113	***0.006***	***-10.99***
miR_27a_3p	***0.001***	***0.013***	***-5.27275***	***0.014***	***-5.76***
miR_186_5p	***0.003***	***0.048***	***-2.02413***	***0.027***	***-10.03***
miR_18a_5p	***0.01***	0.885		***0.021***	***-8.72***
miR_191_5p	***0.002***	0.122	-1.60763	***0.005***	***-19.87***
miR_192_5p	***0.003***	***0.05***	***-7.18242***	***0.021***	***-5.18***
miR_148a_3p	***<0.001***	***<0.001***	***-74.0857***	***0.026***	***-3.98***
miR_194_5p	***0.01***	0.276	-1.97295	***0.02***	***-5.62***
miR_195_5p	***<0.001***	***0.001***	***-22.8883***	***0.003***	***-19.62***
miR_196a_5p	***0.152***				
miR_19a_3p	***0.003***	0.238	-1.32691	***0.007***	***-9.76***
miR_19b_3p	***0.001***	0.116	-2.36763	***0.003***	***-14.51***
miR_200b_3p	***0.001***	***0.004***	***-6.73932***	0.118	-5.34
miR_200c_3p	***<0.001***	***0.001***	***-25.1103***	0.093	-8.44
miR_202_3p					
miR_10a_5p	***0.01***	***0.024***	***-5.07663***	0.227	-2.96
miR_205_5p	***0.115***				

miR_206					
miR_20a_5p	***0.015***	1		***0.023***	***-10.97***
miR_20b_5p	***0.004***	0.776		***0.009***	***-8.04***
miR_21_5p	***0.004***	0.158	-2.33306	***0.01***	***-3.58***
miR_210_3p	***0.052***				
miR_214_3p	***0.001***	***0.013***	***-12.4123***	***0.011***	***-8.81***
miR_215_5p	***0.006***	0.107	-6.01519	***0.025***	***-5.43***
miR_22_3p	***0.023***	***0.048***	***-7.09379***	0.333	-1.91
miR_221_3p	***0.161***				
miR_222_3p	***0.064***				
miR_223_3p	***0.002***	0.24		***0.017***	***-12.89***
miR_23a_3p	***0.004***	0.168	-1.94819	***0.009***	***-8.84***
miR_23b_3p	***<0.001***	***0.013***	***-4.15095***	***0.002***	***-9.52***
miR_24_3p	***0.001***	***0.031***	***-2.90708***	***0.005***	***-5.96***
miR_25_3p	***0.007***	1		***0.005***	***-13.55***
miR_26b_5p	***0.012***	1	-1.04132	***0.01***	***-10.73***
miR_27b_3p	***0.001***	***0.002***	***-12.9222***	0.077	-6.49
miR_29a_3p	***<0.001***	***0.001***	***-7.20084***	0.024	-3.00
miR_29b_3p	***<0.001***	***0.002***	***-5.29576***	0.051	-3.95
miR_29c_3p	***<0.001***	***0.001***	***-10.0439***	***0.018***	***-3.96***
miR_30b_5p	***<0.001***	***0.003***	***-9.23627***	***0.008***	***-13.81***
miR_30c_5p	***<0.001***	***0.008***	***-4.68652***	***0.005***	***-15.83***
miR_30d_5p	***<0.001***	***0.004***	***-6.76264***	***0.002***	***-13.24***
miR_31_5p	***0.05***	0.741		***0.049***	
miR_34a_5p	***0.007***	***0.023***	***-2.75659***	0.16	-1.98
miR_423_5p	***0.014***	0.192	-2.44845	***0.042***	***-7.27***
miR_7_5p	***<0.001***	***0.001***	***-25.1126***	0.128	-2.60
miR_9_5p	***<0.001***	***0.001***	***-14.8621***	***0.004***	***-21.01***
let_7e_5p	***0.005***	0.173	-1.84597	***0.014***	***-6.04***
miR_92b_3p	***0.002***	0.058	-4.17145	***0.009***	***-8.46***
miR_93_5p	***0.011***	0.92		***0.023***	***-7.44***
miR_99a_5p	***<0.001***	***<0.001***	***-58.2561***	***0.011***	***-13.68***

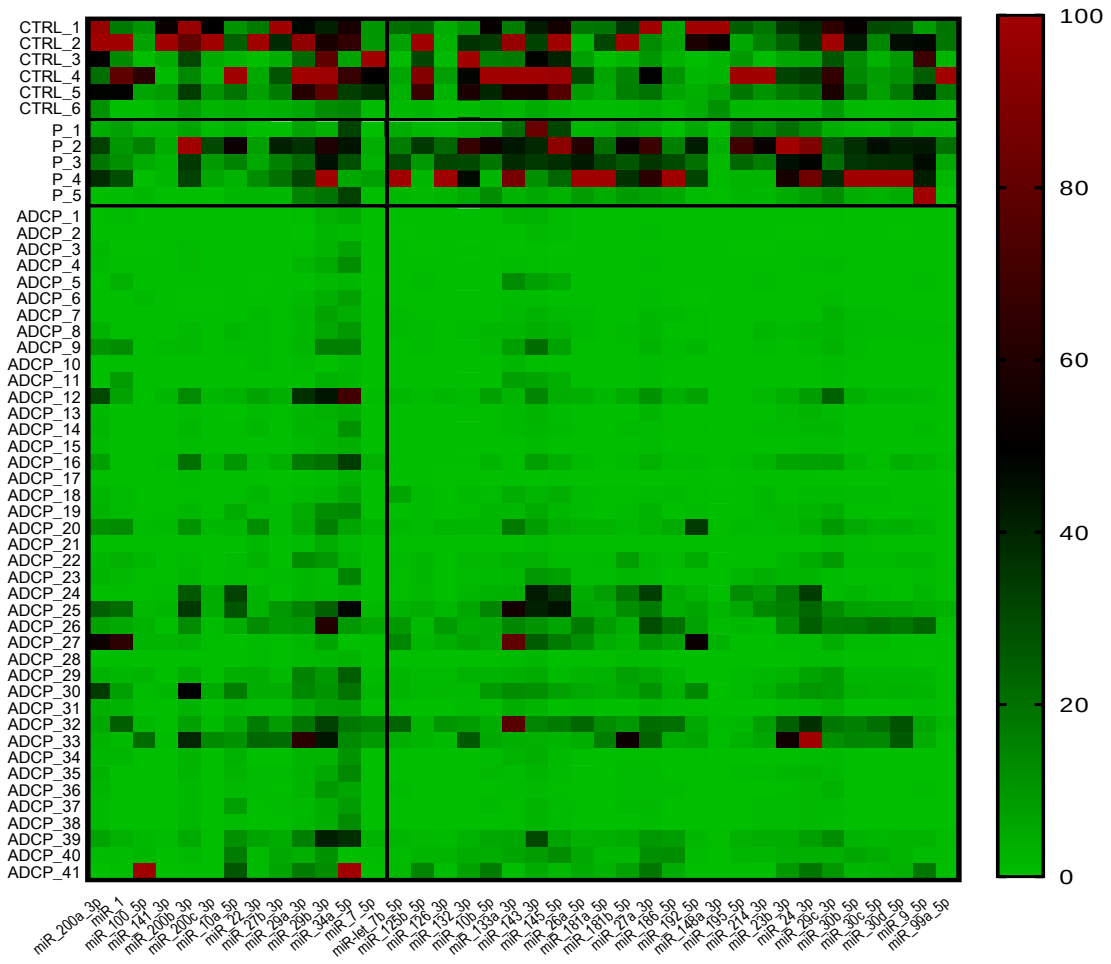

Figure 6. Heat map. Expression pattern of 38 miRNAs (out of 84 miRNAs analyzed) altered in pancreatic adenocarcinoma compared to control and comparison groups, p-value < 0.005 and FR ≥ |2|. Heat map indicates overexpression (RED), underexpression (GREEN) and average expression (BLACK). Data were distributed scalar considering the highest value as 100% and the lowest value as 0%. Rows represent individual tissue samples, while columns represent miRNA symbols.

Pair-wise analysis demonstrated reduced expression of 25 miRNAs in the study group compared to the control and comparison groups. We can observe that the lowest values of miRNAs in the study group (the most powerful from the study) were: miR-148a, miR-99a, miR-125b, miR-132, miR-195 (ordered in descending order corresponding to fold regulation).

In the pair-wise analysis we determined 13 miRNAs that showed decreased expression in the study group compared to the control group, but not to the comparison group. miR-141, miR-200c, miR-7 present the lowest values within PDAC according to fold regulation.

Malnutrition remains an poor prognostic factor in PDAC. In our study we detected statistically significant differences between miR-143, miR-29b, miR-34b and miR-99a levels among undernourished patients. We observed that patients with BMI below 18.5kg/m2 had lower levels of miR-143 (*p=0.034*), miR-29b (*p=0.036*), miR-34a (*p=0.023*), and miR-99a (*p=0.020*) (Image 7).

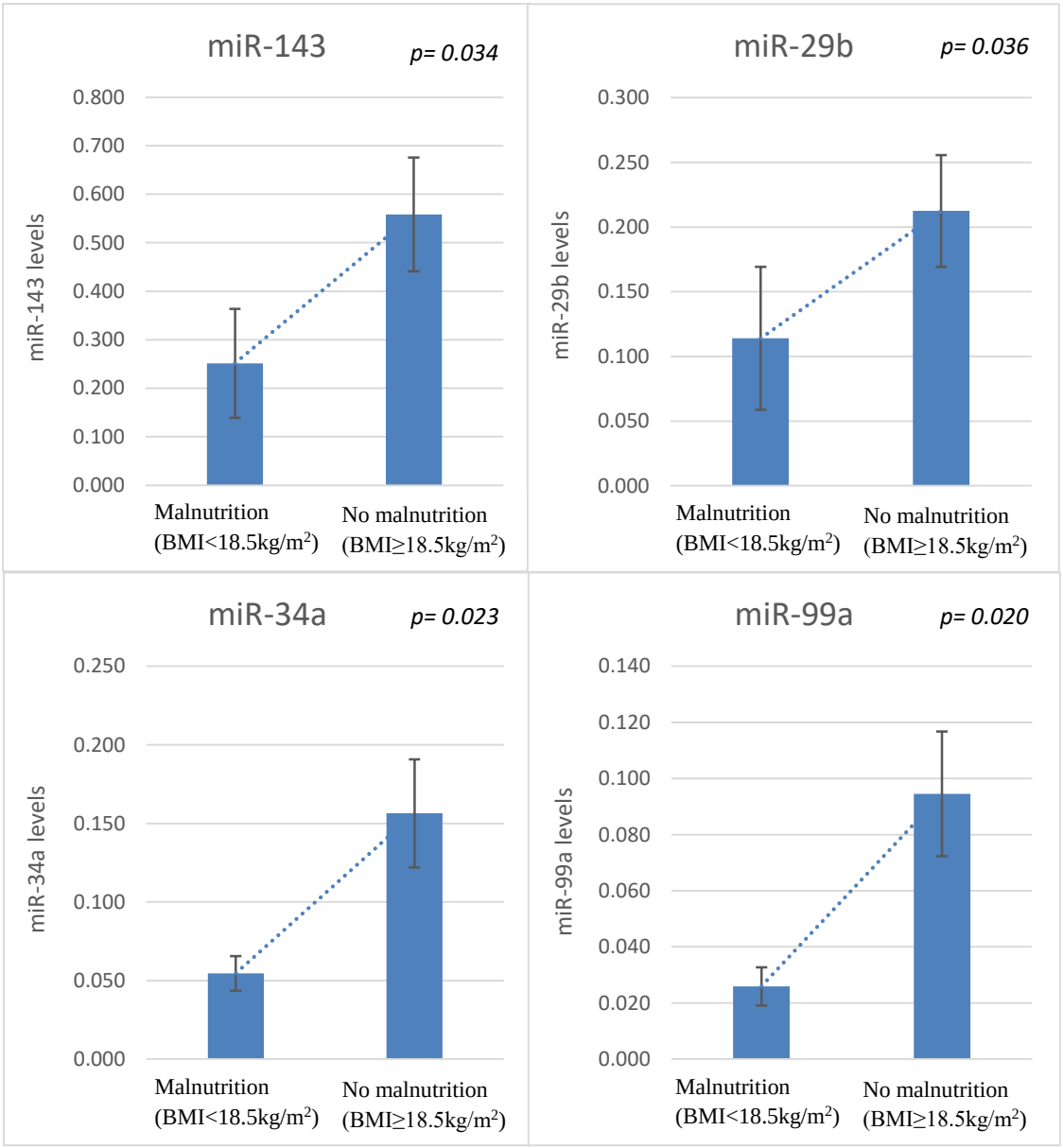

Image 7. Statistically significant differences in miR-29b, miR-34a, miR-99a and miR-143 expression according to the presence of malnutrition (BMI ≥ 18.5kg/m2)

On Mann-Withney analysis we observed that the presence of pain (which would suggest advanced disease) associated lower levels of miR-1 (*p=0.003*), miR-100 (*p=0.046*), miR-126 (*p=0.023*), miR-133 (*p=0.005*), miR-143 (*p=0.026*), miR-145 (*p=0.028*) and let-7 (*p=0.042*).

There was a statistically significant trendline between low miR-100 values and increased tumor size (p=0.060 on Kruskall-Wallis analysis).

Vascular invasion is a negative prognostic marker, and when analyzing miRNA profile we observed statistically significant differences between miR-10a levels among patients with vascular invasion and without vascular invasion (*p=0.028*). Thus patients with vascular invasion have lower miR-10a levels (Image 8).

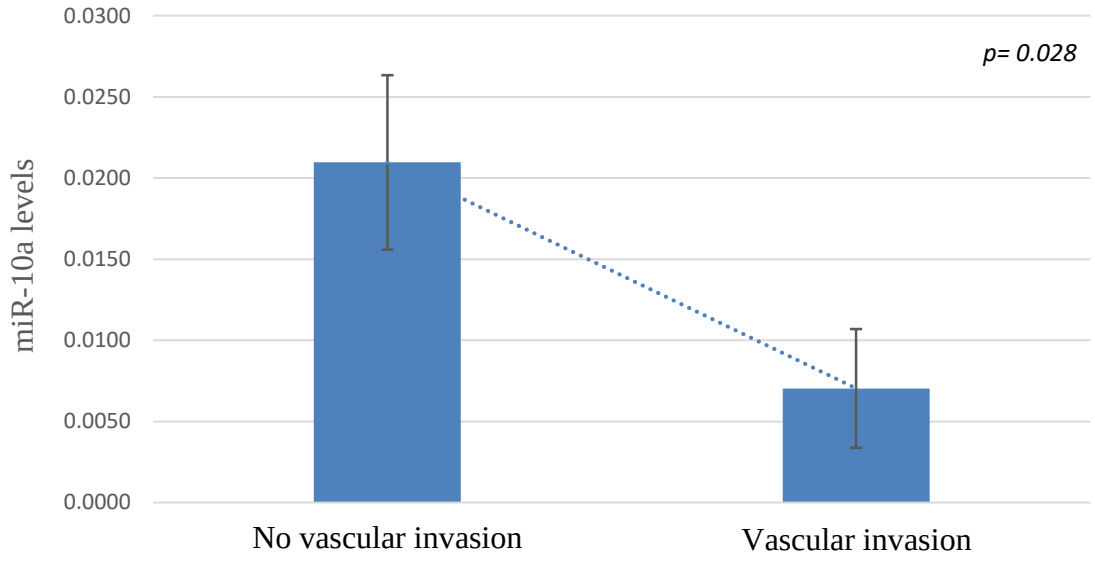

Image 8. Significantly differences of miR-10a within patients with and without vascular invasion in PDAC

Negative tredline of statistical significance was observed in miR-192 expression among patients with and without metastases (p=0.065). Patients with metastases have lower levels of miR-192, which can be evaluated in larger groups of patients to see if it will play a prognostic role.

Using bioingormatics (DIANA TarBase) we can investigate signaling pathways involved in miRNA function. Through this system we identified the involvement of insulin and IGF/IGF1-R signaling pathways in PDAC progression. From our research identified 6 miRNAs (miR-1, miR-100, miR-9, miR-145, miR-29c și miR-195) involved in the IGF/IGF-1R pathway, underexpressed in the study group compared to the control and comparison groups. Furthermore, there are differences in expression of 9 miRNAs involved in the IGF/IGF-1R pathway compared to the control group, which show low levels in PDAC: let-7b-5p, miR-126-3p, miR-133a-3p, miR-145-5p, miR-181a-5p, miR-181b-5p, miR-195-5p, miR-29c-3p, miR-9-5p.

In the analysis of the study group, we observed that there are elevated miRNAs only in the chronic pancreatitis group compared to the control group. miR-103a-30, miR-106a,b-5p, miR-16-5p, miR-17-5p, miR-18a, miR-20a,b-5p, miR-223-3p, miR-93-5p are overexpressed, showing FR up to 73.88. They may play an important role in inflammatory pathways. More studies that would certify miRNA roles in inflammatory mechanisms in chronic pancreatitis may help in determining therapeutic targets for the treatment of this pathology.

There are limitations to our study. Since the study cohort, control group and comparison group consist of small number of patients, further studies following miRNAs already found in the present research as underexpressed would be useful, performed on larger groups of patients to strengthen the value of the data obtained. As the information obtained for the diagnosis of chronic pancreatitis is limited due to FNA sampling, follow-up of patients in the comparative chronic pancreatitis group was essential to strengthen the diagnosis. Studies in the literature demonstrate that FNB or surgical biopsy provides better results in terms of cellularity in chronic pancreatitis. Another limitation of the study is the lack of histopathological analysis of samples directed to miRNA examination, but for this problem we performed qualitative analysis of the tissue submitted and subsequently by bioinformatics programs and statistical analysis we have identified ouliers.

The conclusions have been finalized in **Chapter 8**. Survival in pancreatic adenocarcinoma remains poor due to presentation in advanced disease. The discovery of predictive biological markers is essential in the optimal treatment of the disease.

Finding risk factors involved in the etiopathogenesis of pancreatic adenocarcinoma would help in early diagnosis of the disease and facilitate early presentation of patients to the physician, thus improving prognosis.

As older age remains an important risk factor in pancreatic adenocarcinoma, decreasing survival, proper assessment of the warning signs in this population group is essential for detection of the disease in less advanced forms.

Low accessibility to medical resources should be addressed among patients in rural areas, as the incidence of pancreatic adenocarcinoma is higher for urban patients only due to the increased ease of carrying out the investigations necessary for diagnosis.

The occurrence of diabetes mellitus in a normal-weight adult patient may necessitate further investigations particularly if the patient has other risk factors for

pancreatic adenocarcinoma. Furthermore, the collection of HbA1C as a biomarker may be considered in the composition of early diagnostic formulas in pancreatic adenocarcinoma.

Echoendoscopy has become an essential examination method in pancreatic pathology providing a wide range of information with greater accessibility than in the past. Thus the diagnosis of patients with pancreatic lesions has become easier by the use of elastography, colour Doppler function, contrast enhanced EUS and especially biopsy (EUS-FNA or EUS-FNB). Echoendoscopy or endosonography combined with aspiration punctures and guided echoendoscopic biopsies represent minimally invasive procedures with lower risks compared to previously used investigations (percutaneous echo-guided punctures or intraoperative punctures). Echoendoscopy shows higher sensitivity and specificity than CT examination in the diagnosis and detailed characterisation of tumours under 3cm, thus involved in early detection of PDAC.

miRNAs are among the biomarkers of great importance in pancreatology, with more and more studies looking at their expression in tumorigenesis either as oncomiR (through overexpression) or as tumor suppressor (through underexpression).

Personal contributions to our research were as follows:

1. The role of echoendoscopy in the early diagnosis and prognosis of pancreatic adenocarcinoma derives from the possibility of sampling tumour tissue by FNA or FNB and subsequent molecular analysis of the samples collected. Following this analysis we can determine early diagnostic or unfavourable prognostic markers involved in pancreatic adenocarcinoma.

2. The mean age of the patients in the study was 66.21 ± 10.12 years, with only 11.62% of the group under 55 years of age. The presence of a statistically significant correlation between older age and poor survival was observed.

3. Among the risk factors implicated in the development of PDAC, we found in the study group risk factors that can be modified: smoking (16.7%), alcohol consumption (16.27%) and toxic exposure (19.2%); respectively that cannot be modified: old age, presence of other comorbidities such as diabetes mellitus (44.2%), hereditary history of PDAC (9.5%).

4. 82.9% of patients have altered basal glucose or diabetes, highlighting the importance of closer follow-up of patients with diabetes.

5. Pain in the upper abdominal floor remains the main symptom in pancreatic adenocarcinoma, seen in the majority of patients in the group (88%).

6. One third of patients are underweight (BMI <18.5 kg/m^2), demonstrating the impact of pancreatic adenocarcinoma on nutritional status.

7. 60.5% of the group presented with tumor localized in the head of the pancreas. As these patients present symptoms faster than those with corporeocaudal localization by compressing the main bile duct, higher values of cholestasis markers (total and direct BR, GGT) and hepatic lysis syndrome (ALT, AST) are associated with tumors localized in the head of the pancreas.

8. 90.69% of the patients in the study have inoperable forms, no patient presents resectable form and only 9.31% of the group are borderline resectable.

9. Survival is negatively correlated with advanced ECOG status, increased tumor size and existence of metastases, so early detection of lesions remains a goal in pancreatic adenocarcinoma research.

10. Echoendoscopy provided essential information through biopsy sampling and molecular analysis, laying the foundation for all research. Our study confirms the literature data and shows that tissue miRNA is differentially expressed in pancreatic adenocarcinoma compared to healthy pancreatic tissue and chronic pancreatitis. So from the first study group of 43 patients, the cohort considered for examining miRNA expression consisted of 41 patients, 6 patients formed the control group (normal peritumoral pancreatic tissue) and 5 patients formed the comparison group (chronic pancreatitis). The miRNA signature of pancreatic adenocarcinoma in our research followed the expression of 84 miRNAs, detecting 75 differently expressed miRNAs between the 3 groups.

11. In the comparative analysis, 25 miRNAs were underexpressed in PDAC compared to the control and comparative groups, and 13 miRNAs were underexpressed in PDAC compared to the control group.

12. Although there are limited studies, predominantly in cell lines or animal models, in our research we identified underexpression of miR-1, miR-133, miR-143, miR-195 and tissue miR-22 in pancreatic adenocarcinoma, adding value to literature data.

13. The underexpression of miR-10b, miR-125b, miR-145, miR-24 and miR-27b is found in the study group, but there are discrepancies in the literature regarding the expression of these miRNAs.

14. Malnutrition decreases survival and quality of life and is a prognostic marker of poor outcome. There are differences in the expression (low expression) of miR-143, miR-29b, miR-34a and miR-99a among patients with BMI below 18.5kg/m2 in the study group.

15. Increased tumor size is a negative prognostic factor in PAC, and the present study demonstrates a statistically significant trend between large tumor size and miR-100 underexpression.

16. Among patients with vascular invasion we observed lower values of miR-10a, being considered as a negative prognostic marker.

17. Further data are needed on larger groups of patients but our research shows a trend of statistical differentiation of miR-192 values among patients with metastases compared to patients without metastasis.

18. In bioinformatics analysis (DIANA TarBase) we determined the role of insulin and IGF-1R signaling pathway in PDAC progression. 6 miRNAs involved in IGF/IGF-1R pathway are underexpressed in PDAC unlike control and comparison group. Another 9 miRNAs have decreased expression in PDAC compared to the control group.

Future study directions in the field of miRNA biomarkers in PDAC have been described in the last subchapter. Defining biomarkers for benign pancreatic pathology at risk of malignant transformation would help in laying the foundation for preventive medicine in PDAC. Different miRNA expression expressed in benign versus malignant pancreatic lesions is important in the preventive treatment and differential approach to these patients. Underexpression of miR-214 and miR-148 occurs in pancreatic adenocarcinoma compared to benign pancreatic lesions according to studies from the literature thus deriving the early diagnostic role of these markers.

Circulating miRNAs remain the most attractive target due to their abundance, stability and ease of sample procurement.

Initially used for biomarker and prognostic roles, miRNAs are beginning to be studied for their therapeutic role in controlling miRNA expression. Therefore new studies are needed to target not only miRNAs but also their target molecules, as an important step in the personalized treatment of pancreatic cancer. MAP4K4 is the molecular target of miR-141 and may be a taget gene taken into consideration for a new personalized treatment in pancreatic adenocarcinoma.

Personalised treatment in pancreatic adenocarcinoma may target miRNA to increase sensitivity to chemotherapy for molecules involved in increasing resistance to chemotherapeutic treatment, i.e. radiotherapy (miR-23b underexpression is associated with radioresistance).

Further steps are underway to develop more accessible markers for the detection of molecules involved in pancreatic adenocarcinoma. Thus immunohistochemical examination may be considered for the detection of ADAM9, involved in the miR-126/ADAM9 axis. Although E-cadherin expression levels can be identified immunohistochemically, specific antibodies will have much lower values compared to circulating or tissue miRNA levels.

LIST OF PUBLISHED SCIENTIFIC PAPERS

Published papers

- ISI articles
  - Dobre M, Herlea V, **Vlăduț C©**, Ciocîrlan M, Balaban VD, Constantinescu G, Diculescu M, Milanesi E. Dysregulation of miRNAs targeting the IGF-1R pathway în pancreatic ductal adenocarcinoma. Cells 2021, 10(8), 1856. DOI: 10.3390/cells10081856.
    https://www.mdpi.com/2073-4409/10/8/1856
  - Ciocîrlan M, Gheorghiu A, Bilous D, Cruceru M, Mănăila, Tianu E, **Vlăduț C**. Connect your needle to a smartphone to increase EUS FNA sample quality and diagnostic accuracy of solid pancreatic masses: a randomized trial. Endoscopy 2021; eFirst 2021 May. PMID: 33940637; DOI 10.1055/a-1497-6532. *https://www.thieme-connect.com/products/ejournals/abstract/10.1055/a-1497-6532*.
  - **Vladut** C, Ciocirlan M, Bilous D, Sandru V, Stan-Ilie M, Panic N, Becheanu G, Jinga M, Costache RS, Costache DO, Diculescu M. "*An Overview on Primary Sclerosing Cholangitis*". Journal of Clinical Medicine 2020; 9, 754: 1-16. *https://www.mdpi.com/2077-0383/9/3/754.*
  - **Vladut** C, Ciocirlan M, Costache RS, Jinga M, Balaban VD, Costache DO, Diculescu M. "*Is mucosa-associated lymphoid tissue lymphoma an infectious disease? Role of Helicobacter pylori and eradication antibiotic therapy (Review)*". Experimental and Therapeutic Medicine 2020; 20: 3546-3553. *https://www.spandidos-publications.com/10.3892/etm.2020.9031*
  - **Diaconu C**, Ciocîrlan M, Ilie M, Sandru V, Balaban DV, Plotogea O, Diculescu M. "*Gurvitis syndrome: the dark shade of hematemesis*". Endoscopy 2019; DOI: 10.1055/a-1059-9268.
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  - **Diaconu C**, Ciocîrlan M, Jinga M, Costache RS, Constantinescu G, Ilie M, Diculescu M. "*Ectopic pancreas mimicking gastrointestinal stromal tumor în the stomach fundus*". Endoscopy 2018; 50(07): E186-E187. DOI: 10.1055/a-0605-2996.
    https://www.thieme-connect.com/products/ejournals/html/10.1055/a-0605-2996

Papers presented at scientific events organised by national and international professional associations:

- Rinja E, Plotogea O, Sandru V, **Vladut** C, Ilie M. "*Endoscopic ultrasound with 'bite' fine needle biopsy în a patient with pancreatic cyst*". ESGE Days 2020 ePoster presentation. April 2020. DOI: 10.1055/s-0040-1705026.
- Plotogea O, lie M, Sandru V, **Diaconu C**, Ilie M, Constantinescu G. "*Endoscopic ultrasonography with fine needle aspiration în the diagnosis of pancreatic tumors – retrospective, unicentric study*". National Conference of Gastroenterology, Hepatology and Endoscopy 2019. XXXIX edition, Bucharest 6-8th June 2019
- Sandru V, Ilie M, Plotogea OM**, Diaconu C**, Ungureanu B, Rosianu C, Constantinescu G. „*Are plastic stents expandable în the treatment of biliary strictures after othotopic liver transplantation?*" UEGW Vienna 2018.

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