"CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST

DOCTORAL SCHOOL

FIELD OF MEDICINE

LIQUID BIOPSY FOR MOLECULAR CHARACTERIZATION OF PANCREATIC DUCTAL ADENOCARCINOMA

DOCTORAL THESIS ABSTRACT

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Introduction

Globally, pancreatic ductal adenocarcinoma (PDAC) continues to be a major public health concern. Its subtle onset, which frequently results in vague symptoms in later stages, makes early detection difficult and restricts the number of curative therapy options. The 5-year survival rate is still low despite improvements in systemic therapies, including targeted therapy in specific molecular subgroups.

Improving chemotherapeutic treatment based on specific biomarkers is the current paradigm of clinical oncology for patients with PDAC. The recent development of liquid biopsy opens up new possibilities for tumor prognosis and early diagnosis. The use of liquid biopsy in tumor identification has gained more attention lately, currently experiencing rapid progress and having significant potential for future applications.

I. GENERAL PART

Chapter 1. Pancreatic ductal adenocarcinoma: trends in epidemiology, risk factors, treatment principles, and outcomes

Globally, pancreatic cancer is the 12th most common cancer and the 7th most common cause of cancer-related death [1]. To enhance the prognosis for PDAC patients, early diagnosis is crucial. The onset of pancreatic cancer is insidious, and most patients are diagnosed at an advanced stage of disease, limiting curative treatment options [1,2]. Advances in the molecular characterization of PDAC have identified potential therapeutic targets [3]. A key development has been the identification of targetable molecular factors that contribute to PDAC progression.

Chapter 2. Liquid biopsy in pancreatic ductal adenocarcinoma

Liquid biopsy is a minimally invasive method of collecting samples that focuses on blood or body secretions for the detection of molecular changes, tumor cells, and metabolites [4]. Several molecular markers can be detected by liquid biopsy, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor-derived extracellular vesicles (EVs), tumor-educated platelets (TEPs), and circulating free RNA (cfRNA) [5]. Circulating free DNA (ccfDNA) represents DNA fragments that circulate in the bloodstream and can be obtained through a simple blood sample.

Measuring the amount of circulating plasma/serum free DNA (ccfDNA) and identifying tumor-derived genetic aberrations, including point mutations, allelic imbalances, microsatellite instability, genetic polymorphisms, loss of heterozygosity, and methylation, are the two main approaches used in the study of ccfDNA. Among these, numerous reports have demonstrated the detection of genetic and epigenetic alterations in ccfDNA in the bloodstream of cancer patients [6–9].

The genetic landscape of pancreatic tumor genomes is characterized by the presence of four frequently mutated genes, represented by KRAS (90%), CDKN2A (p16, 90%), TP53 (70%), and SMAD4 (55%). These four genes (KRAS, TP53, CDKN2A, and SMAD4) are considered driver genes for PDAC.

Overall, liquid biopsy has the potential to enable us to diagnose at an early stage; predict prognosis; track current status, such as therapeutic efficacy or resistance to treatment; and provide optimal and individual treatment strategies for cancer patients, i.e., personalized treatment. The development of liquid biopsy could provide numerous benefits to cancer patients, especially PDAC patients. Recently, liquid biopsy techniques have been gradually moving from laboratory to clinical practice.

II. PERSONAL CONTRIBUTIONS

Chapter 3. Research hypothesis and general objectives

This thesis's working premise is that patient prognosis, treatment response, and PDAC recurrence can be assessed using quantitative analysis of ccfDNA and liquid biopsy detection of tumor-specific mutations.

The general objectives include:

(a) comparing the efficacy of chemotherapy regimens available in our country (FOLFIRINOX, gemcitabine monotherapy and gemcitabine-based regimens) and identifying prognostic factors for overall survival (OS) and progression-free survival (PFS) in a real setting, as well as establishing the recruitment dynamics of patients with PDAC and clinical particularities. For this purpose, the pancreatic tumor database of the Gastroenterology and Hepatology Clinic (Fundeni Clinical Institute, Bucharest) was updated, and a retrospective study including patients diagnosed with metastatic PDAC (mPDAC) between 2015 and 2017 was conducted.

(b) investigating the correlations between the concentration of plasmatic circulating cell-free DNA (ccfDNA) and clinical and tumor characteristics, as well as the prognostic value of the levels of ccfDNA and the neutrophil-leukocyte ratio (NLR) in patients with PDAC—analysis performed through a prospective cohort study that included patients diagnosed with PDAC at all stages between October 2018 and December 2021;

(c) emphasizing the possible value of integrating molecular alterations with inflammatory biomarkers and liquid biopsy (NLR and ccfDNA levels) to improve risk stratification in PDAC—for this purpose, a clinical case was presented, and a narrative review of the literature was performed, highlighting available treatments and their clinical significance

Chapter 4. Real-Life Results of Palliative Chemotherapy in Metastatic Pancreatic Ductal Adenocarcinoma

In a retrospective cohort study, we compared the efficacy of chemotherapy regimens available in our country (FOLFIRINOX vs gemcitabine-based regimens vs gemcitabine monotherapy). We analyzed data from 83 consecutive patients diagnosed with mPDAC between 2015 and 2017 in the Department of Oncology of the Fundeni Clinical Institute (Bucharest, Romania) and compared the median PFS and OS for each chemotherapy line, as well as the OS rates at 6, 12, 18, and 24 months in each. We subsequently used a Cox regression model to assess the relationship between patient characteristics and survival, taking into account factors such as age, sex, tumor location, diabetes mellitus, performance status according to the European Cooperation Group on Oncology (ECOG-PS), therapeutic regimens administered in the first (L1) and second (L2) line of palliative chemotherapy, carbohydrate antigen 19-9 (CA 19-9), and NLR at the time of diagnosis.

SPSS statistical software (IBM SPSS Statistics 20.0) was used for all analyses. According to the Kaplan–Meier method, a survival analysis was conducted, and survival curves were compared using the log-rank test, considering $p < 0.05$ for statistical significance. The optimal cut-off value for baseline NLR was determined using a time-dependent receiver operating characteristic (ROC) analysis, considering 6-month survival as the reference time point.

The multivariate survival analysis was further conducted using the Cox regression model, submitting into analysis variables that were found to be significant ($p < 0.05$) in univariate analysis and adjusting for age and ECOG-PS.

The study's goals were to assess the effectiveness of the chemotherapy regimens that are used in our nation and to pinpoint the variables that affect OS and PFS in the real world.

The results of this study demonstrated that age >70 years, elevated CA 19-9 levels, NLR >4.15, and tumors located in the pancreatic body negatively influenced progression-

free survival under first-line chemotherapy (PFS-L1), while ECOG-PS 0/1 was associated with prolonged PFS-L1. Male gender, ECOG-PS 0/1, and first-line chemotherapy with FFX (vs. Gem) were associated with better OS, while increased NLR was correlated with decreased OS. At the same time, patients with elevated CA 19-9 levels at diagnosis had a shorter OS.

In this study, around one-third of the patients received L2, and regardless of the L2 regimen, those who received FFX first had significantly greater PFS-L2 and OS-L2 than those who did not. There is very limited information available regarding the treatment strategies for pancreatic cancer in Eastern Europe. As far as we are aware, this is the first study comparing the effectiveness of mPDAC palliative chemotherapeutic options currently available in Romania. We believe that our research's conclusions may be helpful in assisting oncologists in making decisions regarding their choice of chemotherapy regimens, given the difficulties they experience in controlling mPDAC.

There are some limitations in this research, as it is a retrospective study performed in a single center with a small number of patients. Additionally, due to the study's retrospective character, no information on the patients' psychosocial or emotional effects could be gathered, although an increasing amount of research suggests a connection between psychological stress and site-specific cancer mortality. To confirm our findings, we think a multicenter, nationwide prospective validation is required.

Chapter 5. Prognostic Value of Circulating Cell-Free DNA Concentration and Neutrophil-to-Lymphocyte Ratio in Patients with Pancreatic Ductal Adenocarcinoma: A Prospective Cohort Study

This prospective observational study was conducted in a tertiary gastroenterology center in Bucharest, Romania, between October 2018 and December 2021. We aimed to investigate the correlations between plasma ccfDNA and clinical and tumor characteristics, as well as the prognostic value of plasma ccfDNA concentration and NLR in patients with PDAC. Therefore, the relationship between ccfDNA levels and age, sex, tumor characteristics, ECOG-PS, tumor stage, metastatic status, or biological parameters (CA 19-9, NLR) was assessed.

Peripheral blood was collected from patients with pancreatic lesions who had been referred to our department for endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) before any invasive procedure or the administration of chemotherapy. Twenty-five mL of peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 4 h of sampling. The DNA isolation was performed using the QIAamp® MinElute® ccfDNA Kit (Qiagen, Hilden, Germany) from plasma obtained after three steps of centrifugation, according to the manufacturer's protocol (Chapter 5.2).

Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 27.0, Armonk, NY, USA: IBM Corp.) and R Software version 4.0.2. All tests were two-sided, considering $p < 0.05$ for statistical significance. Missing data were automatically excluded from the analyses. Continuous variables were reported as median and interquartile range (IQR), and categorical variables as frequency (%). The Mann–Whitney U test and Pearson's correlation coefficient were applied for categorical and continuous variables, respectively, to determine the statistical association between ccfDNA levels and tumor features, ECOG-PS, or biological parameters (CA 19-9, NLR). When more than two groups (such as tumor stage or metastatic status) were being compared, one-way analysis of variance (ANOVA) was used.

For the survival analysis, we dichotomized the concentration of ccfDNA at a cut-off of 25.79 ng/mL using a maximally selected rank statistic via conditional Monte-Carlo (R Software version 4.0.2, maxstat package) [10,11]. This allowed us to classify observations into two groups based on an ordinal predictor variable. A time-dependent receiver operating characteristic (ROC) analysis was performed using 6-month survival as the reference time point in order to determine the optimal cut-off value for baseline NLR (cut-off 3.31, AUC 0.331, $p = 0.009$). The estimation of survival rates was performed with the Kaplan–Meier method, and survival curves were compared using the log-rank test, considering $p < 0.05$ for statistical significance. The multivariate survival analysis was further conducted using the Cox regression model.

The multivariate survival analysis was further conducted using the Cox regression model, submitting into analysis variables that were found to be significant ($p < 0.05$) or near significant in the univariate analysis and adjusting for age and ECOG-PS. Baseline characteristics (age, gender, tumor location, size, stage, metastatic status, ECOG-PS, presence of diabetes mellitus, CA 19-9, NLR), as well as ccfDNA concentration, were analyzed with respect to their prognostic significance.

The combination of NLR with ccfDNA was also analyzed, and positive and negative values were assigned when NLR or ccfDNA were above (positive) or below (negative) the cut-off for prognostic value in OS; we defined three groups: both positive (PP), one positive (PN), and both negative (NN) [12]. Overall, survival was defined as the time from diagnosis to death due to pancreatic cancer-related complications or the end of follow-up. Survival data were censored at the last follow-up. The cut-off date for follow-up was 15 May 2023.

Results

Patients were followed for a median of 7 months (ranging from 1 to 47 months). The Cox regression model was used to assess the relationship between patients' or tumor' characteristics and overall survival. Both univariable and multivariable Cox regression analyses were performed to estimate the prognostic role of ccfDNA levels and NLR on

survival, relative to other clinicopathological parameters. This analysis took into account factors like age, gender, presence of diabetes, tumor location, tumor size, tumor stage, metastatic status, baseline CA 19-9 levels, and NLR (dichotomized < 3.31 and ≥3.31), as well as ccfDNA concentration (both continuous and dichotomized < 25.79 and ≥25.79 ng/mL). The prognostic value of combining NLR with ccfDNA concentration was also tested in the univariate and multivariate analyses, using the classification system described above. Multivariate survival analyses were performed by submitting into analysis variables that were found to be significant ($p < 0.05$) in univariate analysis and by adjusting for age and ECOG-PS, including dichotomized NLR and ccfDNA levels or combination of NLR with ccfDNA concentration.

Our findings indicated a correlation between elevated plasma ccfDNA levels and age over 55 years, venous and arterial invasion, and increased number of metastases.

In univariate analyses, tumor size (HR 1.02, 95% CI 1.01–1.04, $p = 0.004$); metastatic status: M0 vs. oligo-metastatic PDAC (HR 2.09, 95% CI 1.13–3.84, $p = 0.018$) and M0 vs. multi-metastatic PDAC (HR 2.53, 95% CI 1.49–4.29, $p = 0.001$); stage I/II vs. IV (HR 2.08, 95% CI 1.16–3.73, $p = 0.013$); NLR ≥ 3.31 (HR 0.58, 95% CI 0.37–0.91, $p = 0.017$); ccfDNA levels ≥ 25.79 ng/mL (HR 0.46, 95% CI 0.25–0.86, $p = 0.015$) and the combination of NLR with ccfDNA concentration: NN vs. PN (HR 1.62, 95% CI 1.01–2.62, $p = 0.048$) and NN vs. PP (HR 3.21, 95% CI 1.51–6.83, $p = 0.002$), were independent prognostic factors for OS.

Using the Kaplan–Meier method and the log-rank test, we found that an elevated ccfDNA level (≥25.79 ng/mL) was highly associated with shorter OS (4 vs. 8 months; log-rank $p = 0.009$). Analyses of NLR demonstrated that higher values (≥3.31) were associated with lower OS (4 vs. 10 months; log rank $p = 0.011$).

In the multivariate Cox regression model including dichotomized NLR and ccfDNA concentration, the independent prognostic factors of OS were: tumor size (HR 1.02, 95% CI 1.00–1.03, $p = 0.049$), metastatic status: M0 vs. oligo-metastatic PDAC (HR 3.78, 95% CI 1.17–12.19, $p = 0.026$) and M0 vs. multi-metastatic PDAC (HR 5.37, 95% CI 1.33–21.62, $p = 0.018$), and ccfDNA levels ≥ 25.79 ng/mL (HR 0.45, 95% CI 0.21–0.97, $p = 0.041$).

The combination of NLR with ccfDNA levels was also analyzed and we found an improvement in the overall survival (NN vs. PP: 10 vs. 3 months, p = 0.0003 and NN vs. PN: 10 vs. 6 months, p = 0.038). The multivariate analysis, including the combination of ccfDNA levels with NLR, revealed that metastatic status and the combination of ccfDNA levels with NLR were the independent prognostic factors of OS. Metastatic (oligo- or multi-metastatic) PDAC was associated with worse OS compared to non-metastatic disease (M0 vs. oligometastatic PDAC: HR 3.78, 95% CI 1.17–12.21, p = 0.026 and M0 vs. multi-metastatic PDAC: HR 5.73, 95% CI 1.40–23.37, p = 0.015). Moreover, when ccfDNA levels and NLR were combined, patients with NN showed significantly longer OS than patients with PN or PP (NN vs. PN (HR 1.56, 95% CI 0.93–2.63, p = 0.092) and NN vs. PP (HR 2.81, 95% CI 1.11–7.15, p = 0.030)).

In summary, our study supports ccfDNA concentration and NLR as promising tools for the non-invasive prognostic assessment of PDAC patients and demonstrates that the combination of NLR with ccfDNA levels significantly improves prognostic ability and provides accurate survival risk stratification. Subsequent studies should validate this combination as a prognostic indicator in PDAC patients and assess its utility for guiding therapeutic decisions.

Chapter 6. Unexpected long-term survival in resected pancreatic ductal adenocarcinoma harboring KRAS G12D/SMAD4 co-mutation: a case report and mini-review

The potential value of integrating molecular alterations with inflammatory biomarkers and liquid biopsy (NLR and ccfDNA levels) was highlighted by a rare clinical case of incidentally diagnosed PDAC presenting with a KRAS G12D/SMAD4 co-mutation, which achieved prolonged stable disease and overall survival, and sustained by a narrative review of the literature. Through this case, we aimed to demonstrate the prognostic significance of NLR and ccfDNA concentration, highlighting that low values of these biomarkers at the time of diagnosis can have a positive impact on patient prognosis even in the presence of pathogenic mutations.

The co-occurrence of KRAS G12D and SMAD4 mutations defines a high-risk molecular subtype, commonly associated with poor prognosis, enhanced metastatic potential, and limited responsiveness to standard chemotherapy.

This article reports a rare case of incidentally detected PDAC harboring a KRAS G12D/SMAD4 co-mutation that achieved a prolonged period of stable disease and OS and presents a narrative review of the literature, emphasizing available treatments and their clinical significance.

In this chapter, we reported the case of a 71-year-old woman with a medical history of hypertension, prior cholecystectomy for gallstone disease, and a family history of rectal adenocarcinoma who had a screening computed tomography (CT) colonography in June 2019 that showed a 25 mm mass in the pancreatic body. The tumor was invading and stenosing the portal vein confluence without arterial invasion or adenopathy. No abnormality was found upon physical examination, and laboratory tests showed normal values of carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP) levels, and total bilirubin. EUS-FNA of the pancreatic lesion confirmed well-differentiated PDAC.

Genomic DNA (gDNA) and ccfDNA were isolated using the same protocols that we detailed in our prior research [13,14]. The ccfDNA concentration was quantified using a Qubit 3.0 Fluorometer (Life Technologies) and measured in ng/μL, eluted in 25 μL of ultra-pure water, and measured 6.3 ng/mL. The NLR was calculated as the ratio between the peripheral blood neutrophil and lymphocyte counts and was 4.49. Subsequently, targeted amplicon-based next-generation sequencing (NGS) was carried out using the Illumina NextSeq500 platform. NGS analysis revealed a pathogenic mutation in KRAS (G12D), along with alterations in SMAD4, ARID1A, TSC2, TP53, EGFR, and ERBB2 (HER2), identified in FNA or in both FNA and ccfDNA samples.

Following multidisciplinary tumor board evaluation, the disease was classified as resectable PDAC, and surgical resection was recommended. On July 23, 2019, the patient underwent a distal pancreatectomy with splenectomy and lateral resection of the portal vein. Pathological examination confirmed PDAC (pT2N1Mx, lymphovascular invasion positive, perineural invasion positive, stage IIB, AJCC 8th edition) [15], subsequently presenting numerous postoperative complications.

Adjuvant chemotherapy with gemcitabine monotherapy was started in November 2019 due to the patient's low performance status (ECOG 2-3), and it was continued until April 2020, when the restage CT scan showed progressing disease.

Second-line treatment with gemcitabine plus capecitabine was administered from May 2020 to October 2021, achieving stable disease during the initial six cycles. Third-line therapy with nano-liposomal irinotecan (Onivyde®) plus 5-fluorouracil (5-FU) was initiated in December 2021 and continued until August 2022, when disease progression involved the superior mesenteric vein, superior mesenteric artery, and celiac trunk, accompanied by rising CA19-9 (114 U/L). In September 2022, fourth-line therapy with mFOLFOX6 was started following a multidisciplinary tumor board discussion.

Disease stabilization was maintained until April 2023, when clinical deterioration occurred, accompanied by a marked increase in CA19-9 (555 U/L). Imaging revealed progression of liver metastasis and new peritoneal nodules. The patient's condition progressively declined, and she died in November 2023, after a 53-month OS.

The co-occurrence of KRAS G12D and SMAD4 mutations defines a high-risk molecular subtype, commonly associated with poor prognosis, enhanced metastatic potential, and limited responsiveness to standard chemotherapy [16–20].

TP53 mutation, frequently observed in PDAC, adds to the aggressive biology, reflecting a high degree of genomic instability and resistance to apoptosis [17,21–23]. Alterations in ARID1A and TSC2 further suggest epigenetic dysregulation and mTOR pathway activation, respectively, both of which are linked to tumor progression and treatment resistance [24–27].

Most variants detected in this case (except KRAS G12D) were identified at low variant allele frequencies (VAFs), likely reflecting the ultra-deep sequencing strategy used. Despite their low abundance, such mutations may hold significant prognostic and therapeutic relevance, particularly when monitored over time.

In this case, the patient's PFS during the first line of chemotherapy (gemcitabine-based regimens) was 18 months, which is comparable to the ESPAC4 trial [28,29]. Under the second line of chemotherapy (Onivyde® +5-FU), the PFS was 9 months (in contrast to the NAPOLI-1 study, where the median PFS was 6.1 months [30]). Additionally, the patient's tumor marker values were noticeably decreased throughout the second regimen. Notably, treatment was well tolerated despite the patient's postoperative course.

The incidental diagnosis of an early-stage (resectable) PDAC with KRAS G12D and SMAD4 mutations, which experienced multiple postoperative complications and a prolonged period of stable disease under adjuvant chemotherapy, both on the first and second lines of chemotherapy, exemplifies this case's uniqueness. Many clinical trials are currently evaluating the safety and effectiveness of KRAS G12D therapy in solid tumors, including PDAC. Enrollment in a trial investigating KRAS G12D inhibitors should be considered if available. However, early diagnosis and surgery remain the cornerstone of increased OS and PFS, even in the presence of negative molecular markers.

Chapter 7. Conclusions and personal contributions

7.1. Personal contributions

The available information on treatment strategies for pancreatic cancer in Eastern Europe is very limited. In a retrospective cohort study, we comparatively analyzed the efficacy of chemotherapy regimens available in our country (FOLFIRINOX, gemcitabine-based regimens, and gemcitabine monotherapy) and identified prognostic factors of OS and PFS in a real-world setting.

Median PFS and OS for each chemotherapy line were compared, as well as OS rates at 6, 12, 18, and 24 months in each group (subchapter 4.3.2, paragraphs 1 and 2). A Cox regression model was then used to assess the relationship between patient characteristics and survival, taking into account factors such as age, sex, tumor location, diabetes mellitus, ECOG-PS, L1 and L2 therapeutic regimens, CA 19-9, and NLR at diagnosis.

We observed a significant improvement in OS in patients treated with FFX or GB regimens compared to patients receiving Gem (12 months and 14 months, respectively, versus 8 months). In our cohort study, multivariate analysis showed that ECOG-PS 0/1 was associated with prolonged PFS-L1, while age over 70 years, elevated CA 19-9 levels, NLR > 4.15, and tumors located in the pancreatic body negatively influenced PFS-L1.

Longer overall survival was associated with male gender, ECOG-PS 0/1, and first-line chemotherapy with FFX (vs. Gem), while shorter survival was associated with higher NLR. Lower survival was also predicted by higher CA 19-9 levels at diagnosis, although this prediction was not statistically significant (HR 0.41, 95% CI 0.17–1, p = 0.05). In addition, the use of FFX as first-line chemotherapy (vs. Gem) was an independent predictor of PFS-L2 (HR 6.91, 95% CI 1.5–31.9, p = 0.013) and OS-L2 (HR 6.95, 95% CI 1.12–43.07, p = 0.037).

In the analyzed cohort, patients who received FFX as a first-line regimen had the best progression-free survival (9 months). Patients who underwent chemotherapy with FFX or GB had a similar overall survival (14 months and 12 months, respectively).

Our results demonstrate that age over 70 years, increased levels of CA 19-9, NLR > 4.15, and tumors located in the pancreatic body negatively influenced PFS-L1, while ECOG-PS 0/1 was associated with prolonged PFS-L1. None of the following factors was statistically significantly linked with PFS-L1: gender, diabetes, or chemotherapy regimen. Male gender, ECOG-PS 0/1, and L1 with FFX (vs. Gem) were associated with better OS, while increased NLR was correlated with decreased OS. At the same time, patients with elevated CA 19-9 values at diagnosis had lower OS. In this study, around one-third of the patients received L2, and regardless of the L2 regimen, those who received FFX first had significantly greater PFSL2 and OS-L2 than those who did not. As far as we are aware, this is the first study comparing the effectiveness of mPDAC palliative chemotherapeutic options currently available in Romania.

This first study allowed us to establish the recruitment dynamics of PDAC patients included in the PANCNGS P4 project and their clinical characteristics. Based on these data, we conducted the second cohort study.

In the second study, we aimed to investigate the correlations between plasma ccfDNA levels and clinical and tumor characteristics (subchapter 5.2), as well as the prognostic value of plasma ccfDNA concentration and NLR in patients with PDAC (subchapter 5.3.4). In this regard, 82 patients with histopathologically confirmed PDAC were enrolled in a prospective observational study, and plasma DNA isolation was performed using peripheral blood collected before any invasive procedure or chemotherapy administration. The extraction protocol (detailed in subchapter 5.2) led to the successful isolation of ccfDNA. The isolated DNA fragments were 150-200 bp in size, and there was little or no contamination with larger DNA fragments (fragmented DNA from lysed leukocytes) (subchapter 5.2).

In this study, the relationship between ccfDNA levels and age, sex, tumor characteristics, ECOG-PS, tumor stage, metastatic status, or biological parameters (CA 19-9, NLR) was evaluated. Our findings indicated a correlation between elevated plasma

ccfDNA levels and age over 55 years, venous and arterial invasion, and an increased number of metastases. We also demonstrated that the association of NLR with ccfDNA levels significantly improves prognostic power and provides accurate survival risk stratification. We demonstrated that, independent of tumor stage, quantification of ccfDNA levels provides a noninvasive and simple technique for predicting clinical prognosis in patients with PDAC.

To our knowledge, this is the first study conducted in the Romanian population to evaluate the prognostic role of ccfDNA concentration in PDAC patients. Additionally, this is among the first studies to demonstrate the predictive value of systemic inflammation in combination with ccfDNA concentration. Future research is needed to further explore the therapeutic applicability of these findings.

We decided to report a rare case of incidentally diagnosed PDAC with a KRAS G12D/SMAD4 co-mutation that achieved a prolonged period of stable disease and overall survival in order to highlight the potential value of integrating molecular alterations with inflammatory biomarkers and liquid biopsy (NLR and ccfDNA levels). Additionally, we presented a narrative review of the literature, highlighting the available treatments and their clinical significance.

We used this case to illustrate the prognostic significance of NLR and ccfDNA concentration, emphasizing that even in the presence of pathogenic mutations, which are frequently linked to a poor prognosis, low values of these biomarkers at the time of diagnosis can have a positive impact on the patient's prognosis.

7.2. Conclusions

PDAC remains a devastating malignancy with limited options for effective therapy. Improving patient outcomes will depend on multidisciplinary advances in imaging, surgical techniques, radiotherapy, and systemic therapies. Although clinical progress is slow, our understanding of the molecular biology of PDAC and the tumor microenvironment continues to expand and will ultimately inform rational therapeutic approaches that will lead to clinical benefit.

Liquid biopsy is a minimally invasive, efficient, and time-saving method that has gradually attracted the attention of researchers as a valuable alternative to tissue biopsy. Liquid biopsy plays an important role in personalized medicine as a non-invasive strategy for tumor detection based on individual characteristics. Advanced methods, including proteomics, NGS, and ddPCR, are employed to analyze biomarkers for liquid biopsy. Moreover, a variety of new technologies are being developed to exploit the complexity of information obtained from blood samples.

In the first study, by comparing chemotherapy regimens available in Romania, we demonstrated that the use of FFX in first-line chemotherapy was an effective option for patients with ECOG-PS score 0 or 1; patients who received FFX as a first-line regimen had the longest PFS (9 months). Patients who underwent chemotherapy with FFX or GB had a similar OS (14 months and 12 months, respectively). In addition, elevated levels of CA 19-9 and NLR > 4.15 were negative prognostic factors in patients with mPDAC.

In the second study, we analyzed the relationship between plasma ccfDNA levels and clinical and tumor characteristics, as well as the prognostic value of plasma ccfDNA concentration and NLR in patients with PDAC. Our study supports ccfDNA concentration and NLR levels as promising tools for noninvasive prognostic assessment of patients with PDAC and demonstrates that the association of NLR with ccfDNA levels significantly improves prognostic capacity and provides accurate survival risk stratification. Further studies should validate this combination as a prognostic indicator in patients with PDAC and evaluate its utility for guiding therapeutic decisions.

Through the clinical case presented, we wanted to highlight the fact that the initial values of ccfDNA concentration and NLR can positively influence patient survival even in the context of a mutational profile associated with an unfavorable prognosis.

Our study supports ccfDNA-based liquid biopsy markers as promising clinical tools for noninvasive prognosis and monitoring of patients with PDAC.

We believe that the results of our research may be useful in assisting oncologists in making decisions regarding the choice of chemotherapy regimens, given the difficulties they face in controlling PDAC. Identification of predictive biomarkers, refining patient

selection strategies, and exploring novel combination regimens will be crucial to increasing the efficacy of targeted approaches in PDAC.

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2. **Varzaru** B, Iacob RA, Bunduc S, Manea I, Sorop A, Spiridon A, Chelaru R, Croitoru A, Topala M, Becheanu G, Dumbrava M, Dima S, Popescu I, and Gheorghe C. Prognostic Value of Circulating Cell-Free DNA Concentration and Neutrophil-to-Lymphocyte Ratio in Patients with Pancreatic Ductal Adenocarcinoma: A Prospective Cohort Study. Vol. 25, International Journal of Molecular Sciences. 2024. p. 2854. Available at: https://doi.org/10.3390/ijms25052854.
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