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PERSONALIZED MEDICINE IN CANCER: LIQUID BIOPSY-BASED BIOMARKERS

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PERSONALIZED MEDICINE IN CANCER: LIQUID BIOPSY-BASED BIOMARKERS

MEDICINA PERSONALIZADA EN CÁNCER: BIOMARCADORES BASADOS EN BIOPSIA LÍQUIDA

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INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

Dª. Marta del Pilar Toledano Fonseca presenta un trabajo original en el que se ha analizado la utilidad de los biomarcadores basados en biopsia líquida como herramientas pronósticas en pacientes con cáncer metastásico de páncreas o colorrectal. Mediante el uso de metodologías avanzadas se han analizado marcadores basados en el ADN libre circulante y micro-ARNs presentes en el plasma de los pacientes. Los resultados obtenidos apoyan el uso de biomarcadores de biopsia líquida como herramientas clínicas para el pronóstico no invasivo, la monitorización de los pacientes y la predicción de la respuesta a terapia. Parte de los resultados obtenidos en este estudio han sido ya publicados en forma de dos artículos en una revista de reconocido prestigio internacional (*Cancers*) y el resto se encuentran en fase de redacción para su publicación. La tesis doctoral presentada se enmarca dentro de las líneas de investigación de nuestro grupo de investigación.

Finalmente, cabe destacar la excelente formación técnica y científica alcanzada por la doctoranda, con la realización de una estancia en un centro internacional de reconocido prestigio (Institut Curie, París), por lo que se presenta la tesis con Mención Internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 01 de Junio de 2021

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I. Abstract

Liquid biopsy is a non-invasive approach that provides tumour molecular profiling and may assist in the management of cancer patients. Therefore, here we investigated the utility of liquid biopsy-based biomarkers as prognostic tools in metastatic pancreatic ductal adenocarcinoma (PDAC) and metastatic colorectal cancer (CRC) patients. Specifically, circulating cell-free DNA (cfDNA) level and fragmentation were determined in plasma, and BEAMing technique was used for quantitative determination of RAS mutation allele fraction (MAF) in cfDNA. Additionally, a TaqMan real-time PCR assay was used to quantify basal circulating levels of 754 microRNAs (miRNAs) in plasma of metastatic CRC patients treated with anti-angiogenic therapy.

For pancreatic cancer studies, plasma was obtained from 61 metastatic PDAC patients. We found that prognosis was more accurately predicted by RAS mutation detection in plasma than in tissue, and RAS mutation status in plasma was a strong independent prognostic factor for both overall survival (OS) and progression-free survival (PFS). Moreover, RAS MAF in cfDNA was also an independent risk factor for poor OS and was strongly associated with primary tumours in the body/tail of the pancreas and liver metastases. Higher cfDNA levels and fragmentation were also associated with poorer OS and shorter PFS, body/tail tumours, and hepatic metastases, whereas cfDNA fragmentation positively correlated with RAS MAF. Remarkably, the combination of CA19-9 with cfDNA-based biomarkers improved the prognostic stratification of PDAC patients. Furthermore, dynamics of RAS MAF better correlated with patients' outcome than standard CA19-9 marker. Of note, PDAC is characterized by a highly inflammatory microenvironment, and we found that neutrophil-lymphocyte ratio (NLR) was significantly associated with both OS and PFS. Remarkably, NLR was an independent risk factor for poor OS. Moreover, NLR and platelet-lymphocyte ratio (PLR) positively correlated, and combination of both inflammatory markers significantly improved the prognostic stratification of metastatic PDAC patients. Notably, NLR showed a positive correlation with cfDNA levels and RAS MAF and we found that neutrophil activation contributed to cfDNA content in the plasma of metastatic PDAC patients. Finally, a multiparameter prognosis model was designed by combining inflammatory markers, cfDNA-based markers and CA19-9, which performs as a promising tool to predict the prognosis of metastatic PDAC patients.

For the study of cfDNA-based biomarkers in CRC, plasma was obtained from 136 metastatic CRC patients. Likewise, RAS mutation detection in plasma rather than in tissue, more accurately predicted the prognosis of these patients. Moreover, RAS MAF in cfDNA was also associated with poor OS and was strongly related with liver metastases and number of metastatic locations. Higher cfDNA

levels and fragmentation positively correlated and they were also associated with worse prognosis and hepatic metastases. Remarkably, the combination of standard CEA marker with plasma RAS status and cfDNA levels improved the prognostic stratification of patients.

For the miRNA profiling study, plasma was obtained from 98 metastatic CRC patients enrolled in a clinical phase II trial before receiving FOLFIRI plus aflibercept treatment. A distinct profile of circulating miRNAs was found between responder and non-responder patients. Remarkably, most of these miRNAs were found to have predicted target genes that are involved in angiogenic processes. Accordingly, some of these miRNAs entered in predictive models of response to therapy, progression of disease and survival of patients treated with FOLFIRI plus aflibercept. Among these miRNAs, circulating levels of hsa-miR-33b-5p efficiently discriminated between responders and non-responders patients and predicted the risk of disease progression.

In conclusion, our study supports the use of cfDNA-based liquid biopsy biomarkers as clinical tools for the non-invasive prognosis and monitoring of metastatic PDAC patients. Besides, the use of systemic inflammatory markers along with circulating tumour-specific markers greatly improves prognostic power and provides accurate survival risk stratification of these patients. Likewise, the use of both cfDNA-based and miRNA-based liquid biopsy markers may greatly help in the non-invasive prognosis of metastatic CRC patients and in the prediction of their response to anti-angiogenic therapy.

Keywords: biomarkers, cell-free DNA, circulating microRNAs, colorectal cancer, liquid biopsy, pancreatic cancer, prognosis, therapy response.

La biopsia líquida es una técnica no invasiva que proporciona un perfil molecular del tumor y que puede ser de gran ayuda en el manejo de los pacientes con cáncer. Por lo tanto, en este estudio se investigó la utilidad de los biomarcadores basados en biopsia líquida como herramientas pronósticas en pacientes con adenocarcinoma ductal pancreático (PDAC) metastásico y cáncer colorrectal (CRC) metastásico. Concretamente, se estudiaron los niveles y la fragmentación del ADN libre circulante (cfDNA) en plasma, y se usó la técnica BEAMing para cuantificar la fracción alélica mutada (MAF) de RAS en el cfDNA. Además, se usó un ensayo de PCR a tiempo real con sondas TaqMan para cuantificar los niveles circulantes basales de 754 microRNAs (miRNAs) en el plasma de pacientes con CRC metastásico tratados con terapia antiangiogénica.

Para los estudios de cáncer de páncreas, se obtuvo el plasma de 61 pacientes con PDAC metastásico. Encontramos que el pronóstico era más preciso mediante la detección de la mutación de RAS en plasma que en tejido, y que el estado mutacional de RAS en plasma era un fuerte factor pronóstico independiente tanto para la supervivencia global (OS) como para la supervivencia libre de progresión (PFS). Además, la MAF de RAS en el cfDNA también fue un factor de riesgo independiente para una peor OS y estuvo estrechamente asociada con la localización del tumor primario en el cuerpo/cola del páncreas y con las metástasis hepáticas. Los mayores niveles y fragmentación del cfDNA también se asociaron con una peor OS y una PFS más corta, con tumores en el cuerpo/cola del páncreas y con las metástasis hepáticas. Además, los niveles de fragmentación del cfDNA se correlacionaron con la MAF de RAS. De forma notable, la combinación de CA19-9 con los biomarcadores basados en cfDNA mejoró la estratificación pronóstica de los pacientes con PDAC metastásico. Asimismo, la dinámica de la MAF de RAS correlacionó mejor con la supervivencia de los pacientes que el marcador estándar CA19-9. Hay que destacar que el PDAC está caracterizado por un microambiente altamente inflamatorio, y en nuestro estudio encontramos que la ratio neutrófilo-linfocito (NLR) estuvo significativamente asociada tanto con la OS como con la PFS. De hecho, NLR fue un factor de riesgo independiente para una OS pobre. Además, NLR y la ratio plaqueta-linfocito (PLR) estaban correlacionados positivamente, y la combinación de ambos marcadores inflamatorios mejoró significativamente la estratificación pronóstica de los pacientes con PDAC metastásico. De forma notable, NLR mostró una correlación positiva con los niveles de cfDNA y la MAF de RAS y también encontramos que la activación de los neutrófilos contribuyó al contenido de cfDNA en el plasma de los pacientes con PDAC metastásico. Finalmente, se diseñó un modelo multiparamétrico para predecir el pronóstico combinando los marcadores inflamatorios, los marcadores

basados en cfDNA y CA19-9, que constituye una prometedora herramienta para predecir el pronóstico de pacientes con PDAC metastásico.

Para el estudio de biomarcadores basados en cfDNA en CRC, se obtuvo el plasma de 136 pacientes con CRC metastásico. De manera similar, la detección de mutación en RAS en plasma en lugar de en tejido predijo de forma más precisa el pronóstico de estos pacientes. Además, la MAF de RAS en el cfDNA se asoció también con una OS pobre y estuvo estrechamente relacionada con metástasis hepáticas y el número de localizaciones metastásicas. Los mayores niveles y fragmentación del cfDNA correlacionaron positivamente y también se asociaron con un peor pronóstico y con metástasis hepáticas. Es de destacar que la combinación del marcador estándar CEA con el estado mutacional de RAS en plasma y los niveles de cfDNA mejoró la estratificación pronóstica de los pacientes con CRC metastásico.

Para el estudio del perfil de miRNAs, se obtuvo el plasma de 98 pacientes con CRC metastásico incluidos en un ensayo clínico fase II antes de recibir tratamiento con FOLFIRI más aflibercept. Se encontró un perfil diferente de miRNAs circulantes entre pacientes respondedores y no respondedores. Es interesante destacar que la mayoría de estos miRNAs tenían genes diana implicados en procesos angiogénicos. En consecuencia, algunos de estos miRNAs fueron candidatos en modelos de respuesta a terapia, progresión de la enfermedad y supervivencia de pacientes tratados con FOLFIRI más aflibercept. Entre estos miRNAs, los niveles circulantes de hsa-miR-33b-5p discriminaron de forma eficiente entre los pacientes respondedores y no respondedores y predijeron el riesgo de progresión de la enfermedad.

En conclusión, los resultados de nuestro estudio apoyan el uso de biomarcadores de biopsia líquida basados en cfDNA como herramientas clínicas para el pronóstico no invasivo y la monitorización de los pacientes con PDAC metastásico. Además, el uso conjunto de marcadores inflamatorios con marcadores circulantes específicos del tumor mejora de forma notable el poder pronóstico y proporciona una estratificación precisa de estos pacientes en función de la supervivencia. Asimismo, el uso tanto de marcadores basados en cfDNA como basados en miRNAs podría ser de gran ayuda en el pronóstico no invasivo de los pacientes con CRC metastásico y en la predicción de su respuesta a terapia antiangiogénica.

Palabras clave: biomarcadores, ADN libre circulante, microRNAs circulantes, cáncer colorrectal, biopsia líquida, cáncer de páncreas, pronóstico, respuesta a terapia.

II. Introduction

1. Liquid biopsy: the importance of minimally invasive cancer biomarkers

Cancer incidence and mortality are rapidly growing worldwide. According to the Global Cancer Observatory, cancer is expected to rank as the leading cause of death in the world in the 21st century [1]. One of the main challenges in the management of cancer patients is to find clinically relevant biomarkers for diagnosis, prognosis and prediction of disease progression or potential response to therapy [2]. The study of tumour-specific biomarkers and tumour molecular profiles has been evaluated in tissue biopsies. However, this strategy conveys invasive procedures that may be painful and potentially risky to the patient and tumour tissues are not always accessible [3]. Besides, solid biopsies are incompatible with longitudinal monitoring of the evolution of the disease. Finally, the analysis of the initial lesions does not consider spatial and temporal heterogeneity of the tumour [3,4]. Due to these disadvantages in the study of tumour tissue, the search for minimally invasive biomarkers have become one of the central goals of oncology research [5]. In this context, **liquid biopsy** has emerged as a promising approach that can deliver a more complete information regarding both primary tumour and metastatic lesions in real time [4,6]. The term "liquid biopsy" refers to the isolation and analysis of cancer-derived components from peripheral blood or other body fluids, including urine, ascites, or pleural effusions [3–6]. Circulating tumour cells (CTCs), circulating nucleic acids, including circulating cell-free DNA (cfDNA) and microRNAs (miRNAs), and extracellular vesicles (EVs) are the main components of liquid biopsy in cancer.

1.1. Circulating tumour cells

CTCs are tumour cells released into the bloodstream from primary tumour or metastatic sites. Tumour cells in circulation have a short half-life and only a small fraction survive to some adverse conditions such as apoptosis, immune system activity or several biophysical conditions before reaching their final destination [6,7]. CTCs are mainly implicated in the spread of the tumour to distant sites for the establishment of metastases, which are the principal cause of cancer-related deaths [7,8]. The leading hypothesis of how CTCs participate in the metastatic process is that they suffer an epithelial-to-mesenchymal transition to increase their migration and invasion capacities favouring their entry in the bloodstream. When CTCs arrive at distant tissues, they reverse to their epithelial phenotype through a mesenchymal-epithelial transition and they establish the metastasis [9]. These disseminated cells in the metastatic niche may remain dormant until they receive appropriate signals triggering tumour development [6–8]. The isolation and characterization of CTCs are a major challenge due to their low frequency and heterogeneity. Hence, CTCs analysis requires extremely sensitive techniques and a combination of enrichment and detection procedures [6]. Most systems employed in CTCs analyses are based on the expression of epithelial markers such as EpCAM. However, some CTC subsets lacking EpCAM expression may be underestimated in these studies. Therefore, CTCs rarity and heterogeneity are the main limitations to translate these studies into clinical practice. Despite these disadvantages, some reports have related a high number of CTCs with poorer prognosis [10,11].

1.2. Circulating cell-free DNA

The cfDNA comprises double-stranded DNA fragments with lengths that have been related to the size of nucleosomes: 147 bp for nucleosome core and 166bp for nucleosome plus histone linker [12]. Accordingly, most studies describe apoptosis as the main source of cfDNA, but other cell death processes such as necrosis, autophagy, NETosis, or active cellular secretion could also release cfDNA into circulation [13]. Moreover, cfDNA arises in the bloodstream from different cells, tissues and organs, and its level in blood is determined by a balance between DNA release and DNA clearance processes. In some malignancies clearance is insufficient and cfDNA is accumulated [14]. The half-life of cfDNA has been estimated from 15 minutes to several hours and this rapid turnover allows to obtain real-time data on cfDNA dynamics [15]. A number of studies have reported that cancer patients have higher levels of cfDNA and with altered fragmentation profiles in comparison to healthy subjects [16,17]. Besides, patients with advanced or metastatic stage have higher levels of cfDNA than patients with benign lesions or early-stage disease [18]. However, non-malignant pathologies such as inflammation, tissue trauma or diabetes, physiological activities such as exercise or special cases such as pregnancy or transplantation can also increase cfDNA levels [14]. cfDNA originating from tumour tissue is a valuable source of biomarkers for the management of cancer patients. The cfDNA bearing genetic and epigenetic alterations that are characteristics of the tumour is termed circulating-tumour DNA (ctDNA). The analysis of ctDNA requires extremely sensitive techniques because is highly diluted by an overwhelming excess of non-tumour cfDNA molecules [13,18].

1.3. Circulating microRNAs

MicroRNAs are single-stranded non-coding small RNA molecules of 17-25 nucleotides in length and their main function is to regulate target genes at the post-transcriptional level. The biogenesis of miRNAs (**Figure 1**) occurs in the nucleus where primary miRNAs are processed by the RNase Drosha into precursor miRNAs, which are transferred to the cytoplasm by exportin-5. MiRNA maturation is performed by Dicer, an endonuclease that cleaves pre-miRNAs leading to double-stranded mature miRNAs. Finally, only one of the strands is integrated into the RNA induced silencing complex (RISC) where the miRNA and its mRNA target interact. The binding between the miRNA and its mRNA target can be canonical, which means a perfect base pairing, or non-canonical, when sequences are not fully complementary [19,20]. About 60% of interactions are non-canonical and this is the reason why a single miRNA can potentially regulate the expression of numerous mRNAs and one mRNA could be targeted by multiples miRNAs. About 30% of the human genome may be regulated by miRNAs and they are involved in the majority of essential cellular processes [19,21].

Although most of miRNAs are inside the cells a high proportion can be found extracellularly in the body fluids. These miRNAs are usually called circulating miRNAs and they are released into the circulation by two different mechanisms: passively released from apoptotic, necrotic or injured cells, and actively secreted in extracellular vesicles, lipoproteins or RNA-protein complexes. Regardless of where circulating miRNAs come from, they need to be packaged into vesicles or bound to proteins in order to avoid their degradation by RNAses present in body fluids. This protection confers

a great advantage for the use of miRNAs as biomarkers because of their increased stability at room temperature, extreme pH values or after several freeze-thaw cycles. About 90% of miRNAs released into circulation are not associated with EVs and bound to proteins such as argonaute 2 (Ago2), nucleophosmin 1 (NPM 1) or high-density lipoprotein (HDL) [19,22].

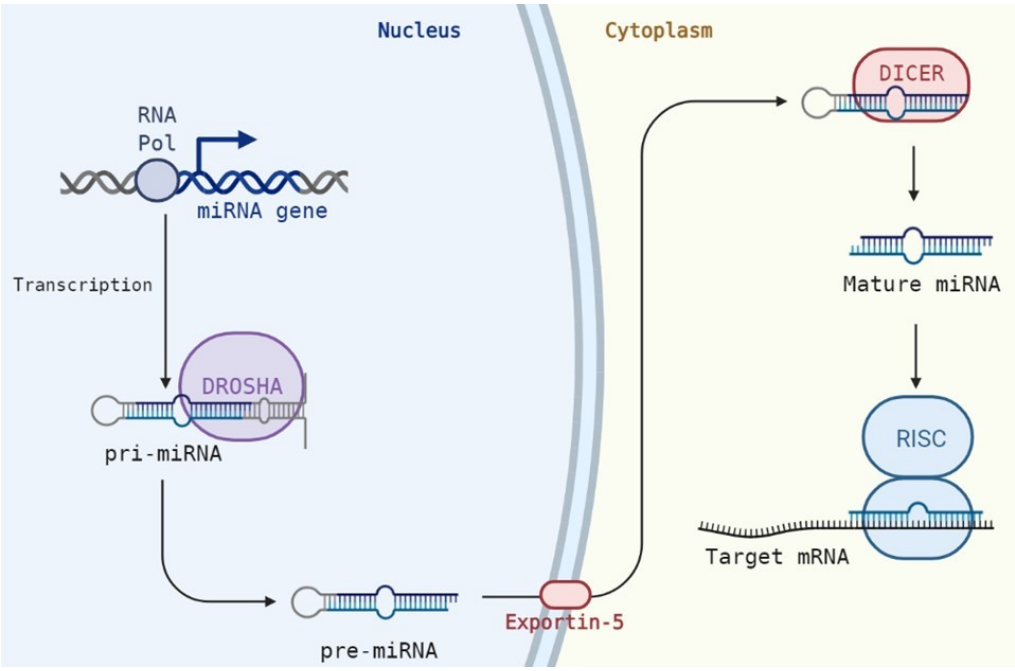

Figure 1. Biogenesis of miRNAs. A schematic diagram of miRNAs biogenesis is shown. After nuclear processing by the RNAse Drosha of the primary miRNA (pri-miRNA), the resulting precursor miRNA (pre-miRNA) is exported into the cytoplasm by the transporter exportin-5. Next, the cytoplasmic ribonuclease Dicer processes the pre-miRNA into the mature miRNA, that is subsequently incorporated into the RNA-induced silencing complex (RISC) for target mRNA recognition. Figure made using BioRender software.

The expression of miRNAs is frequently deregulated in cancer patients compared to healthy individuals. This aberrant miRNA expression is usually associated with molecular and cellular processes involved in human malignancies, including cell proliferation, apoptosis, metastasis, and drug response. The main role of miRNAs in cancer biology is related to the silencing of oncogenes by tumour suppressor miRNAs, and the silencing of tumour suppressors genes by oncogenic miRNAs (oncomiRs*)*. Moreover,

some miRNAs can act both as tumour suppressor miRNAs and oncomiRs [19,20].

1.4. Extracellular vesicles

Extracellular vesicles is the generic term used to define all the heterogeneous cell-derived membranous structures, which are released into the extracellular space [23–25]. EVs have been classified classically into three different groups depending on their size, mechanism of release and composition. Exosomes, which are considered the smallest EVs with approximately 150 nm in diameter, are released into the extracellular space after the fusion of multivesicular bodies with the plasma membrane. On the other hand, microvesicles and apoptotic bodies, which are similar in size (> 200 nm diameter), are both released directly from the plasma membrane of living and dying cells, respectively [26,27].

Exosomes carry several types of cargo molecules, including proteins, nucleic acids and lipids, which are protected from the degradation and can be transferred between cells. The cargo in exosomes is specifically selected through multiple cellular mechanisms. Therefore, exosomes play an important role in intercellular communication and in the maintenance of cellular homeostasis because of their capacity to transfer or eliminate products in a selective manner [26]. Exosomes have been associated with several physiological and pathological processes. In cancer, the release of exosomes is increased and exosomes from cancer cells are capable of modulate the tumour microenvironment [26,27]. Hence, exosomes from cancer cells can be transferred to other cells and stimulate their growth, survival and migration promoting different processes implicated in tumour progression such as angiogenesis, modulation of immune system and pre-

metastatic niche formation. Thus, tumour-exosomes in circulation represent a rich and accessible source of relevant cancer biomarkers [23].

2. Pancreatic cancer

2.1. Epidemiology

Pancreatic cancer is the fourth leading cause of cancer death in Europe in both males and females, with the lowest survival rate of all cancers and responsible for over 95,000 deaths every year [1,28]. While the death rates of the most common cancers have mostly declined over the past decades, the mortality rate of pancreatic cancer remains flat or slightly increases over time [29]. Poor prognosis is associated with diagnosis at advanced stage, due to a lack of early symptoms, detection methods, as well as resistance to therapy [30]. More than 50% of pancreatic cancer cases are diagnosed with metastatic disease and the incidence and mortality correlate with age and sex, being more common in men than in women [31]. There are two type of risk factors that play important roles in the development of pancreatic cancer: non-modifiable risk factors, such as gender, age, diabetes miellitus, family history, genetic factors, chronic pancreatitis and infection with *Helicobacter pylori*; and modifiable risks factors, such as tobacco, alcohol consumption, obesity and exposure to other toxic substances (metals or pesticides) [31,32].

2.2. Genetic classification of pancreatic cancer

Several reports estimate that 10% of pancreatic cancers have a hereditary basis. Predisposition to pancreatic cancer can be inherited in three ways: hereditary tumour predisposition syndromes, which represents 15-20% of familial cases; syndromes related with chronic inflammation of the

pancreas, such as hereditary pancreatitis and cyst fibrosis; and finally, familial pancreatic cancer, which is defined as families with two or more first-degree cases of pancreatic cancer without relation with other hereditary syndromes. In the latter case, the most common are germline mutations in BRCA2 gene [33].

Sporadic pancreatic cancer can be classified into two main types: exocrine tumours, which affect to the exocrine gland of the pancreas and represent 85% of pancreatic cancers, and pancreatic neuroendocrine tumours, which occur in the endocrine tissue of the pancreas and represent less than 5% of all pancreatic cancers [31]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, representing 80-90% of all pancreatic exocrine tumours [34]. PDAC precursor lesions are classified into three types: pancreatic intraepithelial neoplasm (PanIN), which is a microscopic lesion occurring in pancreatic ducts; intraductal papillary mucinous neoplasm (IPMN) which is located in main duct or in one of the lateral branches; and mucinous cystic neoplasm (MCN) which is a pancreatic cystic lesion. PanIN is the most common precursor lesion, and it is estimated that it will take 11-12 years to progress from PanIN to PDAC [32]. PanIN can be classified as low grade PanIN or high degree PanIN and mutations accumulate during the progression from these precursor lesions to PDAC (**Figure 2**). Thus, KRAS mutations are found in low degree PanIN, while CDNK2a, p53 and SMAD4 mutations are additionally found in high degree PanIN [34,35].

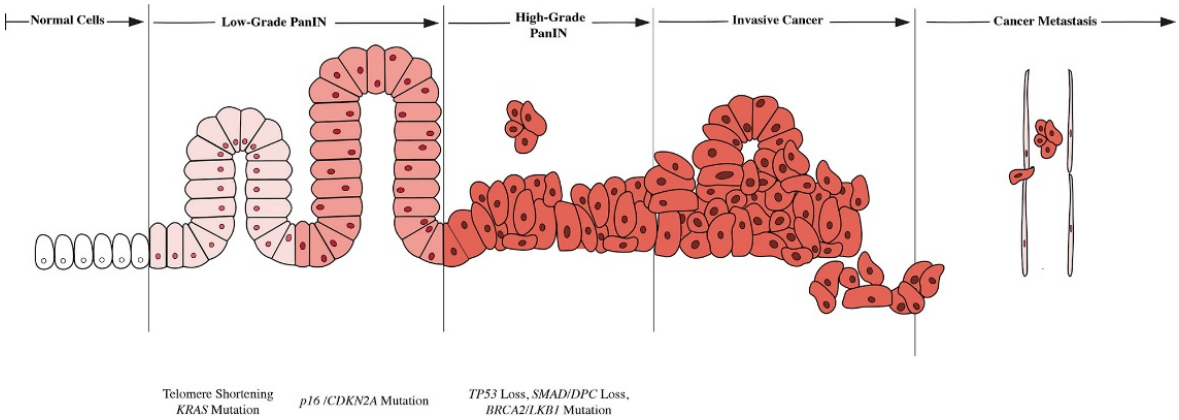

Figure 2. Pancreatic carcinogenesis progression model. Mutations accumulate during the progression from precursor lesions to invasive PDAC. See text for details (Figure from Chhoda et al, 2019).

2.3. Clinical classification of pancreatic tumours

Pancreatic cancer is clinically stratified in different stages through the TNM classification, which takes into account the size and invasiveness of the primary tumour (T), the involvement of regional nodes (N) and the presence of metastasis (M). The combination of these three factors leads to the classification of pancreatic cancer patients in four stages [31,36]:

- Stage I: resectable and pancreas limited tumour, less than 4 cm.
- Stage II: borderline or local dissemination, tumour greater than 4 cm and may disseminate to lymph nodes.
- Stage III: unresectable, tumour invades blood vessels and nervous, but not distant organs.
- Stage IV: unresectable and metastatic, cancer disseminate to distant organs.

PDAC has a worse survival rate than pancreatic neuroendocrine tumours. In general, only 24% or 9% of PDAC patients are alive one or five years, respectively, after diagnosis. According to the tumour stage, the 5-

years survival rate in PDAC is 32%, 12% and 3% for local stages (stage I and II), stage III and stage IV, respectively [31].

2.4. Anatomical location of primary tumour and metastases and prognosis in pancreatic cancer

During human embryogenesis pancreas is developed from the ventral and dorsal pancreatic buds. The head of the pancreas is originated from the ventral bud and the body and tail of the pancreas develops from the dorsal bud. These distinct endodermal origins result in different cellular composition and blood supply and may explain the different behaviour of head and body/tail pancreatic tumours [37]. Pancreatic head tumours have higher incidence than body/tail tumours. Also, head tumours can block biliary ducts causing jaundice and therefore symptoms that may allow an early detection compared with body/tail tumours which are detected at an advanced stage. Body/tail tumours have a more aggressive biology and they are bigger at diagnosis than head tumours. Moreover, body/tail tumours have higher metastasis rates and they are less often resectable than head tumours [37,38]. In regional and metastatic disease (stage III and IV), tumours located in the head of the pancreas have better prognosis. On the contrary, body/tail tumours have a better prognosis in local disease (stage I and II) [38].

Only 15-20% of pancreatic tumours are resectable at the time of diagnosis, whereas 7-10% are borderline, 15-20% are locally advanced and 60-70% are metastatic tumours [39]. Pancreatic cancer first metastasizes to regional lymph nodes, followed by liver and peritoneal metastasis. Although less commonly, metastases are also found in lungs, bones and brain [34]. Patients with multiple metastatic sites and patients with liver or peritoneal

metastasis have a worse prognosis than patients with only one metastatic location and other metastatic locations than liver and peritoneum. Number of metastases varies depending on primary tumour location. Hence, patients with primary tumour located in the tail of the pancreas show more metastatic locations that appear more frequently in peritoneum compared with primary tumours located in the head or the body of the pancreas [40].

2.5. Pancreatic cancer treatments

Although surgery is the only potentially curative treatment, patients with operated tumours still have a poor prognosis, with 5-year survival rates around 20%. Patients with complete resection present local and systemic recurrence in 35-60% and in 80-90% of the cases, respectively [41]. The most common sites of recurrence are liver, lungs, regional lymph nodes and peritoneum. Adjuvant chemotherapy is recommended for all patients with resectable tumours, and the current standard is gemcitabine plus capecitabine, although FOLFIRINOX (folinic acid, fluorouracil, irinotecan and oxaliplatin) treatment can also be used in patients with a good Eastern cooperative oncology group performance status (ECOG 0-1). The role of neoadjuvant chemotherapy in these patients is not clear yet and more clinical trials are warranted [41,42]. Regarding borderline resectable disease, one-third of these tumours are finally resected after neoadjuvant therapy. However, due to the lack of clear consensus data these patients should be treated in a clinical trial context [41]. Most of pancreatic cancer patients are diagnosed with unresectable, locally advanced disease and only a small percentage become eligible for surgery after an excellent chemotherapy response. However, chemotherapy is normally used in these patients to eradicate micrometastases and reduce the size of primary tumour [41,42].

Finally, although metastatic patients present a very low response rate (5-10%) and a short survival, treatments improve quality of life [41]. Therefore, the main objective of systemic chemotherapy in metastatic pancreatic cancer is related with reducing cancer-related symptoms and increasing survival time. Gemcitabine has been the standard treatment, but recently FOLFIRINOX and gemcitabine plus nab-paclitaxel have shown some improvement in the management of pancreatic cancer patients. However, FOLFIRINOX should only be used in patients with a good performance status due to higher toxicity compared to gemcitabine. Gemcitabine plus nab-paclitaxel is used in around 60% of newly diagnosed patients because improves the overall survival (OS) compared to gemcitabine alone but with less toxicity than FOLFIRINOX [41,42].

2.6. RAS mutations in pancreatic cancer

KRAS mutation occurs in approximately 95% of PDAC cases, while mutations in other RAS isoforms such as NRAS and HRAS appear in less than 1% of the cases [43]. KRAS mutations are found in more than 90% of low-grade PanIN and it is considered the initiating genetic event in PDAC tumours, although other mutational hits, such as the activation of p53, CDKN2a or SMAD4, are required for rapid proliferation. Moreover, KRAS is necessary for the progression of PDAC, since oncogenic KRAS activation favours proliferation, migration, invasion, and survival [43,44]. In addition, KRAS is involved in the reprogramming of cellular metabolism, increasing glucose uptake, aerobic glycolysis, and the production of lactate and reactive oxygen species contributing to PDAC growth and metastasis [45,46]. Besides, KRAS mutation in PDAC have been associated with a tumour

inflammatory response due to KRAS-mediated release of immunosuppressor signals leading to tumour growth [46].

Detection of KRAS mutations in tissue or plasma of PDAC patients has been related with poor prognosis regardless of the stage of the tumour [47]. Moreover, several reports have suggested that analysis of KRAS mutation may improves PDAC diagnosis when the cytopathology data are not clear, and KRAS mutation detection may differentiate between chronic pancreatitis and pancreatic tumours at diagnosis. Currently, the determination of KRAS mutation status is not being used for PDAC in clinical practice [46].

2.7. Liquid biopsy in pancreatic cancer

2.7.1. Circulating cell-free DNA in pancreatic cancer

Patients with pancreatic cancer present higher levels of cfDNA than healthy subjects or patients with chronic pancreatitis and neuroendocrine tumours [48,49]. Furthermore, cfDNA concentration is higher in stage IV than in stage I-III disease [50], and higher cfDNA concentration has been related with poorer OS [51]. Fragmentation of cfDNA has been related with tumorigenicity in pancreatic cancer. Thus, shorter fragments of cfDNA have been reported in pancreatic cancer patients than in healthy individuals and there are also shorter fragments in metastatic compared to locally advanced disease. Besides, shorter cfDNA size has been associated with worse OS and progression free survival (PFS) [52].

Because KRAS is a key mutation in PDAC it is considered a good candidate for the analysis of ctDNA. Pancreatic tumours at early stages seem to release less DNA in bloodstream and the study by Bettegowda *et al.*

shows that while more than 90% of patients with metastatic pancreatic cancer had detectable ctDNA, this biomarker was detected in only 48% of patients with localized disease [53,54]. Therefore, the presence of ctDNA is associated with the occurrence of distant metastasis [50]. Moreover, both baseline and after surgery ctDNA positive patients have higher mortality risk [55]. In addition, the detection of KRAS mutations in plasma is associated with a worse prognosis in all stages [53]. Regarding resectable tumours, patients with no detectable ctDNA after surgery have better OS and PFS than those with detectable ctDNA. Moreover, positive ctDNA before surgery is also associated with higher risk of postoperative recurrence [53,56]. Besides, ctDNA may be used for more effective monitoring of recurrence after surgery, and a study have shown better early recurrence detection with ctDNA detection than with imaging alone [57]. In unresectable patients, presence of ctDNA is also a poor prognostic factor for OS and PFS [56]. Furthermore, it is possible to distinguish between chronic pancreatitis and PDAC by combining ctDNA and CA19-9 [53].

3. Colorectal cancer

3.1. Epidemiology

Colorectal cancer (CRC) is the third most incident cancer worldwide and this tumour type comprises 10% of all diagnosed cancers [1]. Higher incidence rates of CRC are observed in developed countries and is the third most common cancer in men and the second most common cancer in women. Moreover, CRC is the second tumour type responsible for cancer-related deaths. There are two types of risk factors for the development of CRC: non-modifiable risk factors, such as sex, age, hereditary mutations or

inflammatory bowel diseases; and modifiable risks factors, such as diet and tobacco or alcohol consumption [58,59].

3.2. Genetic classification of colorectal cancer

CRC is traditionally divided into familial and sporadic. Approximately 20% of CRC patients present a hereditary disease whereas 80% are sporadic cases. There are three main syndromes associated with the familial CRC that represent less than 5% of all cases. Lynch syndrome, also known as hereditary-non-polyposis colorectal cancer, is characterised by germline mutations in mismatch repair genes such as MLH1, MSH2, MSH6 and PMS2, with a penetrance of 80% and occurs in approximately 2.5% of CRC cases. Familial adenomatous polyposis (FAP) is an inherited condition caused by a defect in the adenomatous polyposis coli (*APC*) gene. FAP has an incidence lower than 1% of all CRC and is characterized by the development of many tens to thousands of adenomas and a 100 percent risk of CRC. Finally, the hamartomatous polyposis syndromes involve several rare inherited disorders, which have in common the appearance of polyps in the gastrointestinal tract increasing the risk of CRC [60,61].

Sporadic CRC is caused by the accumulation of somatic gene mutations in colonic epithelial cells in a multi-step process known as the adenoma-carcinoma sequence, which may take more than ten years. Classically, genes contributing to this sequence are classified into three groups: oncogenes, tumour suppressor and mismatch repair genes. The most common altered tumour suppressor genes in CRC are: *APC*, which is related with both familial and sporadic CRC and results in abnormal numbers of chromosomes in daughter cells after mitosis; *DCC (deleted in colorectal carcinoma)*, which seems to prevent tumour invasion and metastasis, and

P53, which is involved in conserving genomic stability by preventing genome mutation. The most frequent altered oncogene in CRC is *KRAS,* which is constitutively activated resulting in the continuous transmission of growth signals to the cell [62]. In sporadic CRC there are three major pathways leading to pathogenesis (**Figure 3**): adenoma-carcinoma pathway, also called chromosomic instability (CIN) or classical pathway, represents approximately 80-85% of all CRC cases and is characterised by a high rate of gains or losses of whole or large portions of chromosomes. In this pathway inactivation of the tumour suppressor *APC* is considered one of the first events, followed by the activation of *RAS* or *PI3K* genes and the inactivation of the tumour suppressor *TP53*. The serrated neoplasia pathway, also called CpG island methylator phenotype (CIMP) pathway, is associated with epigenetic instability silencing tumour suppressor genes due to the hypermethylation of CpG islands in their promoters and leading to inactivation of gene transcription. This pathway is also related with *RAS* and *RAF* mutations and represents 10-20% of all CRC cases. Finally, the microsatellite instability (MSI) pathway (2-7% of all CRC cases) is characterised by a loss of DNA repair mechanisms, especially in repetitive DNA sequence regions (microsatellites), where mutations tend to accumulate leading to inactivation of tumour suppressor genes and promoting tumorigenesis [58,63–65].

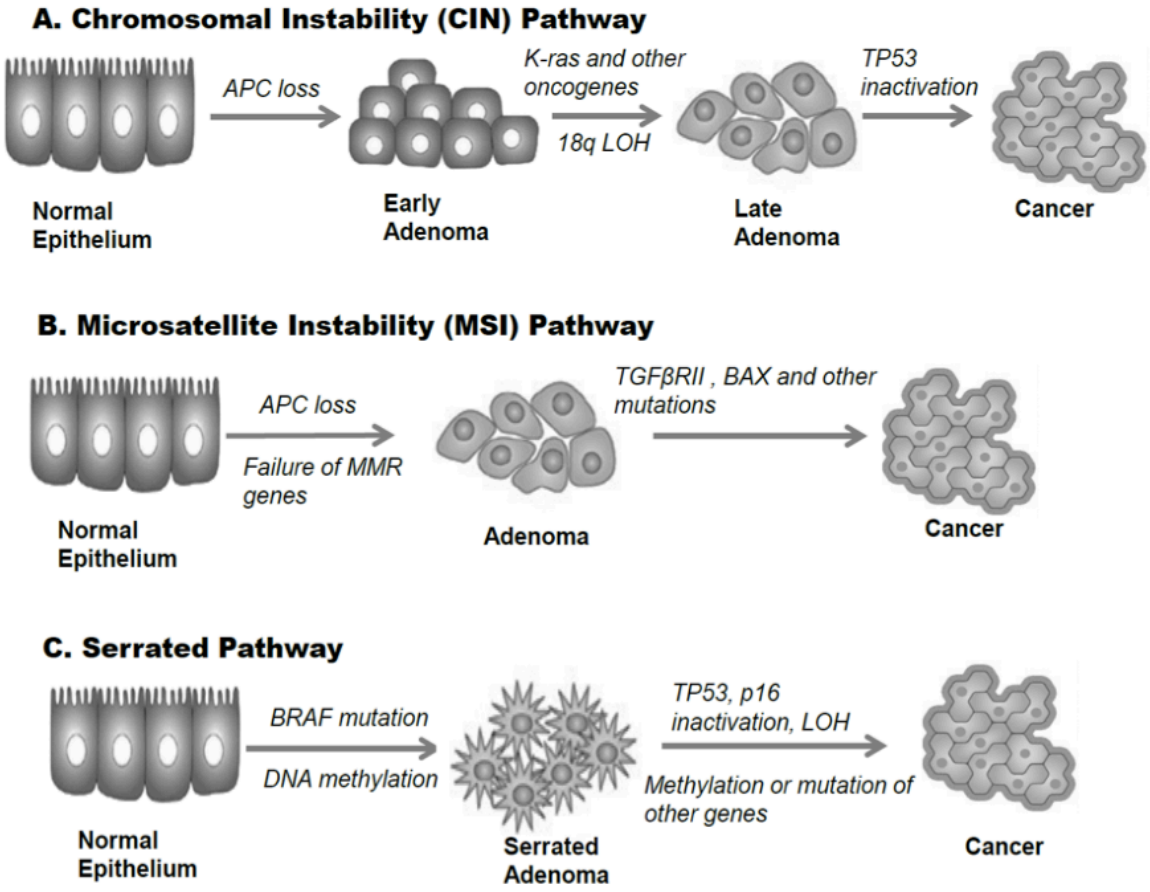

Figure 3. The three major pathways involved in CRC pathogenesis. Accumulation of somatic gene mutations in colonic epithelial cells leads to CRC development. See text for details (Figure from Mundade et al, 2014).

3.3. Clinical classification of colorectal tumours

Tumours of CRC patients at diagnosis are classified into three histological groups: well differentiated (low grade), moderately differentiated (intermediate grade) and poorly differentiated (high grade). Approximately 70% of CRC patients present a moderately differentiate histology, whereas 20% and 10% are well and poorly differentiate tumours, respectively [66]. Moreover, CRC patients are stratified according to the TNM classification [67], which take into account the size and invasiveness of the primary tumour (T), the involvement of regional nodes (N) and the

presence of metastasis (M). The combination of these three factors classifies CRC patients into five stages:

- Stage 0: carcinoma in situ
- Stage I: localized tumours
- Stage II: locally advance tumours, in early stages
- Stage III: locally advance tumour, in advanced stages
- Stage IV: metastatic tumours

The 5-year survival rate is around 90%, when CRC is diagnosed at early stages but drastically decreases to 14% when tumour is diagnosed at advanced stages. The overall 5-year survival rate of CRC patients is 65%, with 90%, 71% and 14% survival rates in patients with localized, regional, and metastatic cancers, respectively [66].

3.4. Anatomical location of primary tumour and metastases and prognosis in colorectal cancer

Right and left colorectal tumours are molecular and histologically different. Right colon includes the appendix, cecum, ascending colon, and transverse colon up to the splenic flexure and left colon includes descending colon, sigmoid colon, and rectum (**Figure 4**). Differences between right and left colon are based on their embryological origins and they do not progress in the same way leading to different survival prognoses [68]. Due to their morphology left tumours are easier to detect in early stage in a colonoscopy than tumours localised in the right side [68,69]. Tumours in the right colon are more frequent in women, and predominantly follow the MSI pathway, although tumours with MSI are more common in stage II (approximately 20% of all CRC cases) than in stage III (approx. 12%) and even less frequent in stage IV (approx. 4%) [70]. Moreover, they are hypermutated tumours

especially in the DNA mismatch repair pathway. However, tumours in the left colon usually follow the CIN pathway. Mutations in *KRAS*, *APC*, *PI3K* and *p53* are more common in this side. Interestingly, several studies show that right tumours in early stages (I and II) have a better survival prognosis than left tumours, however in advance stages (III and IV) left tumours seems to have better survival prognosis than right tumours. In this regard it is interesting to note that the sites where right and left colon metastasize are different. Hence, patients with tumours localized in the right colon often have metastasis in the peritoneum, which commonly have worst prognosis, whereas left colorectal tumours tend to metastasize to the liver and lung [68,69].

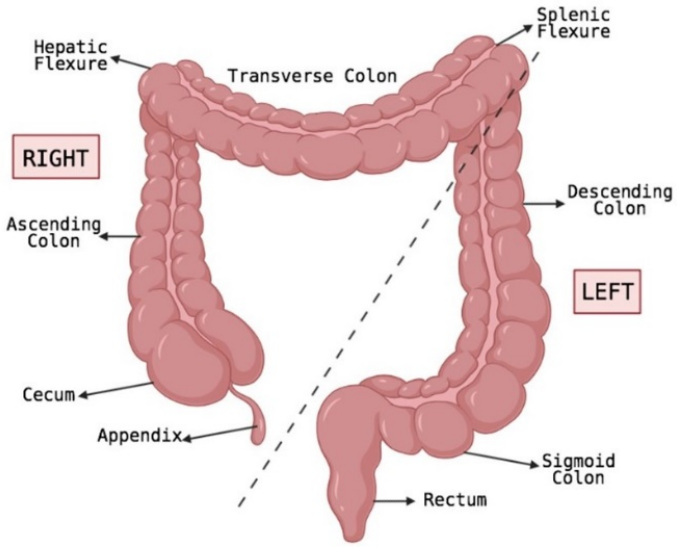

Figure 4. Right and left sides of the colon. Anatomical features defining the left and right sides on colon. Figure made using BioRender software.

Approximately 20-25% of CRC patients are diagnosed with metastatic disease. The main organs where CRC metastasize are liver, lung, peritoneum, distant lymph nodes, bones, and nervous system. Liver metastases appear alone frequently, whereas lung metastases often occur concurrently with liver metastases. However, peritoneum or nervous system

metastases are more frequent in patients with lung metastases [71,72]. Regarding to the relation between site of metastasis and prognosis, patients with lung-only metastases show better outcome than metastases in other sites, followed by patients with liver-only metastases. However, patients with any peritoneum, bone or brain metastases have the worst prognosis [73,74].

3.5. Colorectal cancer treatments

Surgical resection is always the first option for all stages. Therefore, recommendation for patients without metastases or resectable liver or lung metastases is surgery. However, in patients with potentially resectable metastases neoadjuvant therapy is necessary to reduce the size of the primary tumour and metastases before surgery, and in patients with unresectable metastases palliative therapy rather than curative therapy needs to be administrated to reduce symptoms and tumour progression [63].

Currently treatments for CRC include systemic chemotherapy, targeted therapy and immunotherapy. Election of first line of treatment will depend on several tumour and patients-related characteristics. Systemic chemotherapy includes fluoro-pyrimidines (5-fluorouracil (5-FU) and capecitabine), irinotecan and oxaliplatin. In patients with high risk of recurrence adjuvant chemotherapy is important. Approximately 15-50% of CRC patients in stage III experience a recurrence. Adjuvant chemotherapy using fluorouracil reduces the risk of recurrence by 40% and oxaliplatin has demonstrated even greater benefit [75]. Neoadjuvant radiotherapy or chemo-radiotherapy is recommended in patients with rectal tumours and this pre-operative treatment reduces the risk of recurrence in these patients [58].

Targeted therapies have been mainly developed against angiogenic and epidermal growth factor receptor (EGFR) pathways. Most common anti-angiogenic therapies are bevacizumab, which is a monoclonal antibody that targets vascular endothelial growth factor (VEGF)-A, and aflibercept, a recombinant fusion protein that blocks VEGF-A, VEGF-B, and placental growth factors. Anti-EGFR therapies include cetuximab and panitumumab, which are monoclonal antibodies that target EGFR, but only patients with tumours without any RAS mutations are candidates to these EGFR-targeted therapies. Combination of bevacizumab with systemic chemotherapy reduces the mortality in both right and left tumours, whereas anti-EGFR therapies improve the overall survival in patients with left tumours but not in right tumours. Moreover, patients with left tumours have better survival rates with anti-EGFR than with bevacizumab.

Finally, immunotherapy is recommended for those patients whose tumours are MSI-high and have a high mutational load, usually right-sided tumours. Immune checkpoints such as PD1, PDL1 and CTLA-4 are essential in normal condition for autoimmunity, however, tumour cells use these regulators as an immune evasion response. Thus, inhibitors of these immune checkpoints have been developed as cancer immunotherapy [63,68], and two PD1-blocking antibodies, pembrolizumab and nivolumab, have shown efficacy in patients with metastatic MSI-high CRC [76].

3.6. RAS mutations in colorectal cancer

RAS mutations occur in approximately 40-50% of CRC cases. The prognostic value of these mutations in CRC is controversial, although they are diagnostic markers for predicting response to anti-EGFR therapy [77]. KRAS, NRAS and HRAS are the most studied RAS proteins because of

their important role in cancer. Specifically, KRAS mutations are present in approximately 40% of CRC cases, NRAS mutations in about 3-5% of cases and HRAS in 1-2% of cases [78]. Usually, EGFR pathway is activated by the binding of specific ligands to this receptor leading to the activation of multiple downstream genes involved in cell survival, proliferation, metastasis, and angiogenesis. RAS is a downstream GTPase in the EGFR pathway that cycles between its active and inactive form. Mutations in RAS gene increase the intrinsic catalytic rate of GTPase and prevent the hydrolysis from GTP to GDP leading to the accumulation of the activated form of RAS. Therefore, constitutive activation of RAS promotes the survival and proliferation of tumour cells [79]. EGFR-blocking antibodies, such as cetuximab and panitumumab avoid the activation of the receptor, but they do not affect downstream effectors, such as RAS, that can be activated by mutations independently of EGFR activation state. Accordingly, several studies have shown that anti-EGFR therapy efficacy is superior for RAS wild type (WT) tumours compared with RAS-mutated tumours [80] and patients with RAS mutated tumours should not be treated with anti-EGFR therapy [78,81].

Approximately 80% of metastatic CRC patients do not benefit from anti-EGFR therapy suggesting that primary resistance to anti-EGFR treatment is common in CRC. First reports analysing anti-EGFR resistance only had into account mutations in KRAS exon 2, but later, mutations in KRAS exon 3 and 4 and NRAS exons 2, 3 and 4 have been included as diagnostic markers for predicting response to anti-EGFR therapy. Besides, although some patients initially respond to anti-EGFR therapy eventually all of them develop resistance. This progression upon anti-EGFR therapy is known as acquired resistance. The main hypothesis for acquired resistance is

the selection of pre-existing resistant subclones under the pressure of anti-EGFR therapy, rather than the acquisition of novel mutations [78,80].

3.7. Liquid biopsy in colorectal cancer

3.7.1. Circulating cell-free DNA in colorectal cancer

cfDNA can be analysed quantitatively by the measurement of cfDNA concentration, and also qualitatively by the analysis of its genetic alterations, such as RAS mutations in the case of CRC. The concentration of cfDNA is higher in CRC patients than in healthy individuals, as well as the proportion of smaller fragments of cfDNA [82,83]. Moreover, cfDNA has been associated with tumoral stage and higher cfDNA levels have been correlated with worse prognosis and with unresponsiveness to therapy [83,84]. The main problem in cfDNA quantification is the lack of standard protocols, therefore making difficult comparative studies where samples and techniques used are entirely different. The cfDNA may be obtained from plasma or serum with different cfDNA extraction methods and additionally there is no consensus about the technology to quantify cfDNA [84,85].

On the other hand, the ctDNA is characterized by the presence of tumour-specific genetic alterations, thereby providing quantitative information through the mutant allelic fraction (MAF) analysis [85,86]. The presence of ctDNA has been detected in metastatic CRC with 87% of sensitivity decreasing up to 47% in stage I CRC patients. In addition, RAS mutations have shown a high concordance between plasma and tissue analyses [83,84]. The presence of ctDNA in CRC patients is a poor prognosis factor and is also an indicator of minimal residual disease. Thus, ctDNA levels decrease after surgery and increase again in patients during

relapse but not during remission. In fact, patients with detectable ctDNA after surgery usually experience relapse within one year [82,83]. Moreover, ctDNA can be useful to monitor the progression of disease in patients with RAS WT treated with anti-EGFR therapy. The detection of RAS mutations during the treatment could indicate anti-EGFR acquired resistance and therefore, progression can be detected long before clinical manifestation [83,84]. Moreover, the mutated RAS allelic fraction increases at progression but decreases when anti-EGFR therapy is discontinued and replaced with new regimens. This is the reason why some studies have shown the clinical benefit of anti-EGFR rechallenge in those patients which initially responded to anti-EGFR therapy [84].

The only blood-based tumour marker routinely used in clinic for the management of CRC patients is the carcinoembryonic antigen (CEA). However, CEA possesses limited sensitivity and specificity, and its levels are also elevated by other malignancies and even by benign conditions [87]. Several studies have shown an improvement in the prognostic value with the combination of CEA and cfDNA, although cfDNA seems to perform better than CEA alone [84,85]. Moreover, analysis of ctDNA also seems to be a marker with higher sensitivity than CEA in the relapse prediction [83].

3.7.2. Circulating microRNAs in colorectal cancer

Different profiles of circulating miRNAs have been described for the diagnosis, prognosis, and the prediction of response to therapy in CRC. Several studies focused on the diagnosis of CRC have shown distinct circulating levels of some miRNAs in healthy individuals, patients with advanced adenomas and CRC patients, suggesting that the expression of these miRNAs may be altered during early steps of CRC carcinogenesis [88–

90]. Moreover, some miRNAs have been reported to correlate with tumour stage and metastasis, enabling the distinction between early and advanced stages of disease and between metastatic and non-metastatic patients [88,90]. Regarding CRC prognosis, several circulating miRNAs have been associated with overall survival and disease-free survival, and even some of them have been described as independent prognostic factors in metastatic CRC [88,90]. Furthermore, some miRNAs recover their normal circulating levels after surgery, but returned to their altered levels after relapse [90]. Therefore, these miRNAs may be used for sensitive detection of minimal residual disease. Some studies have shown that circulating levels of some miRNAs may also help to differentiate between responder and non-responder patients. For instance, the analysis of circulating miRNAs helped in the prediction of the response of CRC patients to chemotherapy and bevacizumab [88,90]. Finally, other studies have reported that the combination of several miRNAs, rather than individual miRNAs profiles, improves their diagnostic or prediction value. Conceivably, their participation in different tumorigenic processes permits a more comprehensive analysis of the disease [88,90].

III. Aims

General aim

The main objective of this study was to establish the potential clinical utility of liquid biopsy-based biomarkers in the management of advanced pancreatic and colorectal cancer patients.

Specific aims

1. To analyse cell-free DNA-based liquid biopsy biomarkers for the prognosis and monitoring of metastatic pancreatic cancer patients.

2. To explore the combination of biomarkers of systemic inflammation with cell-free DNA-based liquid biopsy biomarkers for prediction of prognosis of metastatic pancreatic cancer patients.

3. To analyse the role of cell-free DNA-based liquid biopsy biomarkers in the prognosis of metastatic colorectal cancer patients.

4. To explore circulating microRNAs as predictors of response to anti-angiogenic therapy in metastatic colorectal cancer patients.

IV. Materials and methods

1. Patients

Three patient cohorts have been analysed in the studies performed in this thesis project. All subjects gave their informed consent for their inclusion in each study. All studies were conducted in accordance with the Declaration of Helsinki, and protocols were approved by the Ethics Committee of Córdoba.

1.1. Cell-free DNA-based liquid biopsy biomarkers in metastatic pancreatic cancer patients

Sixty-one patients diagnosed with metastatic PDAC in the Reina Sofía Hospital (Córdoba, Spain) were enrolled in this study from 2017 to 2019. Eligible patients were 18 years or older with histologically confirmed metastatic PDAC and were not treated by chemotherapy or radiotherapy before the enrolment.

1.2. Cell-free DNA-based liquid biopsy biomarkers in metastatic colorectal cancer patients

One hundred and thirty-six patients diagnosed with metastatic CRC in the Reina Sofía Hospital (Córdoba, Spain) were enrolled in this study from 2015 to 2019. Eligible patients were older than 18 years with histologically confirmed metastatic CRC. These patients were not treated by chemotherapy or radiotherapy before the enrolment.

1.3. MicroRNAs-based liquid biopsy biomarkers in metastatic colorectal cancer patients

Ninety-eight patients from an open-label, single-arm, phase II trial were enrolled in this study from fourteen Spanish hospitals from 2016 to 2017. Eligible patients were 18 years or older with metastatic CRC resistant to or progressive on an oxaliplatin-containing regimen. Patients received aflibercept, followed by FOLFIRI (folinic acid, fluorouracil and irinotecan). Blood samples were drawn within seven days prior to first treatment administration and sent to the Reina Sofia Hospital (Córdoba, Spain) as central laboratory.

2. Blood collection and plasma separation

For liquid biopsy studies (PDAC and CRC patients) plasma was obtained from 20 mL of blood collected using two Streck cell-free DNA BCT^{TM} tubes (Streck Corporate, Omaha, NE, USA). Blood samples were centrifuged at 1600×*g* during 10 min at room temperature to separate plasma, followed by centrifugation at 6000×*g* during 10 min at room temperature to remove any possible remaining cells. Plasma samples were then aliquoted in cryotubes and stored at -80 °C until use. For the miRNA study, 18 ml of blood collected using two EDTA tubes were sent from different hospitals to Reina Sofia Hospital (Córdoba, Spain). Plasma was separated by centrifuging blood at 1650×*g* during 10 min at 4ºC. Plasma samples were then aliquoted in cryotubes and stored at -80°C.

3. Circulating cell-free DNA analysis

cfDNA was extracted from 3 mL of plasma using QIAamp Circulating Nucleic Acid Kit and the QIAvac 24 Plus vacuum system

(Qiagen, Hilden, Germany) following the manufacturer's instructions except for the proteinase K incubation which was 1 hour at 60ºC and the DNA elution step which was repeated twice with 70 µl of buffer AVE from the kit. cfDNA was quantified by fluorimetry using the Quantifluor dsDNA kit in the Quantus fluorometer (Promega, Madison, WI, USA) and cfDNA fragmentation was analysed using the automated electrophoresis system Agilent 2200 TapeStation (Agilent, Santa Clara, CA, USA) with the High Sensitivity D1000 ScreenTape Assay, which allows the analysis of 35 to 1000 bp fragment size DNA. Using the 2200 TapeStation Software we obtained different peaks of intensity, which correlated with the quantity of cfDNA for each fragment size (bp) and selecting all the peaks in the sample the software reported the percentage of the integrated area of each peak. In our analysis we used the percentage of the shortest fragments, therefore, cfDNA fragmentation was defined as the percentage of shortest fragments to total cfDNA.

4. RAS mutation analysis

RAS mutation status in cfDNA and RAS MAF in plasma were determined using BEAMing (*beads, emulsion, amplification and magnetics*) technology with the OncoBEAM™ RAS Assay (Sysmex Inostics GmbH, Hamburg, Germany), which detects 34 mutations in KRAS/NRAS codons 12, 13, 59, 61, 117, and 146 (**Figure 5**). OncoBEAM™ RAS Assay started with a conventional PCR to amplify a locus of interest, which included 7 amplicons covering 12 codons and 34 mutations in KRAS/NRAS genes. Six replicates for each sample and for the negative and positive controls were carried out. When the PCR had finished replicates for each sample were pooled and two dilution steps were then performed. This diluted cfDNA was

added to magnetics beads together with primers for the different codons analysed. An emulsion was generated and most droplets contained all the necessary reagents for a PCR reaction and a single cfDNA molecule (digital PCR). Droplets from the emulsion were broken with different buffers and magnetics beads were captured with a magnet and hybridized with fluorescent probes. Three different probes were used: mutation-specific, which recognized mutations in the different codons analysed, wild type, which recognized sequences without mutations and universal, which was bound to all cfDNA molecules to distinguish magnetics beads with or without PCR product. Then, some washes were made to eliminate excess of probes. Finally, a flow cytometry analysis allowed the detection and quantification of mutated and wild type alleles. Flow cytometry data was analysed using the FCS Express™ software (De Novo Software, Pasadena, CA, USA) and a report for each codon in each sample was provided with the RAS mutational status and the MAF analysed. This approach allows reliable detection of MAF<0.1% in cfDNA [91].

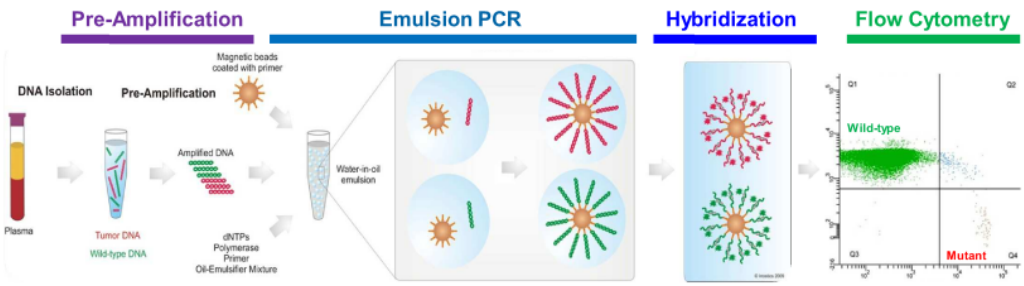

Figure 5. Scheme of BEAMing (*beads, emulsion, amplification and magnetics*) technology and the OncoBEAM™ RAS Assay. In brief, OncoBEAM™ RAS Assay started with a conventional PCR to amplify a locus of interest (pre-amplification), followed by a digital PCR (emulsion PCR) and the hybridization with specific fluorescent probes to detect both RAS mutant and wild type molecules by flow cytometry.

RAS mutations in tumour tissue were analysed by standard-of-care procedures validated in our hospital. Specifically, therascreen KRAS RGQ

PCR Kit (Qiagen) or the Idylla™ platform (Biocartis, Malinas, Belgium) were employed. If the results indicated WT KRAS, testing for NRAS mutations was mandatory.

5. Tumour and inflammation biomarkers

Serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels were measured using a standard radioimmunoassay test in the Clinical Laboratory Department of our hospital. Platelets, neutrophils, and lymphocytes values were also obtained in the Clinical Laboratory Department and neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) were calculated by dividing absolute neutrophil count and platelets count by the absolute lymphocyte count, respectively.

The levels of neutrophil elastase in plasma were quantified using the Human PMN ELISA Kit (Abcam, Cambridge, UK) following the manufacturer's instructions.

6. Circulating microRNA analysis

6. 1. Circulating microRNA extraction from plasma

Circulating miRNAs were extracted from 0.2 ml of plasma using the miRNeasy Serum/Plasma Kit (Qiagen) following manufacturer instructions. During miRNA extraction 5 pM of phosphorylate ath-miR159a from *Arabidopsis thaliana* (Thermo, Waltham, MA, USA) was added (spike-in) as exogenous control for miRNA levels normalization.

6.2. Circulating microRNA analysis in the screening cohort

First, 30 patients were selected as screening cohort for the miRNA-based liquid biopsy study. Circulating levels of 754 miRNAs in these 30

patients were determined using the TaqMan OpenArray MicroRNA Panel with the advanced chemistry. This chemistry enables the use of universal primers instead of miRNA-specific primers due to the modification of mature miRNAs by the addition of a poly (A) tail (3´) and an adaptor (5´). Preparation of miRNAs for the analysis was carried out using TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). In brief, the first steps in this process were a poly (A) tailing reaction and an adaptor ligation reaction. Then, a retrotranscritation reaction was made using universal primers that bind to 3´poly (A) tail and miRNAs were reverse transcribed. The product was pre-amplified (miR-Amp reaction) using universal forward and reverse primers. Protocol was optimized by using 22 cycles of PCR in the pre-amplification reaction. Real-time PCR was carried out in a TaqMan OpenArray Human Advanced microRNA Panel (Applied Biosystems), which is a 3072-well microfluidic plate containing dried TaqMan primers and probes that enables quantification of the miRNA levels of up to 754 miRNAs and controls in three samples. The pre-amplified cDNA product was mixed with TaqMan OpenArray Real-Time PCR Master Mix (Applied Biosystems) and loaded into the array using the OpenArray Accufill System (Applied Biosystems). Real-time PCR was carried out in the QuanStudio 12K Flex Real-Time PCR System (Applied Biosystems).

6.3. Circulating microRNA analysis in the validation cohort

The remaining sixty-eight patients of the miRNA study cohort were selected for the validation assay and using TaqMan Array MicroRNA Cards with the advanced chemistry. Forty-seven miRNAs were selected based on the screening assay. Preparation of miRNAs for the analysis was carried out

using TaqMan Advanced miRNA cDNA Synthesis Kit as described above. In this case, protocol was optimized increasing to 18 cycles for the pre-amplification reaction (miR-Amp reaction). Pre-amplification product was diluted 1:10 with 0.1X TE Buffer, mixed with TaqMan Fast Advanced Master Mix and loaded into the Custom TaqMan Array MicroRNA Cards (Format 48), which contain dry primers and probes for the forty-seven miRNAs selected plus the spike-in control and they can accommodate up to 8 individuals samples. Real-time PCR in the Cards was carried out in the ViiA7 Real-Time PCR System (Applied Biosystem).

7. Circulating microRNA normalization and transformation

Circulating miRNAs levels were normalized using the exogenous control and the mean levels of the three most stable miRNAs using the NormFinder algorithm [92]. Relative circulating levels were calculated using the $2^{-\Delta\Delta Ct}$ method [93]. Those miRNAs not detected in at least one third of the patients were excluded in further analyses. Data of circulating miRNA levels from 98 patients were $\log_2$ transformed for the construction of predictive models of response to treatment, progression-free and overall survival. In those patients in which a circulating miRNA was not detected, a value resulting of dividing by 10 the lowest circulating miRNA level was used.

8. Characterization of microRNAs target genes and biological functions

The Ingenuity Pathway Analysis (IPA) software (Qiagen) was used to perform a network analysis of miRNAs with their mRNA targets in the colorectal metastasis signalling pathway. The online miRNA set enrichment

tool TAM 2.0 [94] was used to further analyse the relation between miRNAs and biological functions and diseases.

9. Statistical analysis

Statistical analyses were performed using SPSS Statistic 22.0.0, GraphPad Prism 6.0 Software, R Software 4.0.0, and MetaboAnalyst web server [95]. Overall survival was calculated from the date of diagnosis to the date of death. Progression-free survival was calculated from the start date of therapy until disease progression or death. The survival rates were estimated using the Kaplan-Meier method and the Log-Rank test was used to identify the prognostic variables. Non-parametric Mann-Whitney and Kruskal-Wallis tests were used to compare two and more than two groups, respectively. Correlation analyses were performed using Pearson's correlation coefficient. Chi-square test was used for testing the association between categorical variables. Multivariate analysis with Cox proportional hazards regression was used to determine independent prognostic factors. Graph data are mean ± standard deviation. All statistical tests were considered significant when $p<0.05$.

The optimal cut-off values were selected with the SurvivalROC package based on the time-dependent ROC curve and were selected by minimalizing the sum of false negative and false positive rates [96]. The cut-off value with prognostic relevance for OS was also tested for prognosis of PFS. When the optimal cut-off from ROC curves did not separate statistically the groups in the Kaplan-Meier analysis, the median, average, first quartile and third quartile were tested in this order. When none of these values were able to separate the groups the Kaplan Scan (KaplanScan) feature in the R2 Genomics Analysis and Visualization Platform

(http://r2.amc.nl) was used to find a cut-off value. The Kaplan scanner separates the samples of a dataset into two groups based on values of variable of interest. In the order of values, it uses every increasing value as a cut-off to create 2 groups and test the p-value in a Log-Rank test.

Selection of variables to build the predictive models was performed using a univariate binary logistic regression. The predictive model was constructed by performing a stepwise regression with bidirectional elimination, which is a combination of forward selection and backward elimination. In each step, a variable is considered for addition to or subtraction from the model using *p* of F-to-enter ≤0.05 and *p* of F-to-remove >0.10. Iteratively adding and removing predictor variables results in the best performing model, that is the model with the lower prediction error. Additionally, the relative risks were calculated.

V. Results

1. Circulating cell-free DNA-based liquid biopsy markers for the non-invasive prognosis and monitoring of metastatic pancreatic cancer

1.1. Clinicopathological characteristics and RAS mutation analysis from plasma and tissue

Sixty-one patients were included in the study between May 2017 and December 2019 (baseline characteristics are summarized in **Table 1**). Patients included in the study ranged in age from 40 to 84 years, with a median of 65 years of age. When patients were stratified according to age, no differences in OS and PFS were found (**Table 2**). Most of patients (78.7%) had a good baseline ECOG performance status and the majority of them (75.4%) received first line gemcitabine-based regimes. ECOG was related with better OS ($p<0.0001$) and PFS ($p=0.0404$) (**Table 2**). Thirty-four patients were men and 27 were women, and there was a trend towards females having better OS than males (157 versus 241 days; $p=0.1038$) and also better PFS (96.5 versus 161.5 days; $p=0.0569$).

All patients had PDAC with distant metastases at diagnosis, and the most frequent site of metastasis was the liver (78.7%). Besides, significant poorer OS and PFS were observed in patients with hepatic lesions compared to patients with metastases affecting other organs (OS 157 versus 339 days; $p=0.0114$; PFS 86 versus 272; $p=0.0048$). On the contrary, there was no significant association between number of metastatic lesions and OS or PFS (**Table 2**). Primary tumour was localized in tail, body and head of pancreas in 27.9%, 41%, and 29.5% of patients, respectively, but primary tumour location was not significantly related with OS or PFS (**Table 2**).

Table 1. **Baseline characteristics of PDAC patients.**

Patient Characteristics		**Number of Cases (*n* = 61)**
Age (median, range)		65, 40-84
Sex	Male	34 (55.7%)
	Female	27 (44.3%)
ECOG	0	17 (27.9%)
	1	31 (50.8%)
	2	10 (16.4%)
	3	3 (4.9%)
1st line treatment	Gemcitabine	3 (4.9%)
	Gemcitabine/nab-paclitaxel	39 (63.9%)
	Gemcitabine/nab-paclitaxel/FOLFOX	4 (6.6%)
	FOLFIRINOX	11 (18%)
	No treatment	4 (6.6%)
Survival	Alive	19 (31.1%)
	Dead	42 (68.9%)
Disease progression	Yes	45 (73.8%)
	No	11 (18%)
	Not valuable (No treatment or surgery)	5 (8.2%)
Tissue availability	Yes	43 (70.5%)
	No (Cytology)	18 (29.5%)
Primary tumour location	Tail	17 (27.9%)
	Body	25 (41%)
	Head	18 (29.5%)
	Body-Tail	1 (1.6%)
Number of metastatic lesions	One location	26 (42.6%)
	More than one location	35 (57.4%)
Metastatic lesion locations	Hepatic lesions	48 (78.7%)
	Non-hepatic lesions	13 (21.3%)
Tissue Biopsy RAS status[1]	RAS mutated	33 (76.7%)
	RAS wild type	10 (23.3%)
Liquid Biopsy RAS status	RAS mutated	47 (77%)
	RAS wild type	14 (23%)

[1] For the analysis of RAS mutational status, primary tumour tissue was available in 70.5% (43/61) of patients.

Table 2. **Overall survival and progression-free survival analysis.**

	OS		**PFS**	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***
Age				
≤65 years	1.00	0.6083	1.00	0.3871
>65 years	1.18 (0.63-2.18)		1.31 (0.71-2.40)	
Gender				
Female	1.00	0.1038	1.00	0.0569
Male	1.67 (0.90-3.10)		1.82 (0.98-3.36)	
ECOG				
0	1.00		1.00	
1	1.83 (0.86-3.89)	<0.0001	1.49 (0.75-2.96)	0.0404
2-3	10.1 (3.18-31.8)		4.56 (1.29-16.1)	
Primary Tumour Location				
Body/Tail	1.00	0.5802	1.00	0.5318
Head	1.22 (0.60-2.49)		1.28 (0.59-2.75)	
Number of Metastasis locations				
1	1.00	0.3380	1.00	0.6304
≥2	1.35 (0.73-2.51)		1.16 (0.63-2.15)	
Metastatic Location				
Non-hepatic	1.00	0.0114	1.00	0.0048
Hepatic	2.40 (1.22-4.74)		2.57 (1.33-4.94)	
RAS mutation status plasma				
WT	1.00	0.0004	1.00	<0.0001
MUT	3.46 (1.74-6.88)		3.84 (1.97-7.47)	
RAS mutation status tissue				
WT	1.00	0.0730	1.00	0.0172
MUT	2.10 (0.93-4.73)		2.50 (1.18-5.30)	
RAS mutation status plasma (with tissue paired samples)				
WT	1.00	0.0068	1.00	0.0019
MUT	3.09 (1.36-7.00)		3.41 (1.57-7.40)	
CA19-9				
≤45500U/ml	1.00	0.0408	1.00	0.0289
>45500 U/ml	2.27 (1.41-4.93)		3.01 (1.12-8.10)	

cfDNA concentration				
≤26.46ng/ml	1.00	0.0057	1.00	0.0107
>26.46ng/ml	2.47 (1.30-4.68)		2.19 (1.20-4.00)	
MAF				
≤0.351%	1.00	0.0261	1.00	0.0556
>0.351%	2.21 (1.10-4.45)		2.02 (0.98-4.13)	
cfDNA fragmentation				
≤38.08%	1.00	0.0297	1.00	0.0101
>38.08%	2.64 (1.10-6.32)		3.14 (1.31-7.49)	
NLR				
≤5.52	1.00	<0.0001	1.00	0.0101
>5.52	5.88 (2.55-13.6)		2.75 (1.27-5.96)	
PLR				
≤90.48	1.00	0.1430	1.00	0.2960
>90.48	1.82 (0.82-4.04)		1.46 (0.72-2.97)	

For the analysis of RAS mutational status, primary tumour tissue was available in 70.5% (43/61) of patients, whereas basal blood samples were obtained from all patients before any treatment. RAS mutation was detected in 76.7% (33/43) of tissue samples and in 77% (47/61) of basal plasma samples. The percentage of patients with RAS mutation was comparable to other cohort studies [97–99]. Mutation in codon 12 of the KRAS gene was found in 93.6% (44/47), and 93.9% (31/33) of plasma and tissue samples, respectively. The overall concordance between plasma and tissue RAS analysis was 79.1%.

1.2. Detection of RAS mutations in cfDNA predicts poor prognosis in metastatic pancreatic cancer patients

The presence of RAS mutations in plasma cfDNA was analysed in 61 metastatic PDAC patients. Detection of RAS mutation in plasma was associated with shorter patient OS (169 versus 372.5 days; p=0.0004; **Table**

2, Figure 6A). Besides, prognosis was more accurately predicted by RAS mutation analysis in cfDNA than by tissue analysis (43 patients, RAS mutation in tissue: p=0.0730; RAS mutation in cfDNA: p=0.0068; **Table 2, Figure 6B-C**).

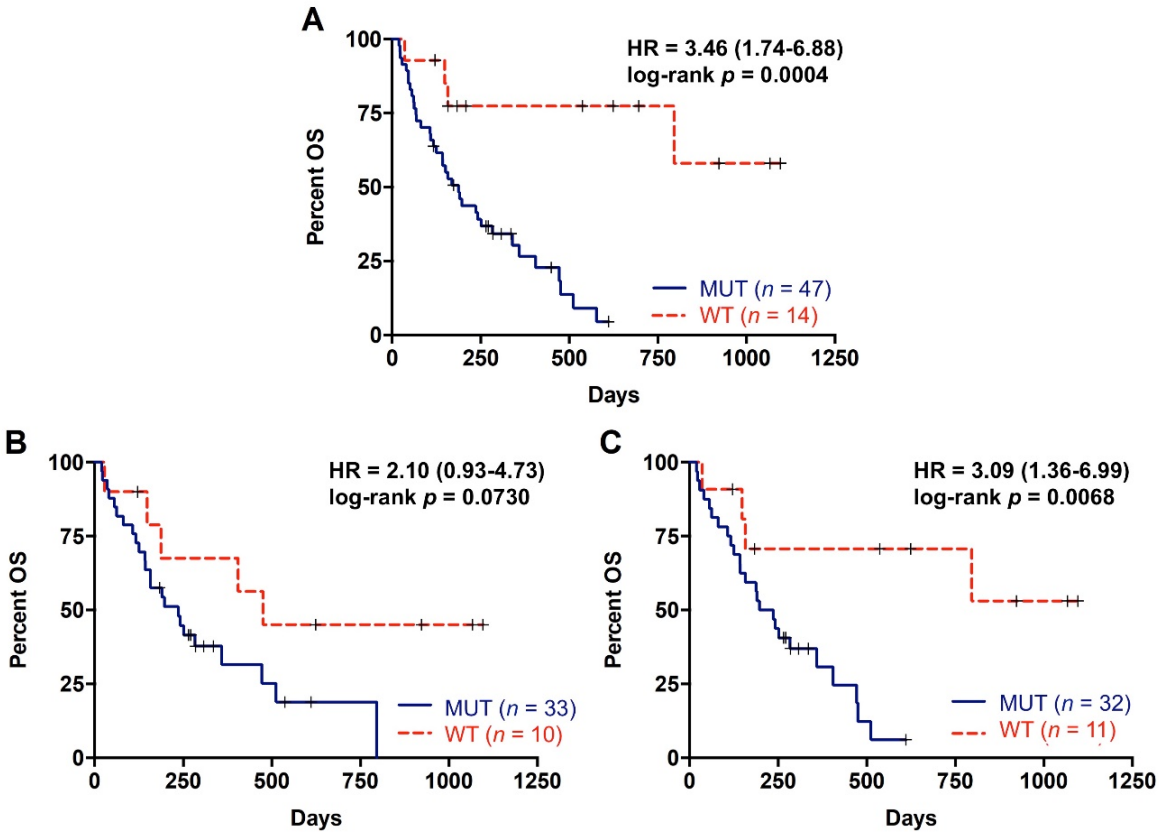

Figure 6. Overall survival rates for patients with metastatic PDAC according to RAS mutation status. (**A**) OS according to RAS mutation status in cfDNA; (**B**) OS according to RAS mutation status in tissue; (**C**) OS according to RAS mutation status in cfDNA of those patients with RAS mutations analysed in tissue.

RAS mutation detection in cfDNA was also a predictive factor of poor PFS in metastatic PDAC patients (93.5 versus 313.5 days; p<0.0001; **Table 2, Figure 7A**). Likewise, tissue analysis was a worse predictive factor of PFS than cfDNA (RAS mutation in tissue: p=0.0172; RAS mutation in cfDNA: p=0.0019; **Table 2, Figure 7B-C**). Finally, multivariate analysis revealed that RAS mutation status in plasma was a strong independent

prognostic factor for both OS (HR 5.692, 95% CI 1.497–21.636; p=0.011) and PFS (HR 8.631, 95% CI 2.311–32.236; p=0.001) (**Table 3**).

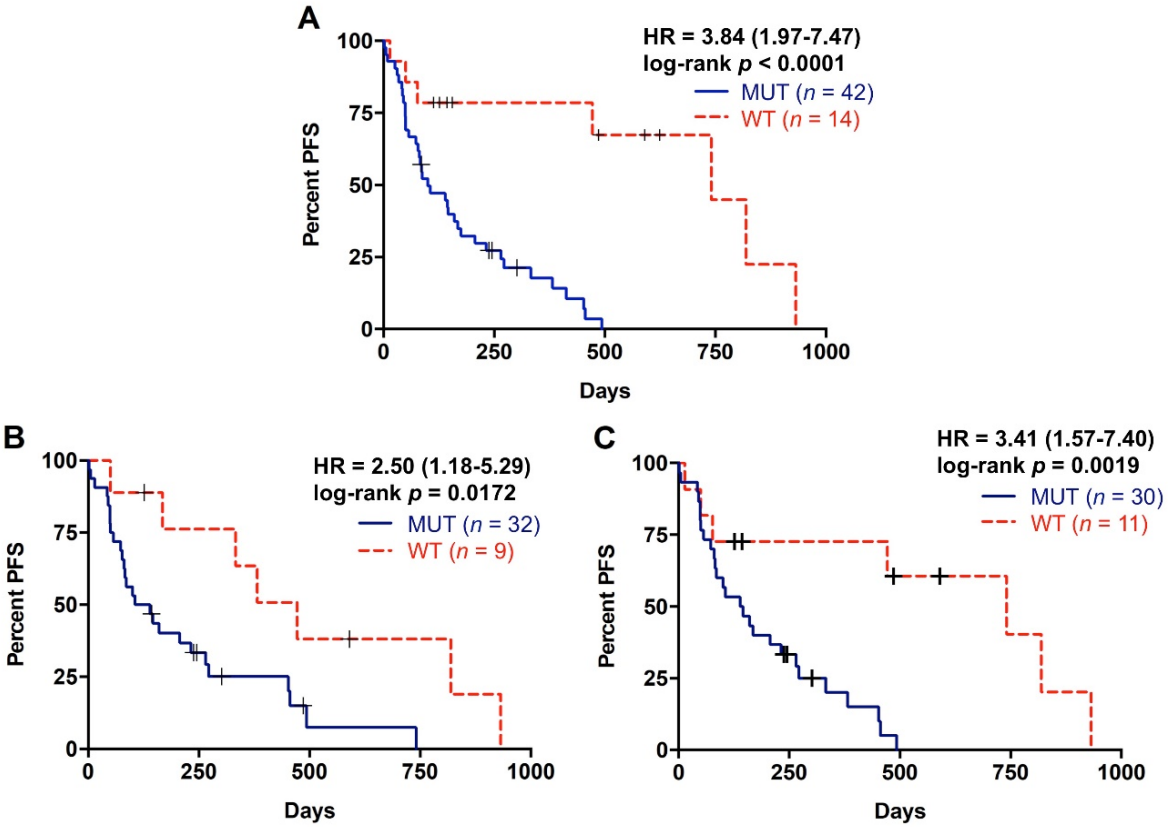

Figure 7. Progression free survival rates for patients with metastatic PDAC according to RAS mutation status. (**A**) PFS according to RAS mutation status in cfDNA; (**B**) PFS according to RAS mutation status in tissue; (**C**) PFS according to RAS mutation status in cfDNA of those patients with RAS mutations analysed in tissue.

Table 3. **Multivariate analysis adjusted for age, sex, ECOG, primary tumour location, metastatic location, number of metastatic locations, CA19-9, RAS mutation status in plasma, MAF, cfDNA concentration and cfDNA fragmentation.**

Variables	OS		PFS	
	HR (95%CI)	*p*	HR (95%CI)	*p*
RAS mutation status plasma				
WT	1.00	0.011	1.00	0.001
MUT	5.69 (1.50-21.64)		8.63 (2.31-32.24)	
MAF	1.07 (1.00-1.14)	0.047	1.04 (0.97-1.10)	0.280

1.3. Higher RAS mutational load in cfDNA is associated with poor prognosis in metastatic pancreatic cancer

For the 47 patients with detectable plasma RAS mutations, the median MAF was 2.92% (range 0.02–29.33%). As shown in **Figure 8** a higher RAS mutational load in cfDNA was associated with poor OS (142 versus 310 days; *p*=0.0261; cut-off value: 0.351%; **Table 2, Figure 8A**) and poor PFS (85 versus 175 days; *p*=0.0556; cut-off-value: 0.351%; **Table 2, Figure 8B**).

Moreover, multivariate analysis identified MAF in cfDNA as an independent risk factor for poor OS (HR 1.070, 95% CI 1.001–1.143; *p*=0.047) (**Table 3**). Although, no differences were observed in the MAF values according to the number of metastatic lesions, higher MAF values were strongly associated with primary tumours located in the body/tail of the pancreas (*p*=0.0281, **Figure 9A**) and liver metastases (*p*=0.0072, **Figure 9B**).

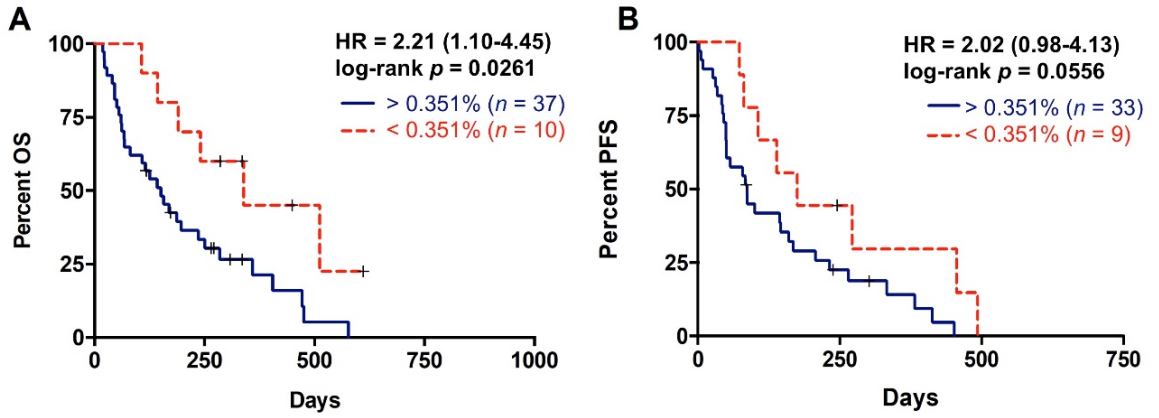

Figure 8. Overall and progression free survival rates according to circulating RAS mutation allele fraction (MAF). (**A**) OS according to circulating MAF (cut-off: 0.351%); (**B**) PFS according to circulating MAF (cut-off: 0.351%).

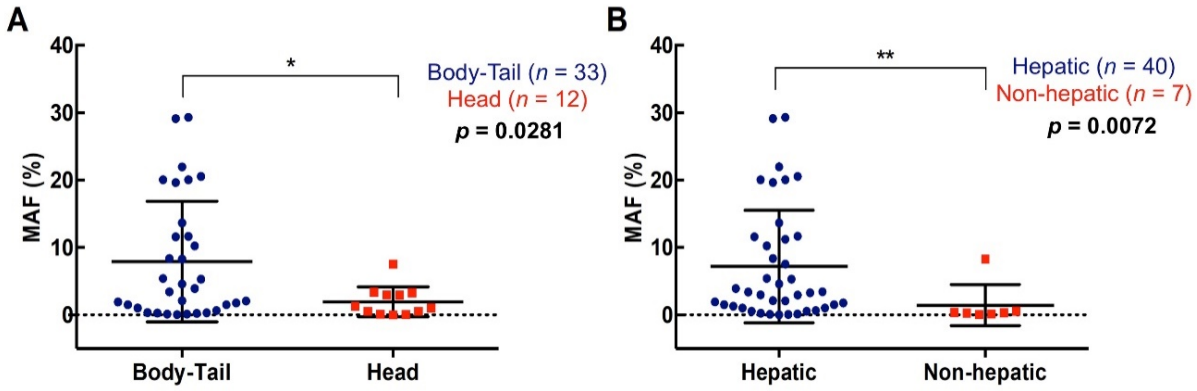

Figure 9. Association of circulating RAS mutation allele fraction (MAF) with primary tumour and metastases location. (**A**) circulating MAF levels in patients with tumour located in the body-tail or the head of the pancreas; (**B**) circulating MAF levels in patients with metastatic lesions in the liver or elsewhere (* $p<0.05$, ** $p<0.01$).

1.4. Higher cfDNA concentration and fragmentation levels are associated with poorer survival in metastatic pancreatic cancer patients

The median cfDNA concentration in plasma of PDAC patients was 33 ng/mL (range 10–700), while the fragment size of plasma cfDNA ranged between 100–1100 bp, with a prominent mode at 135 pb for the shortest fragments detected. In this study cfDNA fragmentation was defined as the percentage of shortest fragments to total cfDNA. As shown in **Figure 10**, cfDNA concentration was significantly higher in those patients in whom

plasma RAS mutations were detected (42.65 versus 24.71 ng/mL, p=0.0057; **Figure 10A**). Although not significant, higher cfDNA fragmentation was observed in RAS mutated patients (**Figure 10B**), and a significant positive correlation between cfDNA fragmentation and RAS MAF was found (r=0.31, p=0.0189).

When metastatic PDAC patients were stratified according to plasma cfDNA concentration, those with higher values (>26.46 ng/mL) had a poorer OS rate (149.5 versus 285 days, p=0.0057; cut-off value: 26.46 ng/mL; **Figure 10C, Table 2**). Also, higher plasma cfDNA concentration was associated with shorter PFS (86.5 versus 149.5 days, p=0.0107, cut-off value: 26.46 ng/mL; **Figure 10D, Table 2**). Similarly, a higher percentage of plasma cfDNA fragmentation in metastatic PDAC patients was significantly associated with a poorer OS (116 versus 197 days, p=0.0297; cut-off value: 38.08%; **Figure 10E, Table 2**) and PFS rates (145 versus 81 days, p=0.0101; cut-off value: 38.08%; **Figure 10F, Table 2**).

Plasma cfDNA concentration or fragmentation were not associated with number of metastatic lesions (p=0.4928; p=0.7735). However, there was an association between cfDNA fragmentation and primary tumour located in body/tail compared to head of the pancreas (15.27 versus 9.27%, p=0.0401) (**Figure 11A**) and a trend towards higher cfDNA concentration in the plasma of metastatic PDAC patients with body/tail tumours (36.17 ng/mL) compared with those with tumours in the head of the pancreas (26.23 ng/mL, p=0.0691) (**Figure 11B**). Also, patients with hepatic metastasis displayed higher cfDNA levels in plasma (38.10 ng/mL), when compared with those patients with other metastatic locations (28.93 ng/mL, p=0.0547) (**Figure 11D**). Similarly, a trend towards higher cfDNA fragmentation was

observed in patients with metastatic lesions in the liver (12.165%), compared with those with metastases elsewhere (10.655%, p=0.3257) (**Figure 11C**).

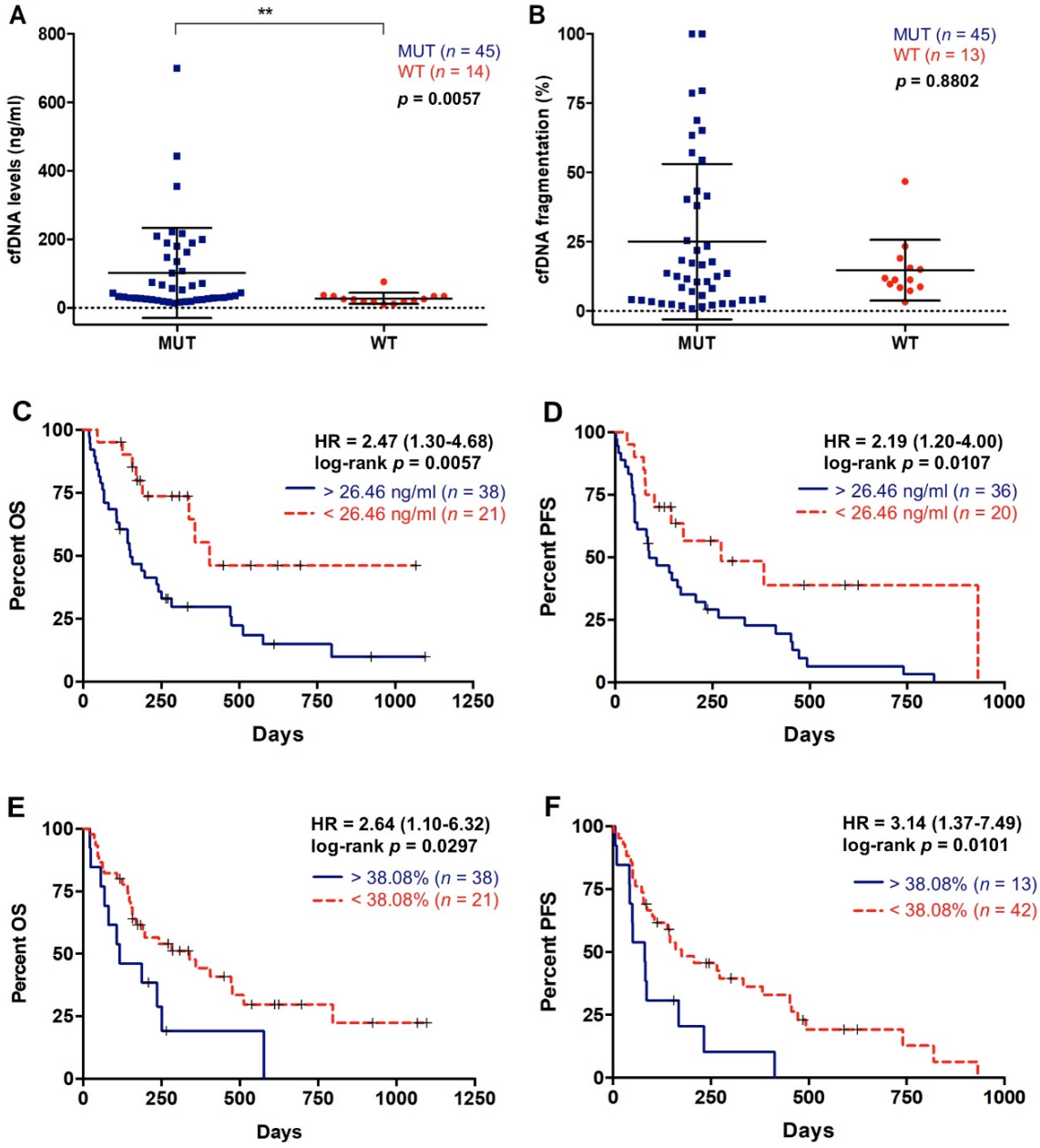

Figure 10. cfDNA concentration and fragmentation in metastatic PDAC patients. (**A**) cfDNA levels and (**B**) fragmentation according to RAS mutational status; (**C**) OS according to cfDNA levels (cut-off: 26.46 ng/mL); (**D**) PFS according to cfDNA levels (cut-off: 26.46 ng/mL); (**E**) OS according to cfDNA fragmentation (cut-off: 38.08%); (**F**) PFS according to cfDNA fragmentation (cut-off: 38.08%). (** p<0.01).

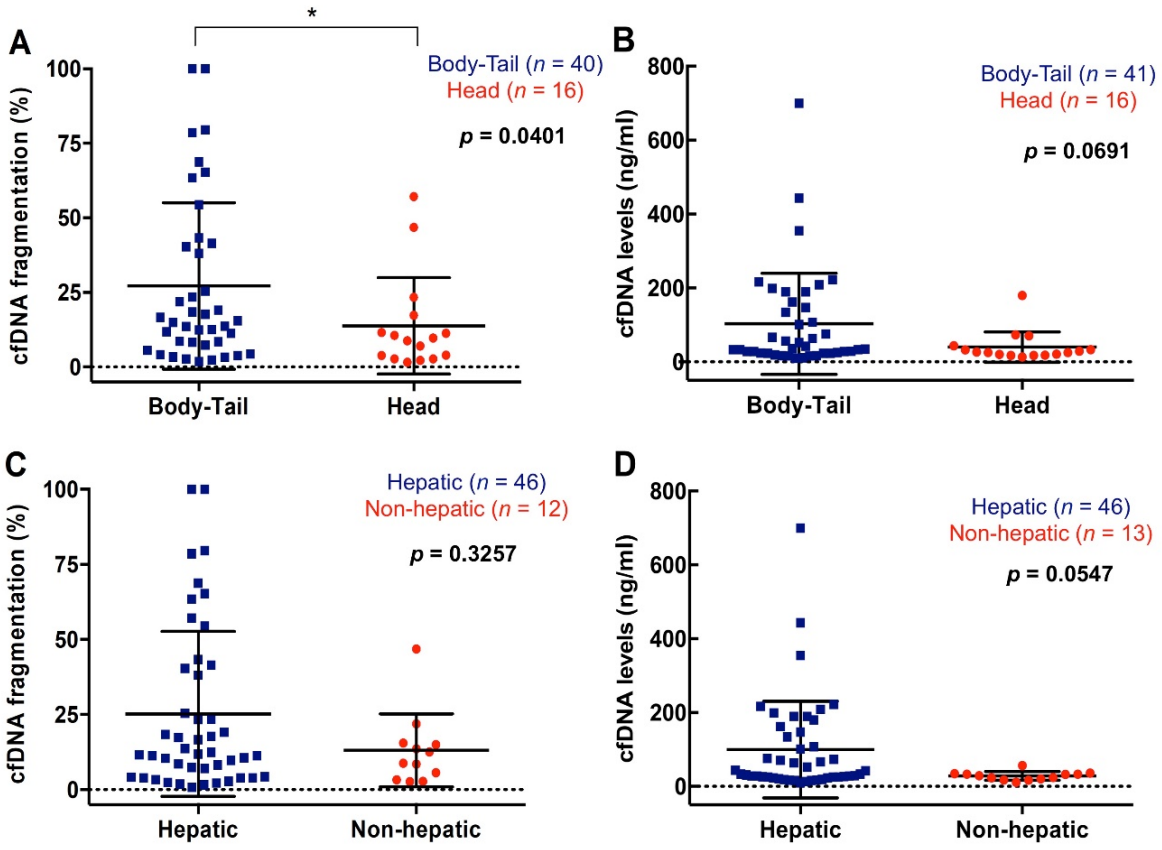

Figure 11. Association between cfDNA concentration and fragmentation and primary tumour and metastasis location. (**A**) cfDNA fragmentation; and (**B**) cfDNA levels according to primary tumour location; (**C**) cfDNA fragmentation; and (**D**) cfDNA levels according to metastatic location (* $p<0.05$).

1.5. Multiparameter liquid biopsy refines prognostic stratification of metastatic pancreatic cancer patients

In our cohort, CA19-9 demonstrated some prognostic value, with higher baseline levels associated with poorer OS and PFS rates (OS 125 versus 202.5 days, p=0.0408; cut-off value: 45,500 U/mL; PFS 72 versus 143 days, p=0.0289; cut-off value: 45,500 U/mL; **Table 2**). No association was found between CA19-9 levels and RAS mutation status (p=0.2909), primary tumour location (p=0.5053), number of metastasis (p=0.4723), location of metastatic lesions (p=0.4908), MAF (p=0.1642), cfDNA levels (p=0.7692) or cfDNA fragmentation (p=0.2769).

Remarkably, the combination of CA19-9 with liquid biopsy improved the prognostic stratification of metastatic PDAC patients. A scoring system was applied by combining CA19-9 with MAF, cfDNA concentration and cfDNA fragmentation. Positive or negative values were assigned depending on whether the corresponding marker was above (positive) or below (negative) the cut-off with prognostic value in OS. Thus, score 0 was defined as negative for all markers; score 1 was defined as positive for 1 marker; and score 2 was defined as positive for 2, 3 or 4 markers. As shown in **Figure 12**, those patients with score 2 displayed poorer survival outcomes in comparison with those patients with score 0 and score 1 in Kaplan-Meier analysis (p=0.0002, and p=0.0072, respectively).

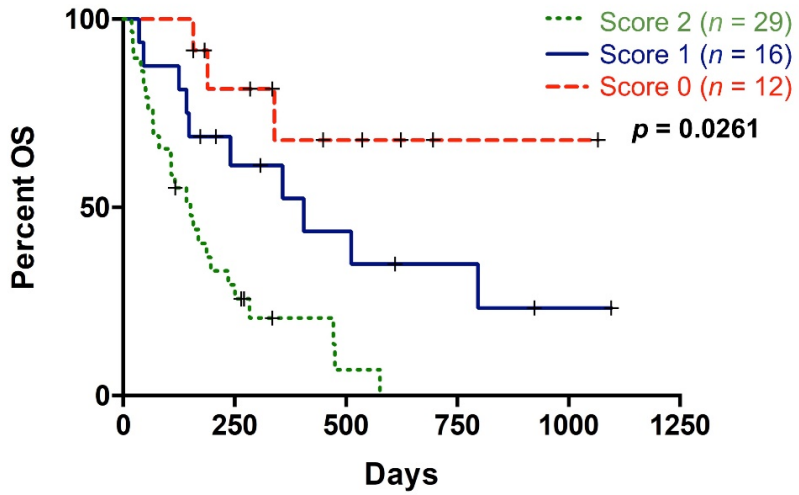

Figure 12. Multi-parametric analysis combining CA19-9 and cfDNA-based liquid biopsy markers (MAF, cfDNA concentration and cfDNA fragmentation). Score 0: no positive markers; Score 1: One positive marker; Score 2: More than one positive markers.

1.6. RAS mutational load in cfDNA enables monitoring of disease progression and response to therapy in metastatic pancreatic cancer patients

Due to the limitations in CA19-9 as a reliable marker of pancreatic cancer, the utility of circulating MAF was compared to CA19-9 in

monitoring disease progression and response to therapy in metastatic PDAC patients. No RAS mutation was detected in blood at baseline in two of the seven monitored patients, but it was detected in disease progression. In patient 1, KRAS codon 12 mutation was found in tissue but not in blood at baseline. Eventually, a novel NRAS mutation was detected during stable disease and a circulating KRAS codon 12 mutation was detected later in blood, along with both elevation of CA19-9 levels and disease progression revealed by radiological criteria and followed by rapid deterioration and death (**Figure 13A**). In patient 2, no RAS mutation was detected at baseline in either tissue or blood, but a KRAS codon 12 mutation was detected later in blood at progression of the disease (**Figure 13B**).

In the three patients (3, 4 and 5) in whom RAS mutation was detected at baseline in blood, circulating MAF dropped following treatment and concurring with lower CA19-9 levels and partial response (PR) to therapy (**Figure 13C–E**). In patient 3, circulating KRAS mutation level markedly declined at PR and rose again at disease progression, along with the detection of a novel circulating NRAS mutation (**Figure 13C**). In patient 4, KRAS mutation remained undetectable in blood, while CA9-19 levels were low and the disease was stable (SD), but unlike CA19-9, MAF was augmented again at the progression of disease (**Figure 13D**). In patient 5, circulating KRAS mutation dropped to undetectable levels in the stable disease. Despite standard criteria and CA19-9 levels in the following monitoring suggested stable disease, KRAS mutation was detected again in plasma anticipating disease progression (**Figure 13E**).

Finally, in patients 6 and 7, circulating RAS mutation levels increased during treatment, compared to baseline levels (**Figure 13F-G**).

Notably, the increase in circulating MAF was associated with a very short survival period (5 months since diagnosis) in these patients.

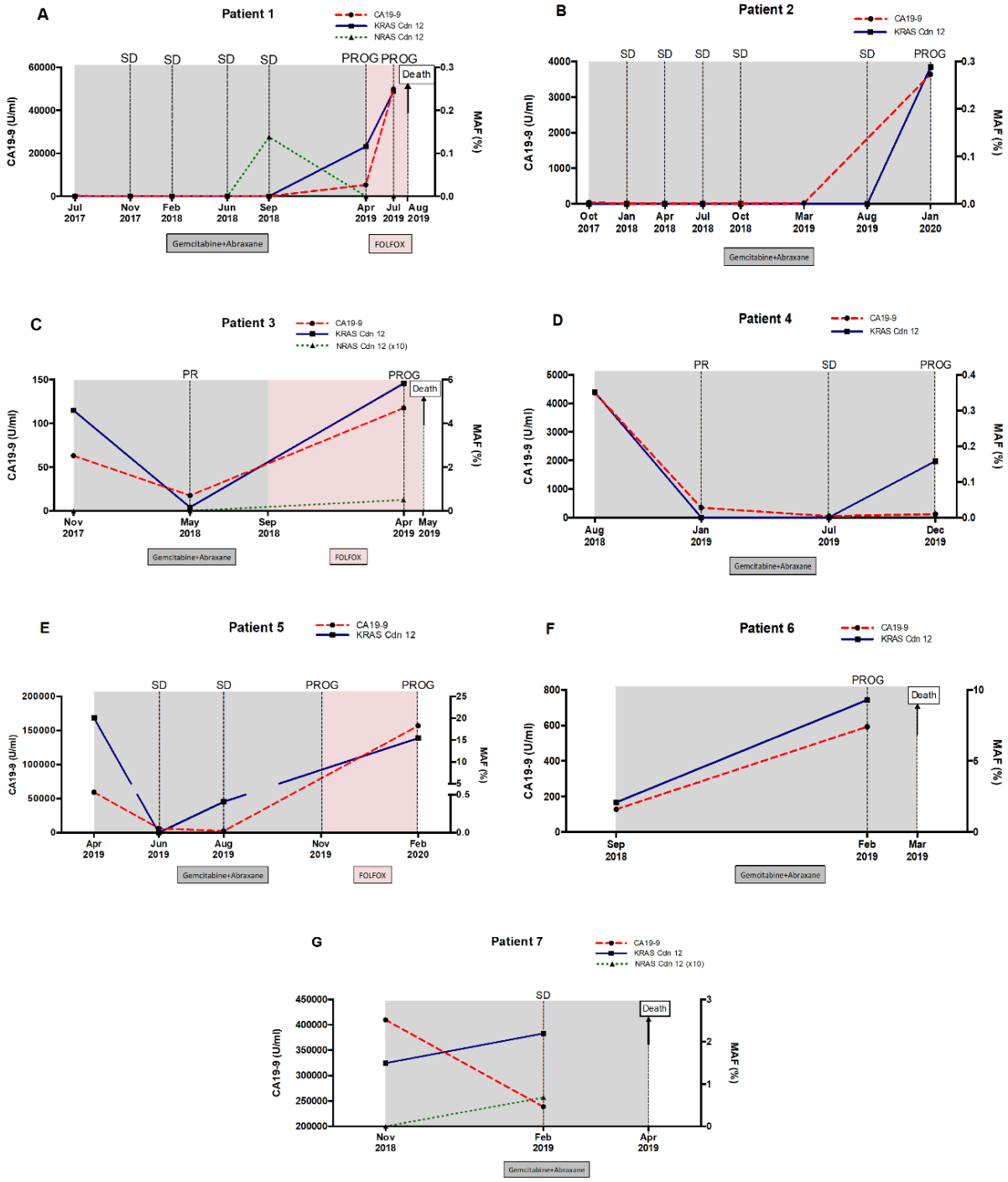

Figure 13. Circulating RAS mutation allele fraction (MAF) enables monitoring of disease progression and response to therapy in metastatic PDAC patients. Circulating MAF (%) was compared to CA19-9 (U/mL), in monitoring response to therapy and disease progression in 7 (**A**–**G**) metastatic PDAC patients.

As a whole, the above results suggest that the dynamics of circulating RAS mutation may better correlate with patients' outcome and survival compared with standard CA19-9 marker. Accordingly, a significant correlation was found between the increase in MAF (r=−0.65, p=0.02), but not in CA19-9 (r=0.09, p=0.78) and survival time (**Figure 14**). Hence, higher increases in circulating RAS mutation during patient monitoring predicted a shorter survival time.

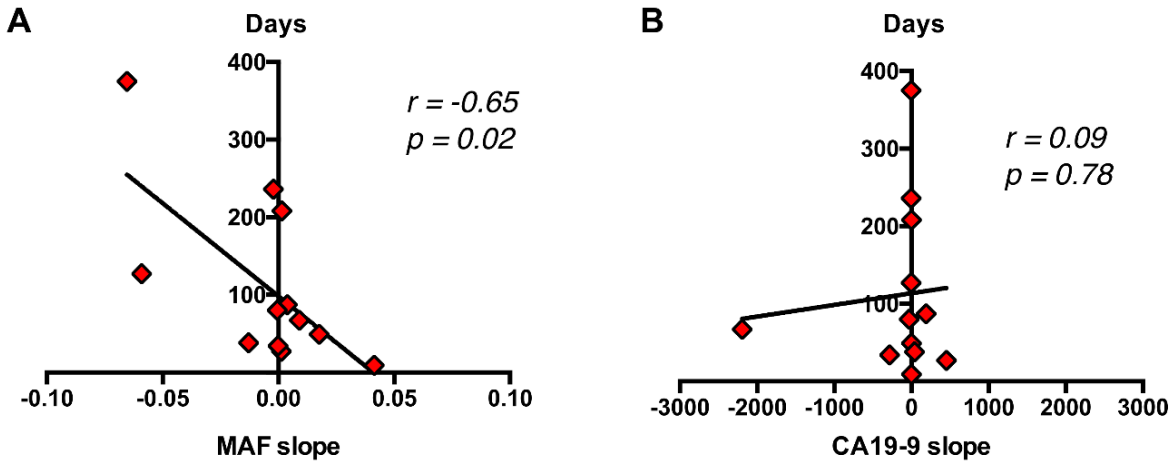

Figure 14. Correlation of dynamics of circulating RAS MAF and CA19-9 with patient's outcome and survival. A significant correlation was found between the increase in MAF (**A**), but not in CA19-9 (**B**) and survival time.

2. The combination of neutrophil–lymphocyte ratio and platelet-lymphocyte ratio with liquid biopsy biomarkers improves prognosis prediction in metastatic pancreatic cancer

2.1. Clinicopathological characteristics of patients

Neutrophil, lymphocyte and platelet count were available for 58 of 61 patients previously described (**Table 1**). Therefore, NLR and PLR were calculated in 58 metastatic PDAC patients. However, one patient with ultra-high platelets count was excluded from the PLR analysis.

2.2. NLR and PLR are prognostic markers in metastatic pancreatic cancer patients

The median NLR in plasma of metastatic PDAC patients was 3.94 (range 0.38-18.8) and the median PLR was 176.07 (range 43.59-492.86). There was a significant association of high NLR with male gender (p=0.0294), while no relation was found between PLR and gender (p=0.2591) (**Figure 15A-B**). On the other hand, although no relation was found between NLR and age (p=0.4891), patients older than 60 years showed a significant lower PLR than those younger (153.39 versus 236.47; p=0.0076) (**Figure 15C-D**). Besides, as shown in **Figure 15E-F**, higher NLR, but not PLR, were associated with worse ECOG (ECOG 2-3) (NLR: p=0.0018; PLR: p=0.6318).

NLR was not significantly associated with primary tumour location (p=0.7859) or number of metastases (p=0.2859), although NLR showed a trend towards higher values in patients with metastatic lesions located in the liver compared with patients with metastases affecting other organs (p=0.1551) (**Figure 16A**). Contrarily, PLR was not associated with metastatic location (p=0.7558) or the number of metastases (p=0.7653), but patients with primary tumour located in the head of pancreas showed higher PLR compared with tumours in the body-tail location (266.2 versus 149.16; p=0.0245) (**Figure 16B**). Patients with higher NLR (>5.52) had significant poorer OS (108 versus 335 days; p<0.0001) and PFS (85 versus 232 days; p=0.0101) rates (**Figure 16C-D**). Moreover, multivariate analysis revealed that NLR was an independent prognostic factor for OS (HR 2.466, 95% CI 1.246-4.880; p=0.010) (**Table 4**). Also, although not significant, patients with higher PLR (>90.48) showed a trend towards poorer OS (236 versus

399 days; p=0.1430) and PFS (145 versus 337 days; p=0.2960) (**Figure 16E-F**).

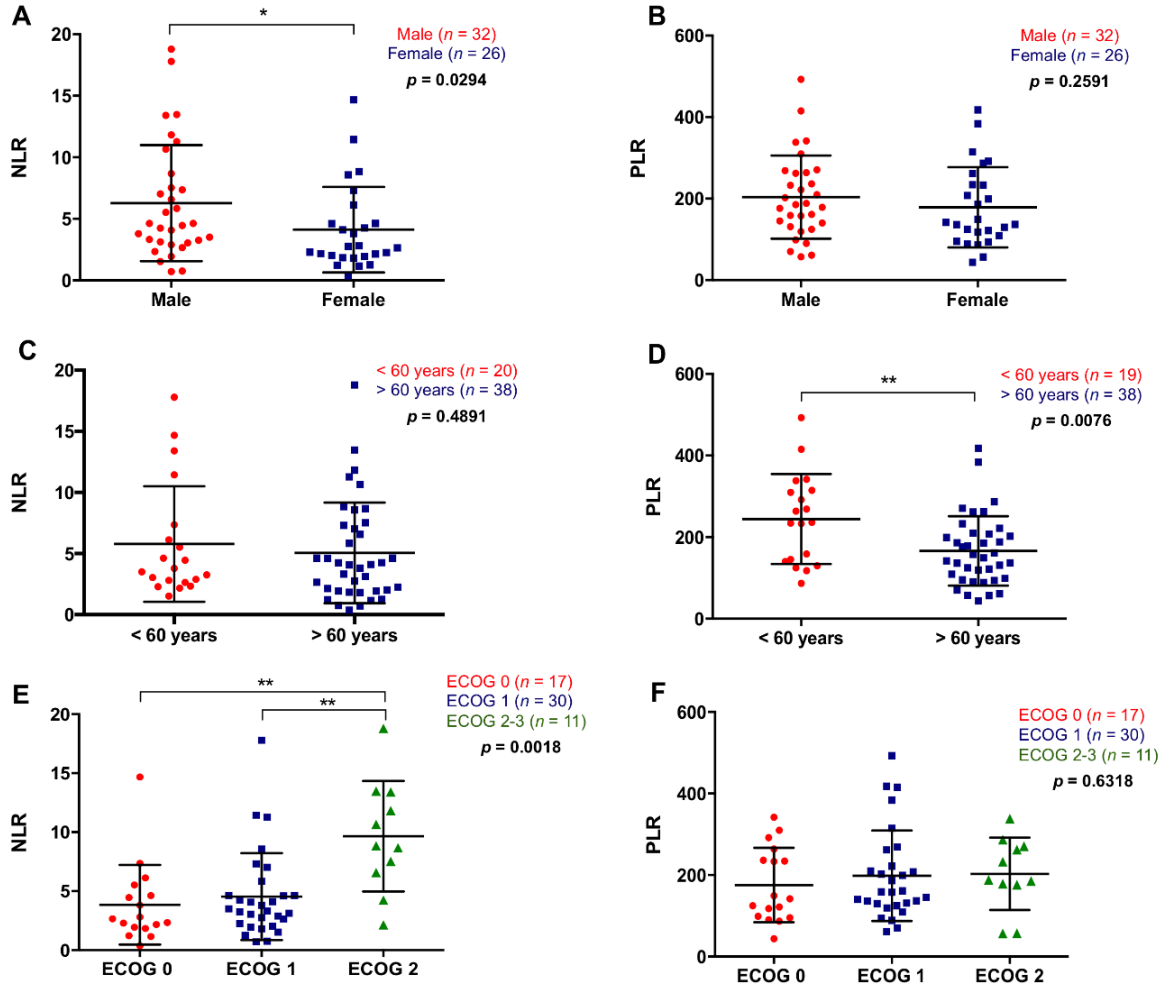

Figure 15. Association between neutrophil–lymphocyte ratio and platelet–lymphocyte ratio with the clinical characteristics of the patients. (**A**) Neutrophil–lymphocyte ratio (NLR) and (**B**) platelet–lymphocyte ratio (PLR) in male or female patients, (**C**) NLR and (**D**) PLR in patients younger or older than 60 years, (**E**) NLR and (**F**) PLR according to the ECOG. (* p<0.05, ** p<0.01).

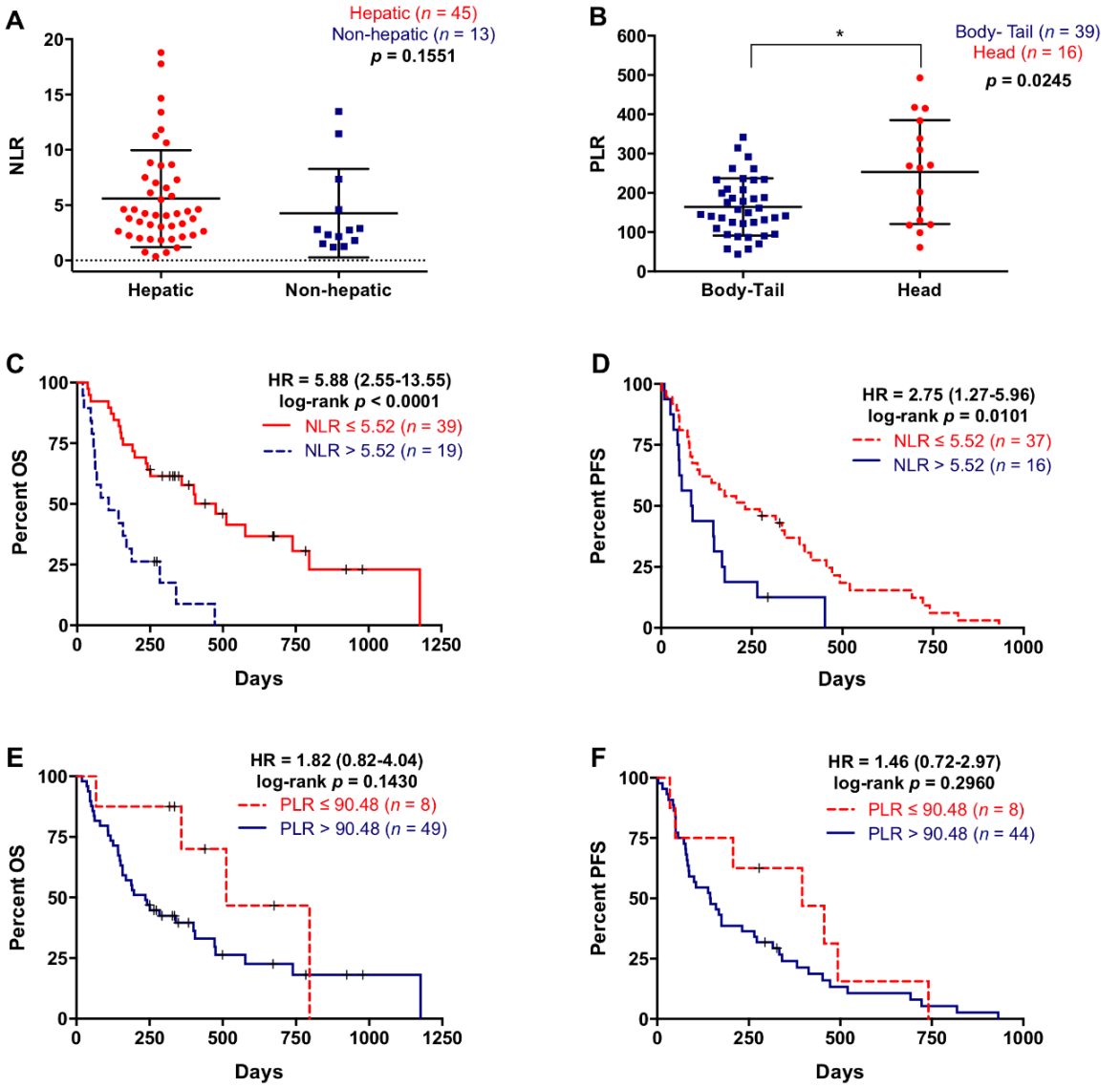

Figure 16. Neutrophil–lymphocyte ratio and platelet–lymphocyte ratio in metastatic pancreatic ductal adenocarcinoma patients. (**A**) Neutrophil–lymphocyte ratio (NLR) in patients with liver metastases or metastases elsewhere, (**B**) platelet–lymphocyte ratio (PLR) in patients with primary tumour located in the body-tail or the head of the pancreas, (**C**) overall survival (OS) according to NLR (cut-off: 5.52), (**D**) progression-free survival (PFS) according to NLR (cut-off: 5.52), (**E**) OS according to PLR (cut-off: 90.48), (**F**) PFS according to PLR (cut-off: 90.48) (* $p<0.05$).

Table 4. **Multivariate analysis adjusted for ECOG, metastatic location, RAS status in plasma and neutrophil-lymphocyte ratio (NLR).**

Variables	OS		PFS	
	HR (95%CI)	*p*	HR (95%CI)	*p*
ECOG	2.02 (1.21-3.39)	0.008	-	-
Metastatic Location	-	ns	3.15 (1.36-7.31)	0.007
RAS mutation status plasma	6.94 (2.03-23.7)	0.002	7.91 (2.48-25.20)	0.0001
NLR	2.47 (1.25-4.88)	0.010	-	ns

2.3. Combined analysis of NLR and PLR improves the prognostic accuracy in patients with metastatic pancreatic cancer

There was a significant positive correlation between NLR and PLR (r=0.35; p=0.0085) (**Figure 17A**). Notably, the combination of NLR and PLR improved the prognostic classification of metastatic PDAC patients. For this combined analysis positive or negative values were assigned when NLR or PLR values were above (positive) or below (negative) the cut-off with prognostic value in OS, and scores 0, 1 and 2 were defined as negative for both markers, positive for one of them and positive for both markers, respectively. As shown in **Figure 17B**, those patients with score 2 showed a highly significant poorer survival than those patients with score 0 or 1 in Kaplan-Meier analysis (p=0.0004 and p=0.0040, respectively). In contrast, the combination of NLR and PLR did not significantly improve prognosis accuracy for PFS (p=0.0856) (**Figure 17C**).

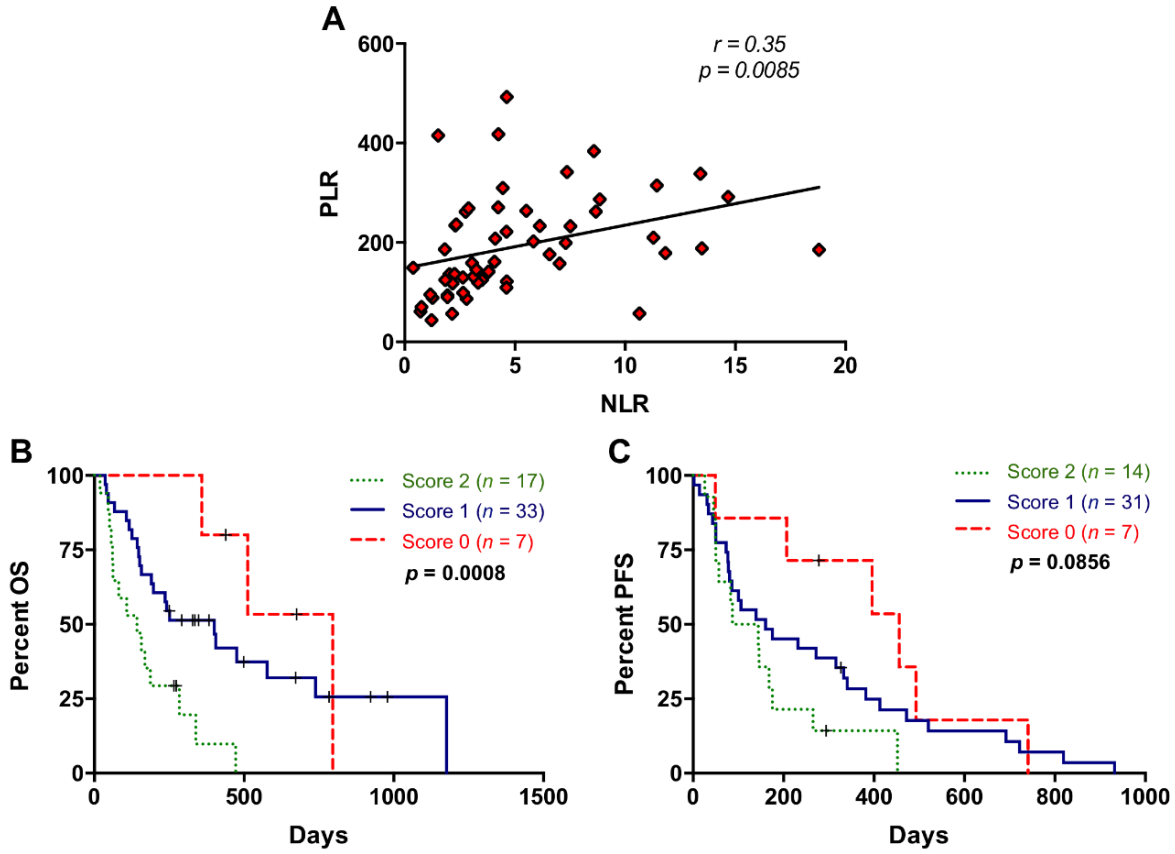

Figure 17. Association between neutrophil–lymphocyte ratio and platelet–lymphocyte ratio. (**A**) Correlation between neutrophil–lymphocyte ratio (NLR) and platelet–lymphocyte ratio (PLR) values; (**B**) overall survival (OS) according to the NLR and PLR combination (score 2 compared to score 0: p=0.0004, score 2 compared to score 1: p=0.0040); (**C**) progression free survival (PFS) according to the NLR and PLR combination (score 2 compared to score 0: p=0.0097; score 2 compared to score 1: p=0.1463) (score 2, positive for both markers; score 1, positive for one of them; score 0: negative for both markers).

2.4. NLR positively correlates with cfDNA concentration in plasma of metastatic pancreatic cancer patients

A high positive correlation (r=0.71; p<0.0001) was found between NLR and cfDNA concentration (**Figure 18A**). No correlation was found between PLR and cfDNA concentration (p=0.8205). However, a negative correlation (r=-0.30; p=0.0244) was found between PLR and cfDNA size. No significant association was found between NLR and cfDNA fragmentation (p=0.4381).

As described above, higher levels of cfDNA were significantly associated with shorter OS and PFS in metastatic PDAC patients and a cut-off for cfDNA concentration (26.46 ng/ml) was determined. When NLR and cfDNA levels were combined according to the scoring system described above, patients with score 2 showed significant shorter OS than patients with score 0 or score 1 (p=0.0001 and p=0.0008, respectively) (**Figure 18B**). We also found an improvement in the prognostic stratification of patients according to PFS when NLR and cfDNA levels were combined (score 2 versus score 0: p=0.0037 and score 2 versus score 1: p=0.0119) (**Figure 18C**).

Since NLR and cfDNA concentration were highly correlated, we next measured neutrophil elastase in plasma as a marker of NETosis to determine whether neutrophil activation contributes to cfDNA content in plasma of metastatic PDAC patients. As shown in **Figure 19A-C**, elastase concentration in plasma positively correlated with NLR (r=0.5618; p<0.0001), cfDNA concentration (r=0.5246; p<0.0001), and CA19-9 (r=0.4995; p<0.0001). Similarly to NLR and cfDNA, elastase concentration was higher in patients with liver metastases (p=0.0423, **Figure 19D**), but there was no relation between elastase levels and primary tumour location (p=0.9890) or number of metastases (p=0.7515). Moreover, high elastase concentration in plasma (>23.15 ng/ml) was a prognostic factor of worse OS (p=0.0110) and PFS (p=0.0241) (**Figure 19E-F**).

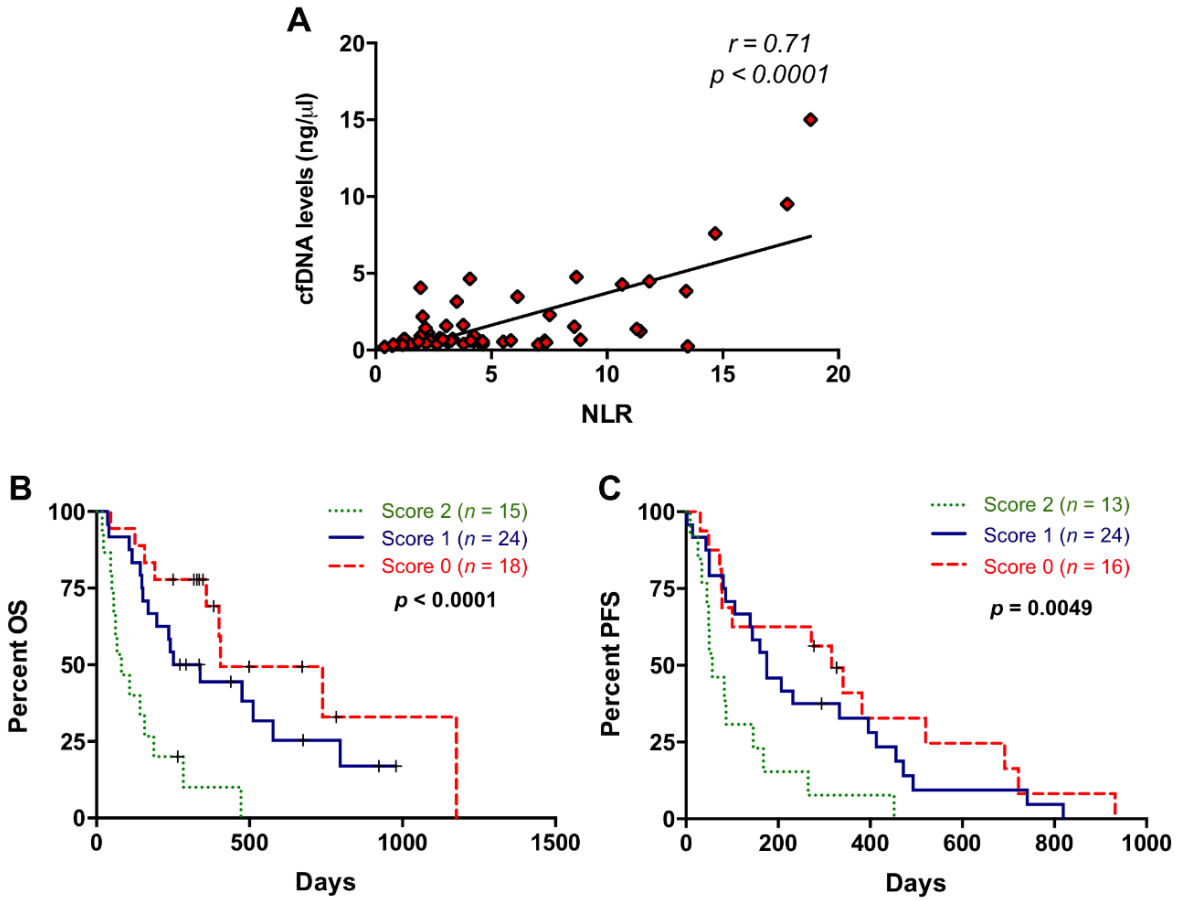

Figure 18. Association between neutrophil–lymphocyte ratio and cell-free DNA concentration. (**A**) Correlation between neutrophil–lymphocyte ratio (NLR) values and circulating cell-free DNA (cfDNA) levels; (**B**) overall survival (OS) according to the NLR and cfDNA combination (score 2 compared to score 0: p=0.0001, score 2 compared to score 1: p=0.0008); (**C**) progression-free survival (PFS) according to the NLR and cfDNA combination (score 2 compared to score 0: p=0.0037; score 2 compared to score 1: p=0.0119) (score 2, positive for both markers; score 1, positive for one of them; score 0: negative for both markers).

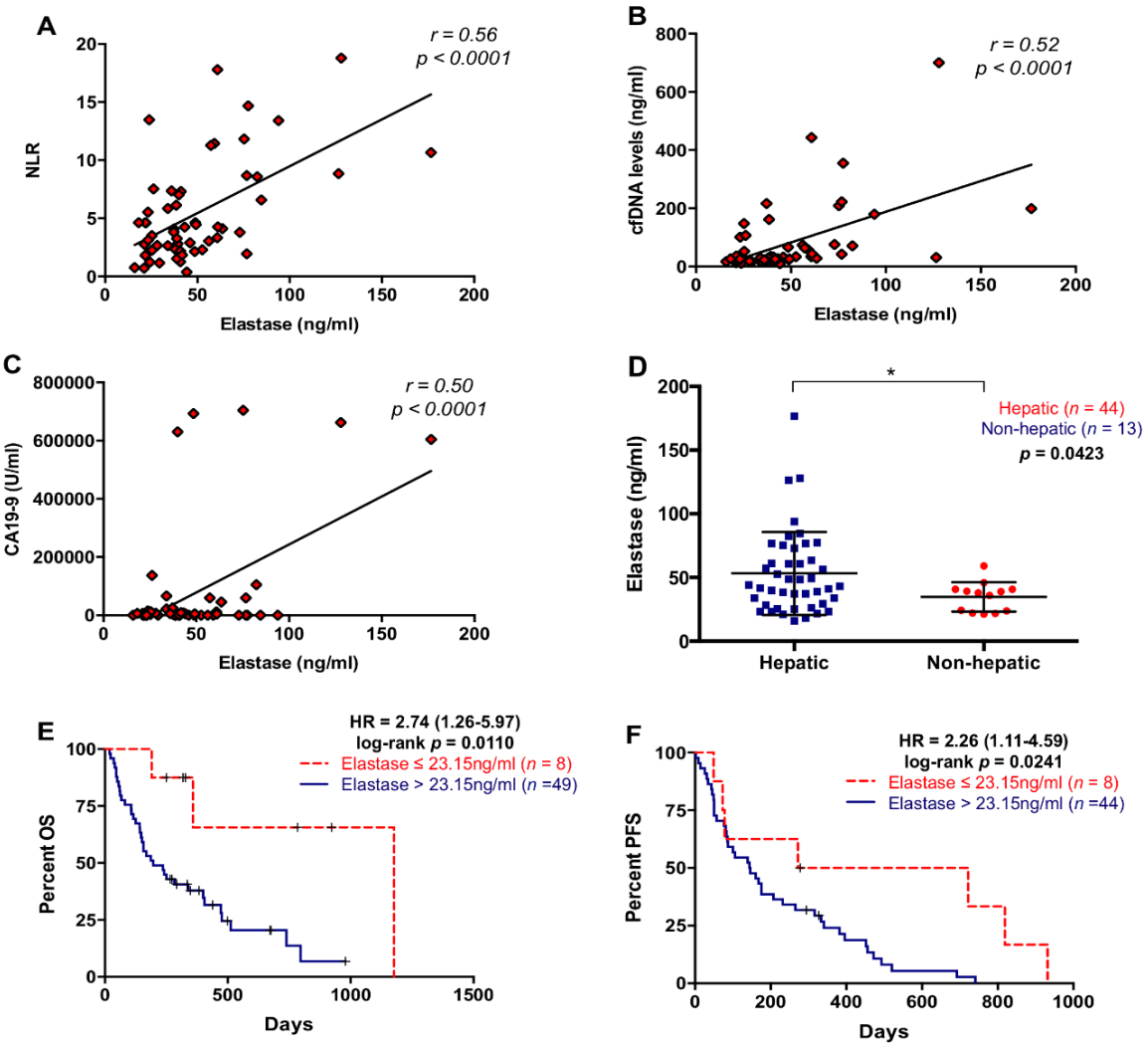

Figure 19. Circulating levels of neutrophil elastase in metastatic pancreatic ductal adenocarcinoma patients. (**A**) Correlation between plasma levels of neutrophil elastase and neutrophil–lymphocyte ratio (NLR); (**B**) correlation between plasma levels of neutrophil elastase and cell-free DNA (cfDNA); (**C**) correlation between plasma levels of neutrophil elastase and CA19-9; (**D**) plasma levels of neutrophil elastase in pancreatic ductal adenocarcinoma patients with metastatic lesions in the liver or elsewhere; (**E**) overall survival (OS) according to circulating levels of neutrophil elastase (cut-off: 23.15 ng/mL); (**F**) progression-free survival (PFS) according to circulating levels of neutrophil elastase (cut-off: 23.15 ng/mL) (* $p<0.05$).

2.5. NLR is related to RAS mutational status in cfDNA of metastatic pancreatic cancer patients

As shown in **Figure 20A**, NLR was significantly higher in those patients in which plasma RAS mutations were detected (4.53 versus 2.24; p=0.0024). Moreover, a positive correlation between NLR and the RAS MAF was found (r=0.4481; p=0.0023) (**Figure 20B**). As described above, RAS mutation and RAS mutational load in cfDNA were related to shorter OS and PFS in metastatic PDAC patients and a cut-off for RAS MAF (0.351%) was determined. As shown in **Figure 20C**, when NLR and RAS mutation status in plasma were combined, patients with score 2 showed poorer OS compared with patients with score 0 and score 1 (p<0.0001 and p=0.0003, respectively). Similarly, this combination of markers also better stratified patients for PFS (score 2 versus score 0: p=0.0003 and score 2 versus score 1: p=0.0533) (**Figure 20D**). When NLR was combined with RAS MAF (**Figure 20E**), an improved stratification of RAS mutated patients for OS (score 2 versus 0, p=0.0029; score 2 versus 1, p=0.0037) but not for PFS (p=0.0869) was observed (**Figure 20F**). No significant association was found between PLR and RAS mutation (p=0.5071) or RAS mutational load (p=0.6854) in plasma.

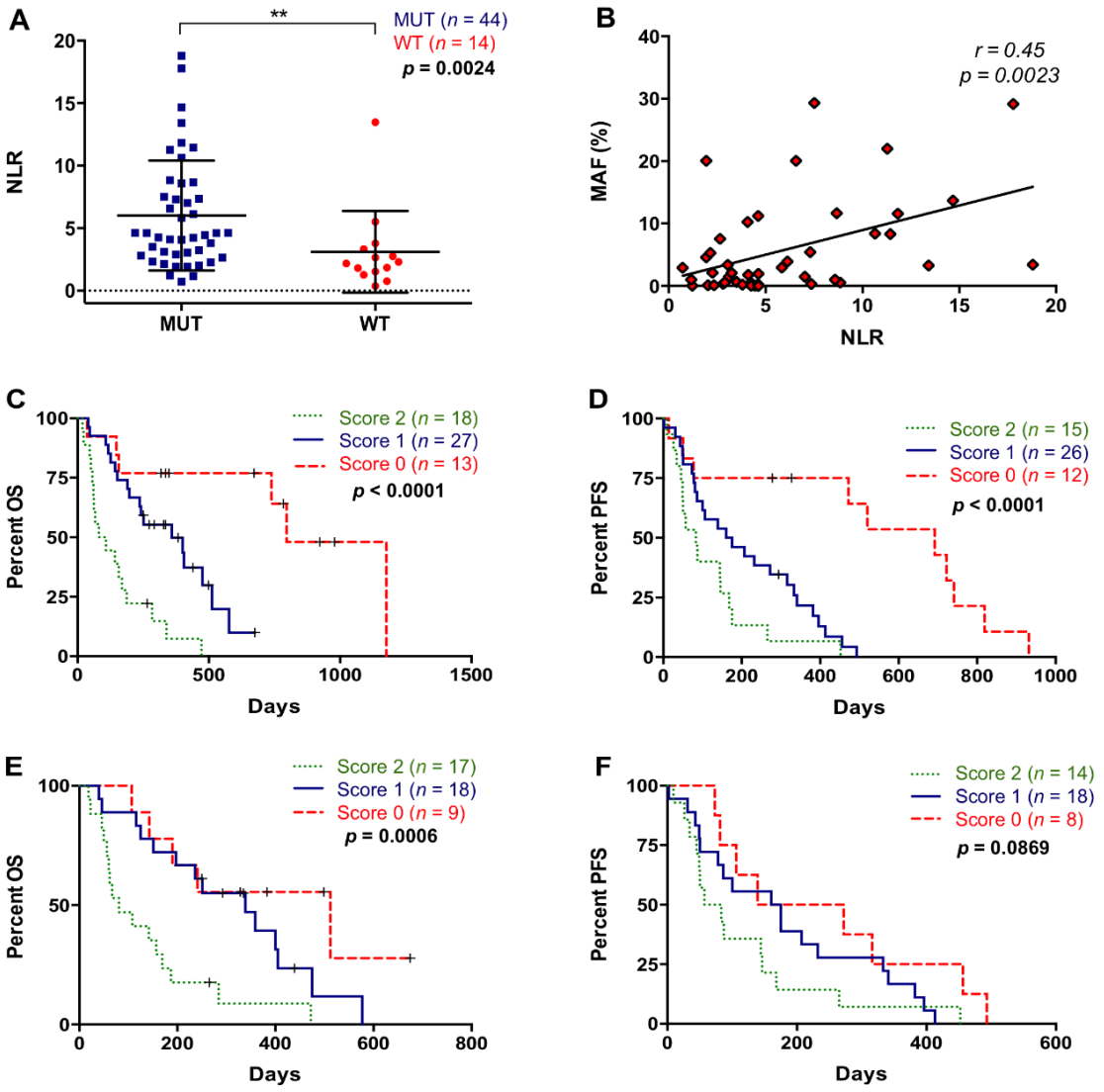

Figure 20. Association between neutrophil–lymphocyte ratio and plasma RAS mutation. (**A**) Neutrophil–lymphocyte ratio (NLR) according to RAS mutational status in plasma; (**B**) correlation between NLR values and RAS mutant allelic fraction (MAF) in plasma; (**C**) overall survival (OS) according to the NLR and RAS mutational status combination (score 2 compared to score 0: $p<0.0001$; score 2 compared to score 1: $p=0.0003$); (**D**) progression-free survival (PFS) according to the NLR and RAS mutational status combination (score 2 compared to score 0: $p=0.0003$; score 2 compared to score 1: $p=0.0533$); (**E**) OS according to the NLR and MAF combination (score 2 compared to score 0: $p=0.0029$; score 2 compared to score 1: $p=0.0037$); (**F**) PFS according to the NLR and MAF combination (score 2 compared to score 0: $p=0.0420$; score 2 compared to score 1: $p=0.3008$) (score 2, positive for both markers; score 1, positive for one of them; score 0, negative for both markers) (** $p<0.01$).

2.6. Multiple blood-based biomarkers improve the prognostic stratification of metastatic pancreatic cancer patients

As described above, higher levels of CA19-9 were significantly associated with shorter OS and PFS in metastatic PDAC patients and a cut-off for CA19-9 (45,500 U/ml) was determined. CA19-9 levels and NLR showed a positive correlation (r=0.3684; p=0.0048) and the combination of both biomarkers showed an improvement in patient stratification for OS (score 2 versus score 0: p<0.0001 and score 2 versus score 1: p=0.0226) and PFS (score 2 versus score 0: p=0.0016 and score 2 versus score 1: p=0.0021) (**Figure 21A-B**).

Next, a combination of multiple blood-based biomarkers (NLR, PLR, cfDNA concentration, RAS status, RAS MAF and CA19-9) was used to improve the prognostic stratification of metastatic PDAC patients. In this case, score 2 was defined as positive for all markers, score 1 positive for 3, 4 or 5 markers and score 0 positive for 1 or 2 marker or negative for all of them. As shown in **Figure 21C**, patients with score 2 had a very short OS outcome compared with patients with score 1 (p=0.0026), and specially with patients with score 0 (p<0.0001). In regard to PFS this combination of multiple blood-based biomarkers also efficiently stratified patients into dismal (score 2), poor (score 1) and good (score 0) prognosis (**Figure 21D**).

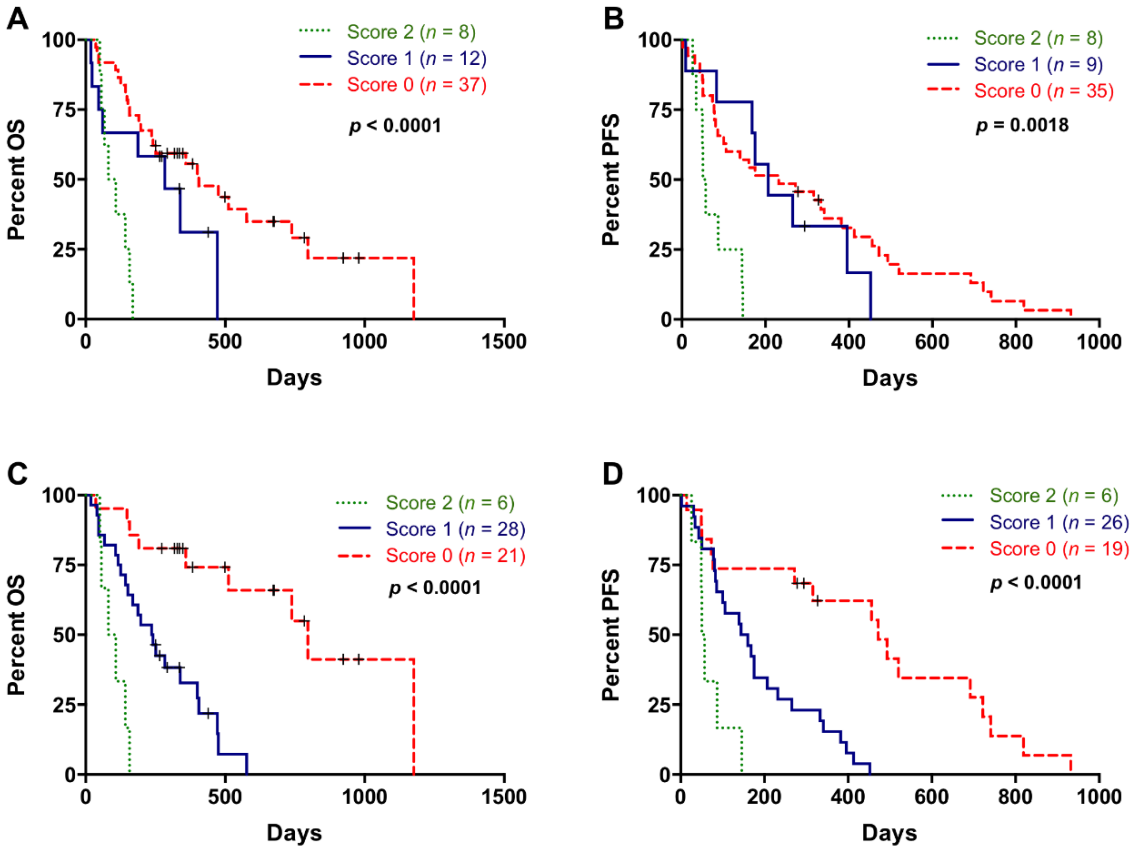

Figure 21. Multiple blood-based biomarkers for the prognosis of metastatic pancreatic ductal adenocarcinoma patients. (**A**) Overall survival (OS) according to neutrophil–lymphocyte ratio (NLR) and CA19-9 combination (score 2 compared to score 0: $p < 0.0001$; score 2 compared to score 1: p=0.0226); (**B**) progression-free survival (PFS) according to NLR and CA19-9 combination (score 2 compared to score 0: p=0.0016; score 2 compared to score 1: p=0.0021) (score 2, positive for both markers; score 1, positive for one of them; score 0, negative for both markers); (**C**) OS according to the combination of multiple blood-based biomarkers (NLR, platelet–lymphocyte ratio (PLR), cell-free DNA (cfDNA) concentration, RAS status, RAS mutant allelic fraction (MAF) and CA19-9) (score 2 compared to score 0: p<0.0001; score 2 compared to score 1: p=0.0026); (**D**) PFS according to the combination of multiple blood-based biomarkers (NLR, PLR, cfDNA concentration, RAS status, RAS MAF, and CA19-9) (score 2 compared to score 0: p=0.0008; score 2 compared to score 1: p=0.0086) (score 2, positive for all markers; score 1, positive for 3, 4 or 5 markers; score 0, positive for 1 or 2 markers or negative for all of them).

3. Circulating cell-free DNA-based liquid biopsy markers for the non-invasive prognosis of metastatic colorectal cancer

3.1. Clinical-pathological characteristics and RAS mutation analysis from plasma and tissue

One hundred and thirty-six patients were included in the study between November 2015 and March 2019. Eighty-six patients were men and 50 were women, ranging in age from 37-85 years, with a median age of 66 years. Patients received as first line of treatment chemotherapy (36%), anti-VEGF (43.4%) and anti-EGFR (17.6%) regimes (**Table 5**). We found differences in the prognosis of metastatic CRC patients according to the gender and age. Males were associated with a better prognosis in OS (p=0.0004) and in PFS (p=0.0247) and patients younger than 60 years were also associated with better OS (p=0.0103) and PFS (p=0.0544) rates (**Table 6**). Most patients (92.6%) had a good baseline ECOG performance status at diagnosis and significant differences in OS (p<0.0001) and PFS (p<0.0001) were found according to this clinical parameter (**Table 6**). All patients had CRC with distant metastases at diagnosis, and the liver was the most frequent site of metastasis (78.7%). Primary tumour was located in the right and left (including rectum) side of the colon in 33% and 64% of patients, respectively, and 3% of the cases had right and left synchronous tumours. Primary tumour location (OS p=0.1189; PFS p=0.3463) and metastatic location (OS p=0.6614; PFS p=0.4791) were not related to OS or PFS. However, significant poorer OS and PFS were observed in patients with metastatic lesions in more than one location (OS 492.5 versus 674.5 days, p=0.0004; PFS 190 versus 297 days, p=0.0009).

Table 5. **Baseline characteristics of metastatic CRC patients**.

Patient Characteristics		**Number of Cases (*n* = 136)**
Age	<60 years	40 (29.4)
	>60 years	96 (70.6)
Sex	Male	86 (63.2)
	Female	50 (36.8)
ECOG	0	43 (31.6)
	1	83 (61)
	2	9 (6.6)
	3	1 (0.7)
1st line treatment	Chemotherapy	49 (36)
	Chemotherapy + anti-VEGF	59 (43.4)
	Chemotherapy + anti-EGFR	24 (17.5)
	No treatment	4 (2.9)
Survival	Alive	44 (32.4)
	Dead	92 (67.6)
Disease progression	Yes	122 (89.7)
	No	10 (7.4)
	Not valuable (No treatment)	4 (2.9)
Primary tumour location	Right	45 (33.1)
	Left	57 (41.9)
	Rectum	30 (22.1)
	Right and left synchronous tumour	4 (2.9)
Number of metastatic lesions	One location	63 (46.3)
	More than one location	73 (53.7)
Metastatic lesions location	Hepatic lesions	107 (78.7)
	Non-hepatic lesions	29 (21.3)
Liquid Biopsy RAS status	RAS mutated	82 (60.3)
	RAS wild type	54 (39.7)
Tissue RAS status[1]	RAS mutated	74 (57.8)
	RAS wild type	54 (42.2)

[1] For the analysis of RAS mutational status, primary tumour tissue was available in 94.1% (128/136) of patients.

Table 6. **Overall survival and progression-free survival analysis.**

	OS		PFS	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***
Age				
≤60 years	1.00	0.0103	1.00	0.0544
>60 years	1.75 (1.14-2.69)		1.45 (1.00-2.12)	
Gender				
Male	1.00	0.0004	1.00	0.0247
Female	2.30 (1.45-3.65)		1.59 (1.06-2.37)	
ECOG				
0	1.00		1.00	
1	1.84 (1.18-2.87)	<0.0001	1.19 (0.81-1.73)	<0.0001
2-3	2354 (369.6-14992)		730.8 (106.7-5007)	
Primary Tumour Location				
Left and Rectum	1.00	0.1189	1.00	0.3463
Right	1.44 (0.91-2.27)		1.20 (0.81-1.80)	
Number of Metastasis				
1	1.00	0.0004	1.00	0.0009
≥2	2.13 (1.40-3.23)		1.85 (1.29-1.67)	
Metastatic Location				
Non-hepatic	1.00	0.6614	1.00	0.4791
Hepatic	1.12 (0.68-1.84)		0.85 (0.54-1.34)	
RAS mutation status plasma (with tissue paired samples)				
WT	1.00	0.0322	1.00	0.1743
MUT	1.61(1.04-2.48)		1.29 (0.89-1.88)	
RAS mutation status tissue				
WT	1.00	0.0657	1.00	0.1932
MUT	1.5 (0.97-2.31)		1.28 (0.88-1.85)	
cfDNA concentration				
≤20.35ng/ml	1.00	<0.0001	1.00	0.0007
>20.35ng/ml	2.76 (1.80-4.24)		1.89 (1.31-2.73)	
cfDNA fragmentation				
≤34.24%	1.00	0.0374	1.00	0.3907
>34.24%	1.84 (1.04-3.27)		1.23 (0.76-1.99)	

MAF				
≤6.95%	1.00	0.0234	1.00	0.7708
>6.95%	1.81 (1.08-3.03)		1.07 (0.68-1.68)	
CEA				
≤9.5U/ml	1.00	<0.0001	1.00	0.0181
>9.5U/ml	2.46 (1.59-3.82)		1.58 (1.08-2.31)	

3.2. Detection of RAS mutations in cfDNA predicts poor prognosis in metastatic colorectal cancer patients

Primary tumour tissue was available for the analysis of RAS mutational status in 94.1% (128/136) of patients. RAS mutation was detected in 57.8% (74/128) of tissue samples and in 60.3% (82/136) of basal plasma samples. Mutations in codons 12 and 13 were the most commonly detected both in tissue (77%) and plasma (79.3%). The overall concordance between plasma and tissue RAS analysis was 93%, increasing to 94% in patients with liver metastases. RAS mutation in plasma, but not in tissue, was significantly associated with shorter OS (540 versus 660 days; p=0.0322) (**Table 6, Figure 22A**). Hence, prognosis was more accurately predicted by RAS mutation analysis in cfDNA than by tissue analysis (551 versus 625 days, p=0.0657) **(Table 6, Figure 22B).** On the other hand, although non-mutated RAS patients had better PFS, non-significant association was found between RAS mutation status in plasma (p=0.1743) or tissue (p=0.1932) and PFS.

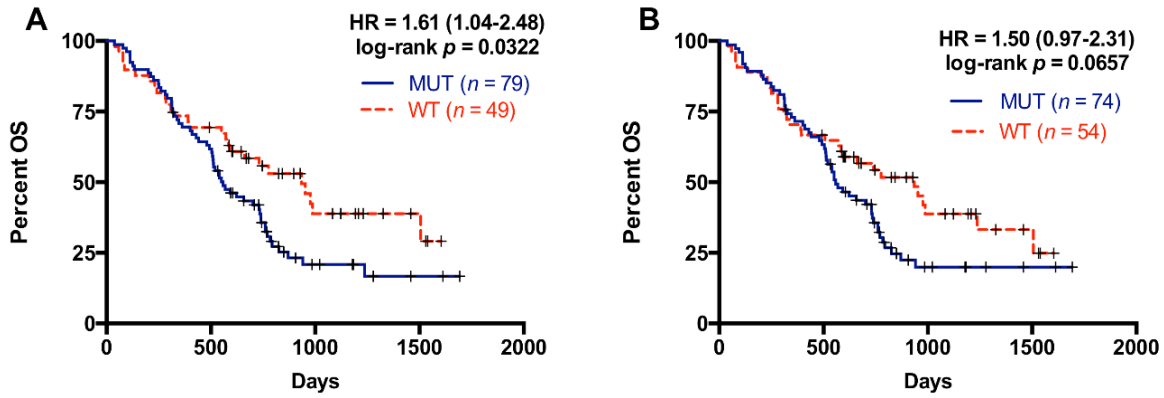

Figure 22. Overall survival according to RAS mutation status. Overall survival (OS) according to RAS mutation status in cfDNA (**A**) and in tissue (**B**).

3.3. Higher RAS mutational load in cfDNA is associated with poor prognosis in metastatic colorectal cancer

For the 82 patients with detectable plasma RAS mutations, the median mutation allele fraction (MAF) was 6.95% (range 0.01-49.45%). A higher RAS mutational load in cfDNA (>6.95%) was associated with poor OS (512 versus 568 days, p=0.0234) (**Figure 23A**), but not with PFS (p=0.7708) (**Figure 23B**). Although no differences were observed in the MAF values according to the primary tumour location, higher MAF values were strongly associated with metastatic location in the liver (p=0.0006) (**Figure 24A**) and a trends towards higher MAF levels in patients with more than one metastatic location was also found (p=0.0921) (**Figure 24B**). Moreover, a higher percentage of patients with metastatic lesions in more than one location showed mutations in RAS gene compared with patients with only one metastatic location (p=0.0339) (**Figure 24C**).

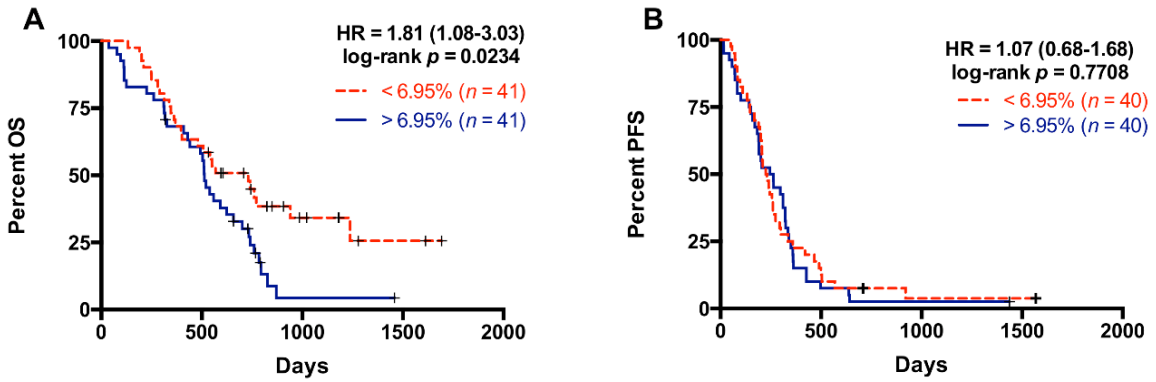

Figure 23. Overall and progression free survival rates according to circulating RAS mutation allele fraction (MAF). (**A**) OS according to circulating MAF (cut-off: 6.95%); (**B**) PFS according to circulating MAF (cut-off: 6.95%).

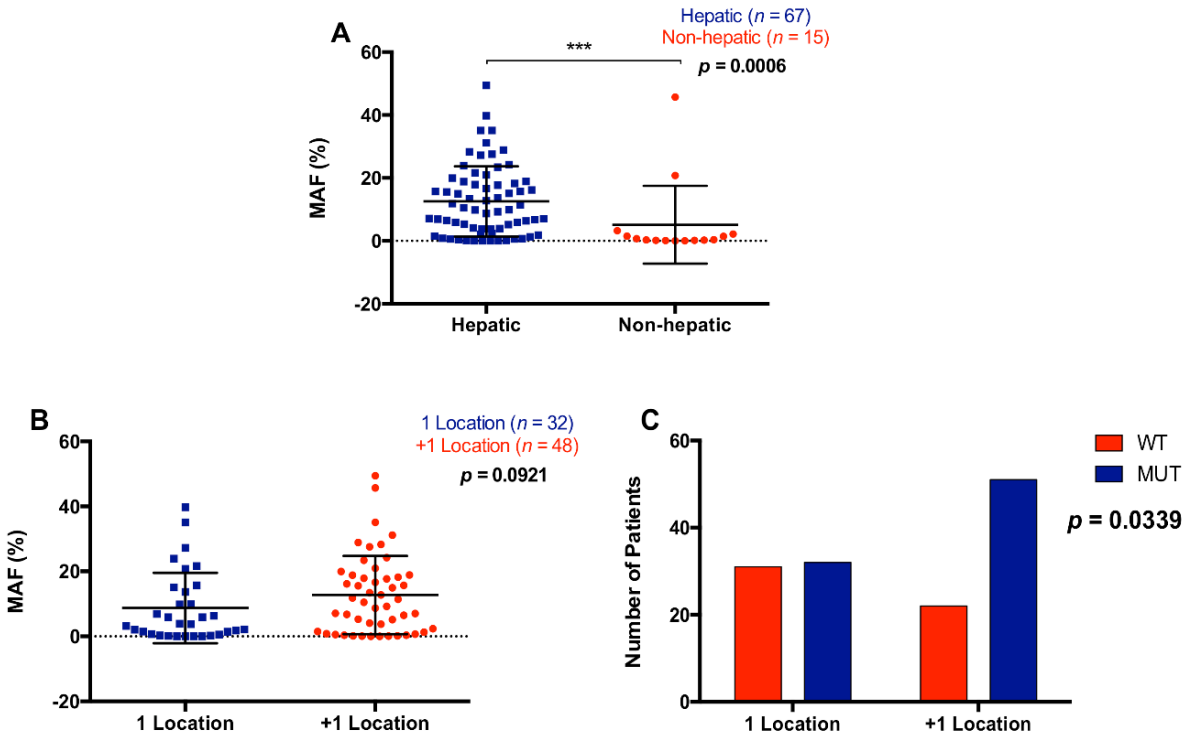

Figure 24. Relationship between RAS mutation and metastatic characteristics. (**A**) RAS mutant allelic fraction (MAF) according to metastatic location in the liver or elsewhere; (**B**) RAS MAF according to the number of metastatic locations; (**C**) RAS mutation status according to the number of metastatic locations.

3.4. Higher cfDNA concentration and fragmentation levels are associated with poor survival in metastatic colorectal cancer patients

The median cfDNA concentration in plasma of CRC patients was 36.3 ng/mL (range 8.3-3266.7 ng/mL) while the fragment size of plasma cfDNA ranged between 100-1100 bp, with prominent mode at 135 pb for the shortest fragments detected. In this study cfDNA fragmentation was defined as the percentage of shortest fragments to total cfDNA. Although not statistically significant, higher cfDNA concentration (41.35 versus 22.91 ng/mL, p=0.0770) and cfDNA fragmentation (35.45 versus 25.45%, p=0.1506) were found in patients with RAS mutated plasma (**Figure 25A-B**). In addition, a significant positive correlation between cfDNA concentration and cfDNA fragmentation was found (r=0.56; p<0.0001) (**Figure 25C**).

When metastatic CRC patients were stratified according to cfDNA concentration, those with higher values (>20.35 ng/mL) had a significant poorer OS rate (512.5 versus 794 days; p<0.0001) (**Figure 26A**). Also, higher cfDNA levels were associated with shorter PFS (216 versus 345.5 days, p=0.0007) (**Figure 26B**). Interestingly, cfDNA concentration was a predicting factor of response to therapy, independently of the first line of treatment (**Table 7**). Moreover, higher percentage of plasma cfDNA fragmentation (>34.24%) in metastatic CRC patients was also significantly associated with poorer OS (527.5 versus 600 days, p=0.0374), but not with PFS (p=0.3907) (**Figure 26C-D**).

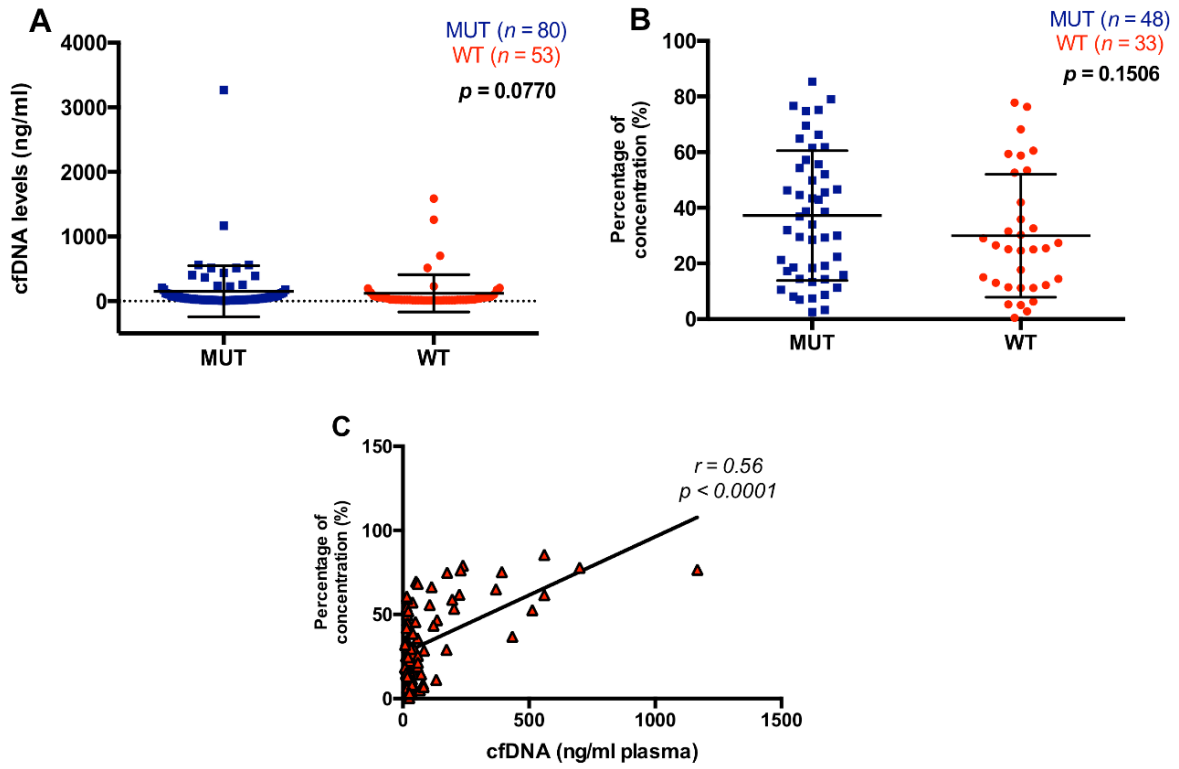

Figure 25. cfDNA concentration and fragmentation according to RAS mutation status and the relation of both factors. (**A**) cfDNA levels according to RAS mutation status; (**B**) cfDNA fragmentation according to RAS mutation status; (**C**) Positive correlation between cfDNA concentration and fragmentation.

Table 7. **cfDNA concentration prognosis according to the first line treatment.**

Treatment	cut-off (ng/ml)	*n*	cfDNA OS		cfDNA PFS	
			HR (95%CI)	*p*	HR (95%CI)	*p*
Chemotherapy	<20.35	13	1.96 (0.95-4.05)	0.0702	2.18 (1.14-4.19)	0.0188
	>20.35	33				
Anti-VEGF	<20.35	18	2.48 (1.29-4.77)	0.0065	1.77 (1.03-3.06)	0.0395
	>20.35	40				
Anti-EGFR	<20.35	11	6.71 (2.16-20.90)	0.0010	1.57 (0.66-3.74)	0.3041
	>20.35	13				

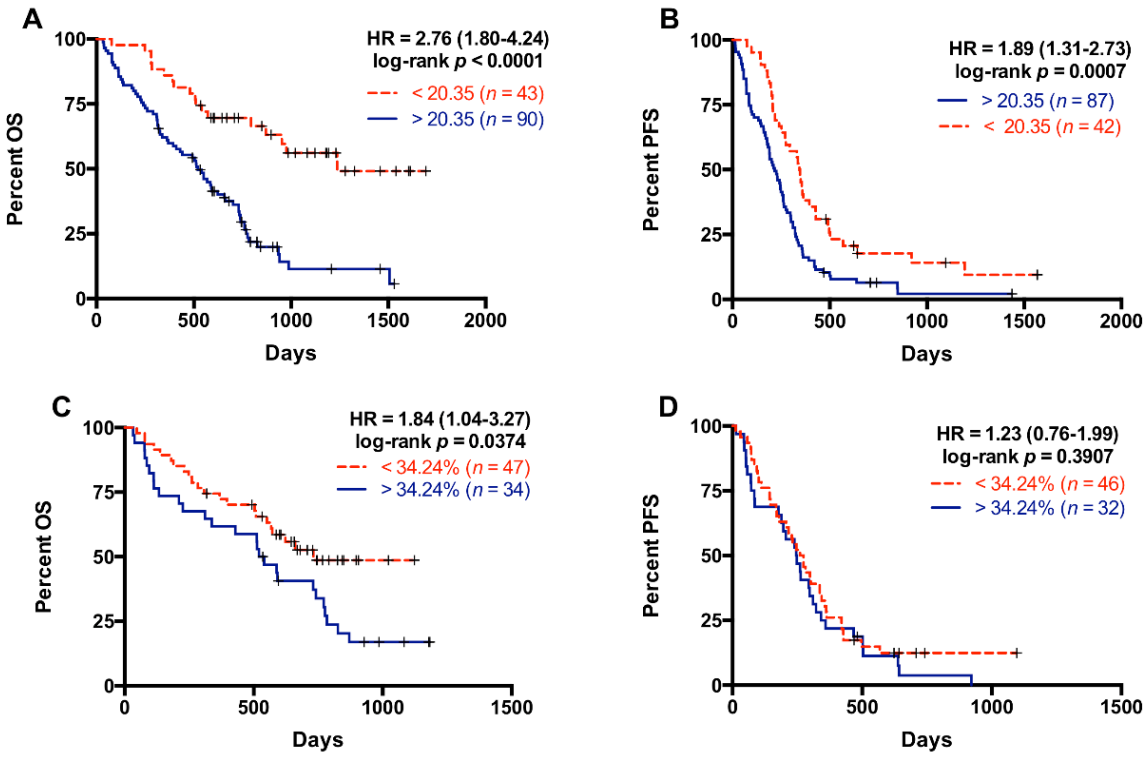

Figure 26. Overall and progression free survival rates according to cfDNA concentration and fragmentation. (**A**) Overall survival (OS) according to cfDNA concentration (cut-off: 20.35 ng/ml); (**B**) Progression free survival (PFS) according to cfDNA concentration (cut-off: 20.35 ng/ml); (**C**) OS according to cfDNA fragmentation (cut-off: 34.24%); (**D**) PFS according to cfDNA fragmentation (cut-off: 34.24%).

Plasma cfDNA concentration and fragmentation were not associated with primary tumour location (p=0.7473; p=0.2307). However, there was a significant association between higher levels of cfDNA concentration and metastatic location in the liver (45.66 versus 21.7 ng/mL, p=0.0049) (**Figure 27A**) and also with more than one metastatic location (53.43 versus 22.63 ng/mL, p=0.0138) (**Figure 27B**). Moreover, higher values of cfDNA fragmentation were also significantly associated with liver metastasis (33.36 versus 15.8%, p=0.0093) (**Figure 27C**).

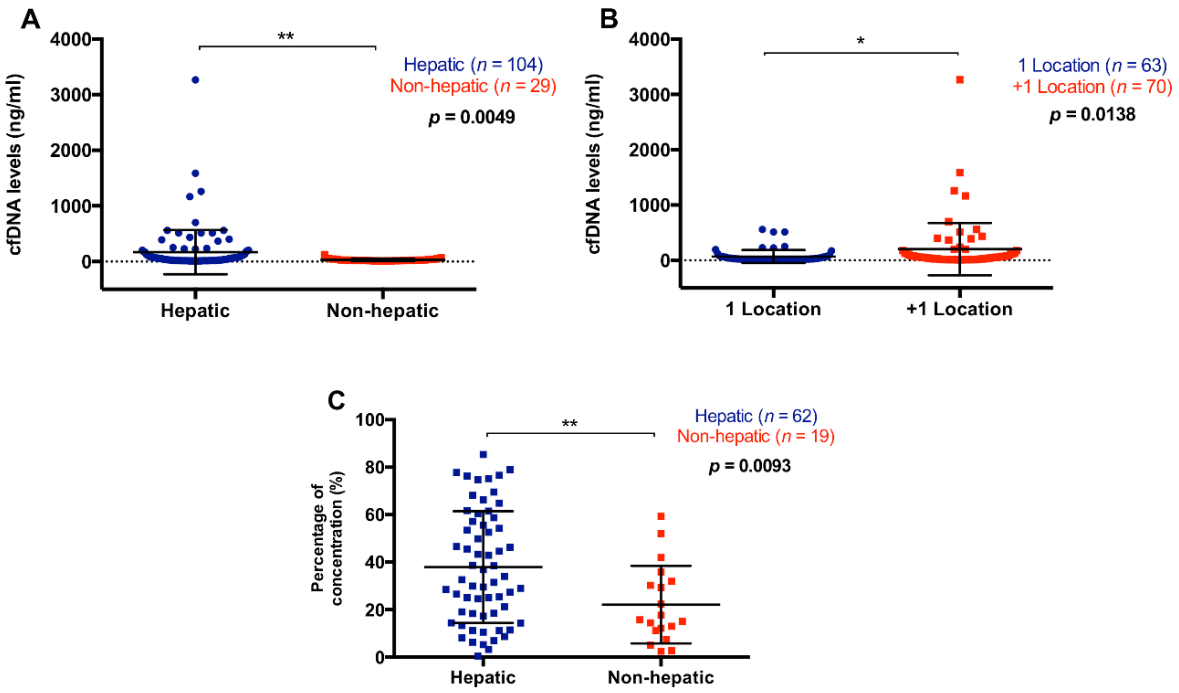

Figure 27. Association between cfDNA concentration and fragmentation with metastasis location. (**A**) cfDNA levels according to metastasis in the liver or elsewhere; (**B**) cfDNA levels according to the number of metastatic locations; (**C**) cfDNA fragmentation according to hepatic or non-hepatic metastasis.

3.5. Multiparameter liquid biopsy improves prognosis stratification of metastatic colorectal cancer patients

In our cohort, CEA had a prognostic value with higher basal levels (>9.5ng/mL) associated with poorer OS and PFS rates (OS 705 versus 520 days, $p<0.0001$; PFS 314.5 versus 227 days, $p=0.0181$) (**Figure 28A-B**). A positive association was found between CEA and RAS mutation status ($p=0.0278$), metastatic location ($p=0.0297$) and positively correlated with RAS MAF ($r=0.3106$; $p=0.0067$), however no association was found between CEA and primary tumour location ($p=0.4094$), number of metastasis ($p=0.3059$), cfDNA concentration ($p=0.2618$) and cfDNA fragmentation ($p=0.4853$).

Remarkably, the combination of CEA with liquid biopsy markers such as RAS status and cfDNA concentration improved the prognostic stratification of metastatic CRC patients. They were classified according to the number of markers they had above (positive) or below (negative) the cut-off determined for prognostic value in OS. Patients with all three positive markers showed worse OS (**Figure 28C**) than patients with two positive markers (p=0.0160), one positive marker (p=0.0002) and specially than patients with all three negative markers (p<0.0001). Regarding to PFS, patients with all three positive markers showed worse PFS than patients with only one positive marker (p=0.0450) and specially than patients with all three negative markers (p=0.0016) (**Figure 28D**).

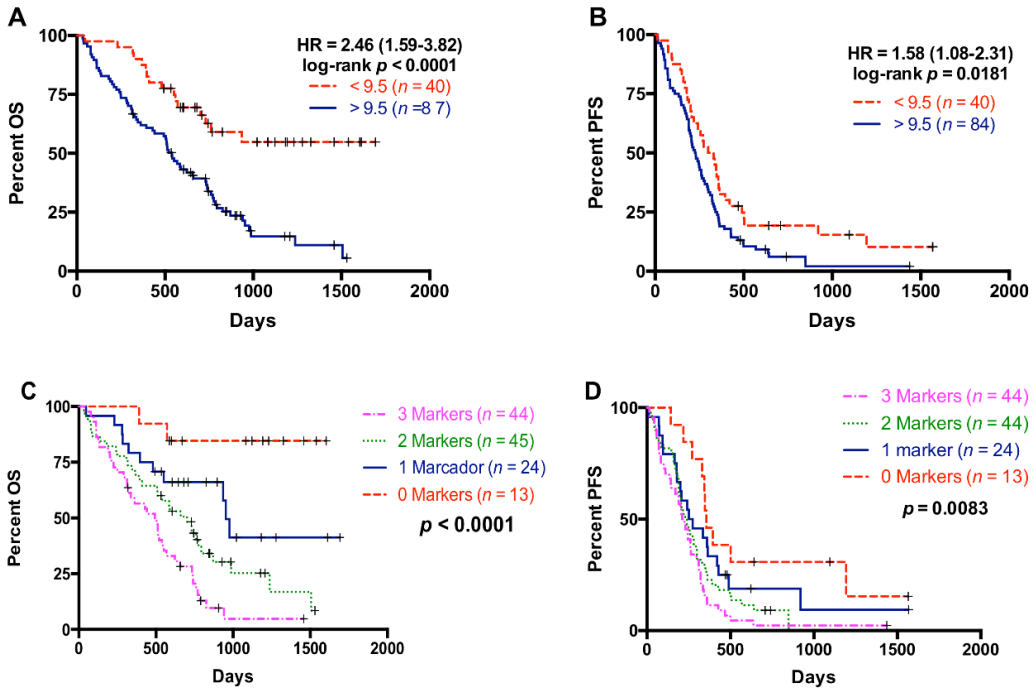

Figure 28. Overall survival and progression free survival according to CEA levels and the combination of CEA with liquid biopsy markers. (**A**) Overall survival (OS) according to CEA levels (cut-off: 9.5 ng/ml); (**B**) Progression free survival (PFS) according to CEA levels (cut-off: 9.5 ng/ml); (**C**) OS combining CEA with cfDNA concentration and RAS mutation status; (**D**) PFS combining CEA with cfDNA concentration and RAS mutation status.

4. Circulating microRNAs as liquid biopsy biomarkers to predict response and prognosis in metastatic colorectal cancer patients treated with FOLFIRI plus aflibercept

4.1. Clinicopathological Characteristics

Ninety-eight metastatic CRC patients (60 men, 38 women) with 63 years median age (range 36-83 years) were included in the study from 2016 to 2017 (clinical characteristics are summarised in **Table 8**). More than 80% of the patients were diagnosed with stage IV CRC and most patients had a primary tumour located in the colon (74.5%) and metastases in the liver (79.6%). RAS mutation was detected in tissue in 57 patients (62.6%). All patients included in the study were metastatic CRC patients resistant to or progressive on an oxaliplatin-containing regimen who were to receive FOLFIRI plus aflibercept.

4.2. Differential circulating miRNA levels between responder and non-responder patients to treatment with FOLFIRI plus aflibercept

Fifteen patients with the longest (responders) time to treatment failure (TTF) and fifteen with the shortest (non-responders) TTF were first included for screening. In each sample 754 circulating miRNAs were analysed and Ct values were obtained. Those miRNAs which circulating levels were not detected in at least one third of the patients were excluded. miRNAs were normalized using an exogenous control and the average level of the three most stable miRNAs using the NormFinder algorithm [92]. Relative circulating levels were calculated using the $2^{-\Delta\Delta Ct}$ method [93]. Seventy-one miRNAs differentially expressed between responders and non-

responders (p<0.1) were selected using the non-parametric Wilcoxon Rank test in the MetaboAnalyst web server [95] for further analysis (**Figure 29**).

Table 8. Baseline characteristics of metastatic CRC patients receiving FOLFIRI plus aflibercept treatment.

Patient Characteristics			**n (%)**
Age (median, range)			63, 36-83
Sex	Male		60 (61.2)
	Female		38 (38.8)
ECOG	0		46 (46.9)
	1-2		52 (53.1)
Primary Tumour Location	Colon	Yes	73 (74.5)
		No	23 (23.5)
	Rectum	Yes	30 (30.6)
		No	66 (67.3)
	Data Not Available		2 (2.0)
RAS status in tissue	RAS mutated		57 (58.2)
	RAS non-mutated		34 (34.7)
	Data Not Available		7 (7.1)
Stage at diagnosis	I+II+III		15 (15.3)
	IV		82 (83.7)
	Data Not Available		1 (1.0)
Histopathological Grade	Well differentiated		16 (16.3)
	Moderately or poorly differentiated		49 (50.0)
	Grade could not be determined		33 (33.7)
Metastatic Location	Liver	Yes	78 (79.6)
		No	20 (20.4)
	Lung	Yes	57 (58.2)
		No	41 (41.8)
	Distant lymph nodes	Yes	23 (23.5)
		No	75 (76.5)
	Peritoneum	Yes	18 (18.4)
		No	80 (81.6)
	Regional lymph nodes	Yes	11 (11.2)
		No	87 (88.8)
Primary tumour surgery prior to study inclusion		Yes	72 (73.5)
		No	26 (26.5)

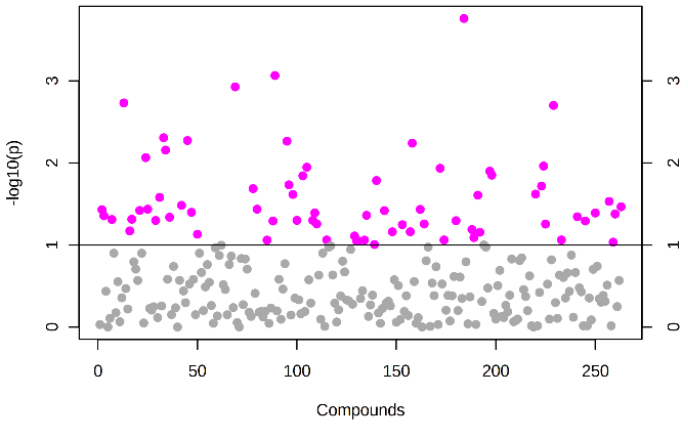

Figure 29. miRNAs differentially expressed between patients responding or non-responding to treatment with FOLFIRI plus aflibercept. Seventy-one miRNAs (purple dots) were differentially expressed between responders and non-responders patients (p<0.1).

Individual evaluation of these 71 miRNAs was then performed and 47 miRNAs were finally selected for the validation step (**Table 9**). As shown in **Figure 30A**, a distinct profile for these miRNAs was found between responder and non-responder patients. Twenty-two and 25 miRNAs were up-regulated and down-regulated in responder patients, respectively (**Figure 30B**).

Table 9. **Forty-seven miRNAs included in the analysis of response to treatment with FOLFIRI plus aflibercept in metastatic CRC patients.**

miRNA ID			
hsa-let-7b-3p	hsa-miR-144-3p	hsa-miR-2110	hsa-miR-375
hsa-miR-101-3p	hsa-miR-16-5p	hsa-miR-22-3p	hsa-miR-409-3p
hsa-miR-106b-5p	hsa-miR-17-5p	hsa-miR-22-5p	hsa-miR-423-3p
hsa-miR-1180-3p	hsa-miR-185-3p	hsa-miR-221-3p	hsa-miR-425-3p
hsa-miR-125a-5p	hsa-miR-186-5p	hsa-miR-224-5p	hsa-miR-425-5p
hsa-miR-125b-5p	hsa-miR-193a-5p	hsa-miR-29c-3p	hsa-miR-432-5p
hsa-miR-127-3p	hsa-miR-193b-3p	hsa-miR-30a-3p	hsa-miR-551a
hsa-miR-128-3p	hsa-miR-19a-3p	hsa-miR-320b	hsa-miR-584-5p
hsa-miR-1301-3p	hsa-miR-19b-3p	hsa-miR-328-3p	hsa-miR-652-3p
hsa-miR-130a-3p	hsa-miR-200b-3p	hsa-miR-33b-5p	hsa-miR-766-3p
hsa-miR-140-3p	hsa-miR-20a-5p	hsa-miR-34a-5p	hsa-miR-93-5p
hsa-miR-142-5p	hsa-miR-21-5p	hsa-miR-361-5p	

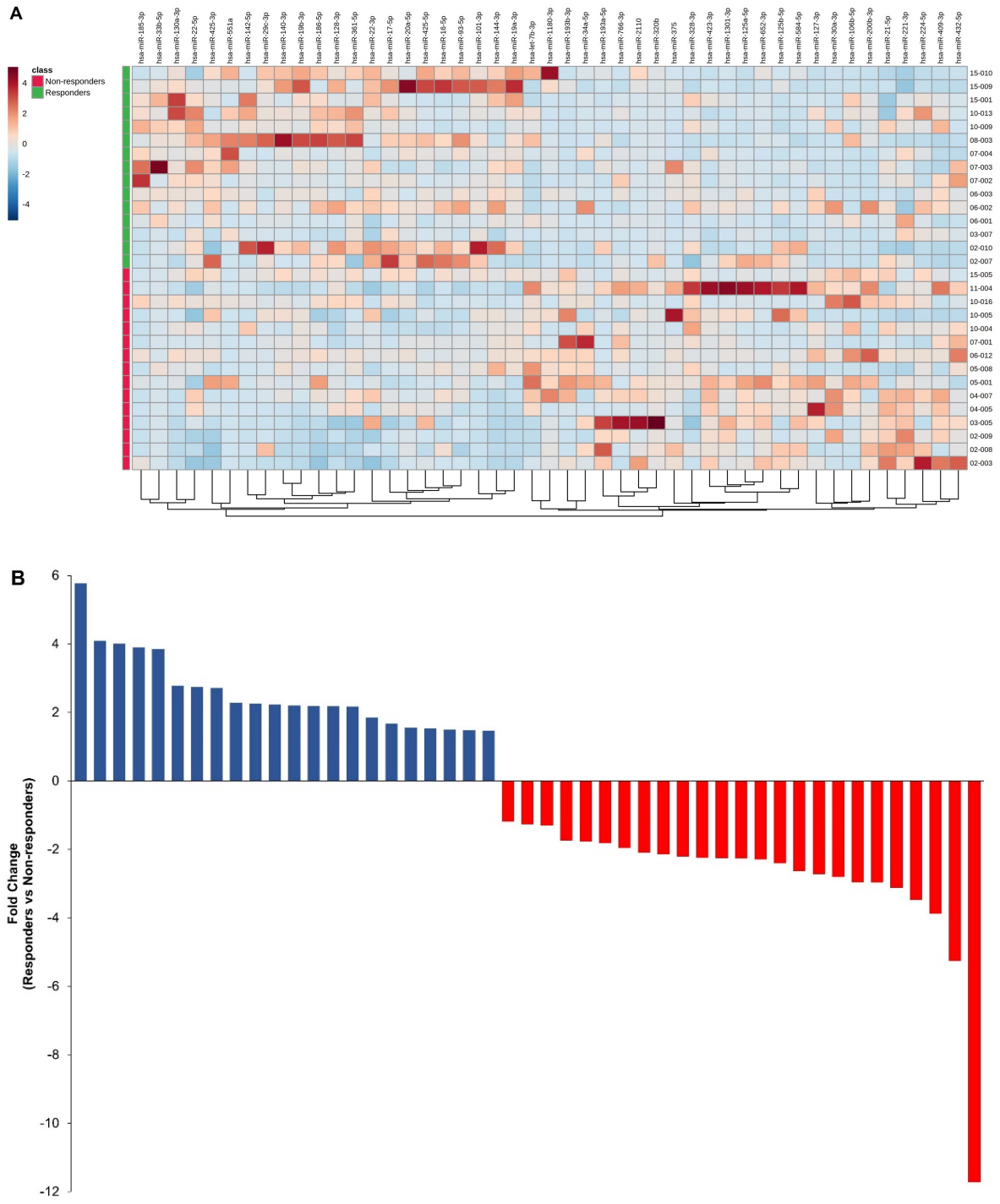

Figure 30. Differential profile between patients responding or non-responding to treatment with FOLFIRI plus aflibercept. (**A**) Heatmap showing the distinct profile of the 47 miRNAs selected between responders and non-responders patients. (**B**) Fold change of the 47 miRNAs selected, 22 miRNAs were up-regulated (blue) in responder patients and 25 miRNAs were down-regulated (red) in responder patients.

To identify those disease phenotypes and functions over-represented in this set of miRNAs, we next performed an enrichment analysis (**Figure 31)** using the online miRNA set enrichment tool TAM 2.0 [94]. Notably, colon carcinoma was the most significant disease-association, with 44 miRNAs. Moreover, angiogenesis ranked among the most significant function-associations in this set of miRNAs, with 19 miRNAs associated with this vasculogenic process.

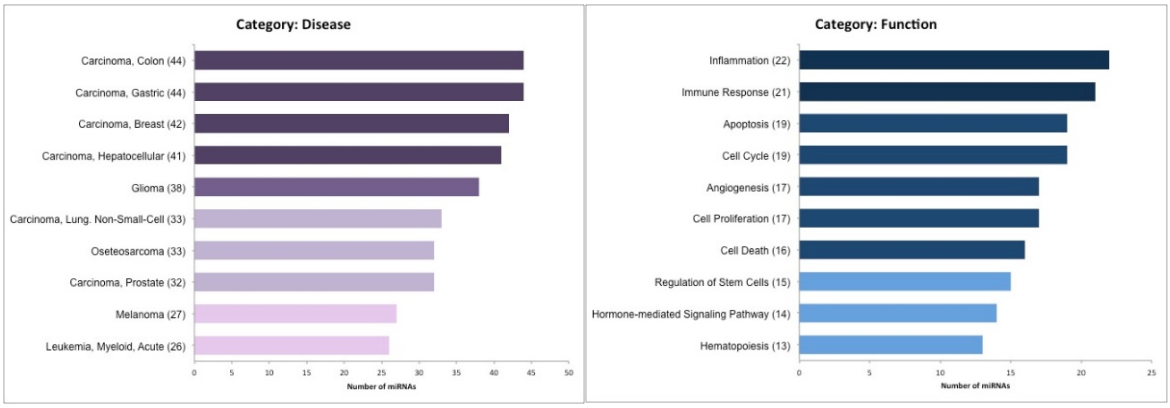

Figure 31. Disease phenotypes and functions associated with the set of miRNAs with distinct profile in patients responding or non-responding to treatment with FOLFIRI plus aflibercept. The top-ten diseases and functions most significantly associated with the set of 47 miRNAs are shown ordered by the number of miRNAs involved.

Interactions between differentially expressed miRNAs and target genes was next analysed using the IPA (Ingenuity Pathway Analysis) software (Qiagen). As shown in **Figure 32**, the set of miRNAs was molecularly interconnected with 91 target genes involved in colorectal metastasis signalling. Moreover, 60.4% of targets (55 genes) were also implicated in angiogenesis, supporting the connection between this set of miRNAs and angiogenic processes in colorectal tumours.

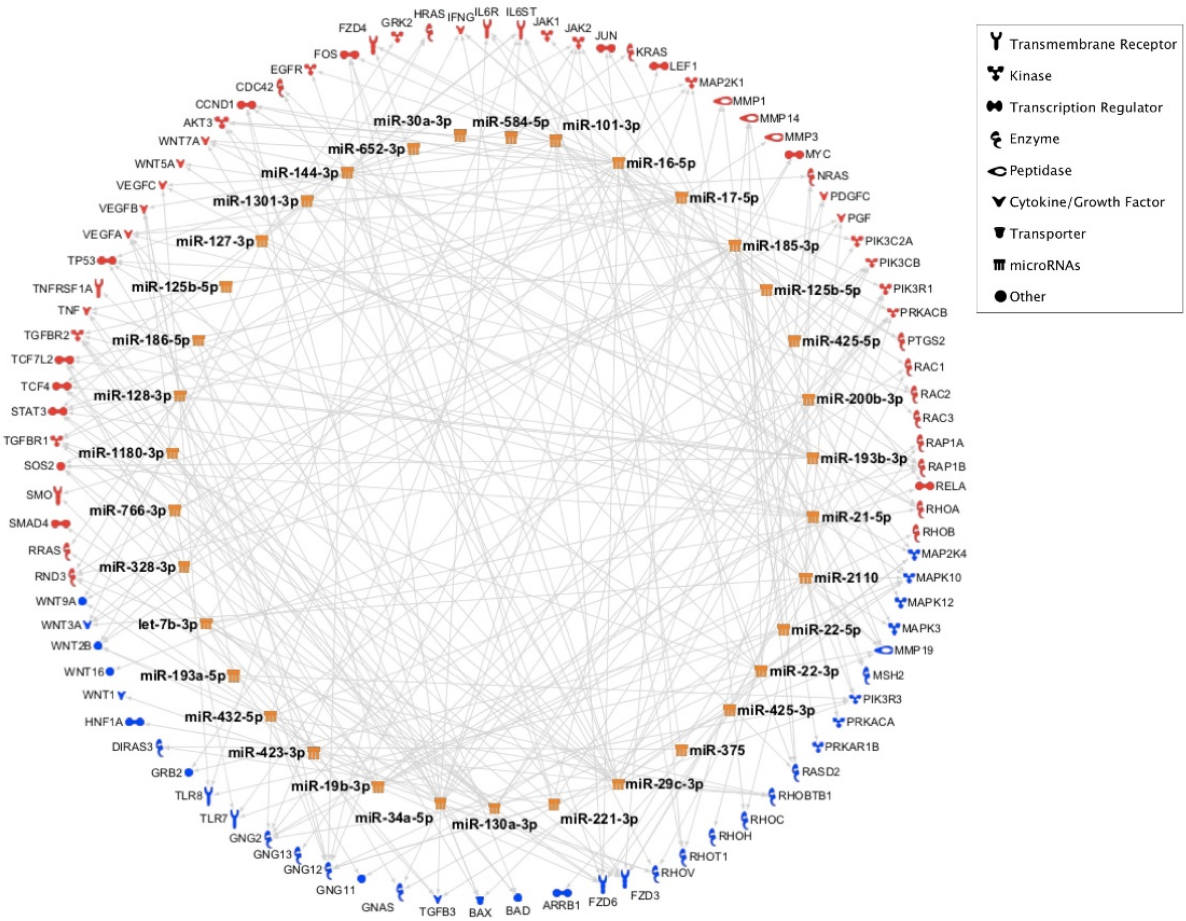

Figure 32. Molecular network of miRNA targets. Interactions between differentially expressed miRNAs (orange) and target genes using the IPA (Ingenuity Pathway Analysis) software. Ninety-one targets (blue and red) were found involved in colorectal cancer signalling pathway and fifty-five of them were also found implicated in angiogenesis (red).

4.3. Predictive model of response to treatment with FOLFIRI plus aflibercept

A predictive model of response was constructed by combining the 47 miRNAs displaying differential profile between responder and non-responder patients (**Table 9**), with the clinical variables (**Table 8**). According to overall response, all the 98 patients included in the study were classified into complete or partial response (19 patients), and non-response to treatment (79). Due to the high number of potential predictors included in the analysis, a univariate binary logistic regression was first performed, and

those variables with $p > 0.15$ for their association with response were excluded. Hence, thirteen variables with a likely higher weight in the model were finally included in the analysis. The predictive model was built by performing a stepwise regression with bidirectional elimination. Primary tumour located in the colon (not in rectum) and two miRNAs (hsa-miR-33b-5p and hsa-miR-30a-3p) were the predictor variables included in the final predictive model (**Table 10**). In brief, patients with primary tumour located in the colon presented a lower probability of response. Higher circulating levels of hsa-miR-33b-5p were also associated with higher probability of response, whereas higher circulating levels of hsa-miR-30a-3p were related with lower probability of response.

Table 10. Predictive model of response to treatment with FOLFIRI plus aflibercept.

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95% C.I. for Exp (B)
Primary tumour located in the colon (Yes versus No (ref.))	-1.258	0.631	3.970	1	0.046	0.284	0.083-0.980
hsa-miR-33b-5p	0.140	0.051	7.517	1	0.006	1.150	1.041-1.271
hsa-miR-30a-3p	-0.087	0.042	4.285	1	0.038	0.917	0.844-0.995

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

Using the predictive values, a ROC curve (AUC=0.782; p=0.000205) was obtained (**Figure 33**), showing that the model efficiently discriminates between responder and non-responder patients. Also, an optimal cut-off of 0.20 was determined by maximizing the sensitivity (72.2%) and specificity (81.8%).

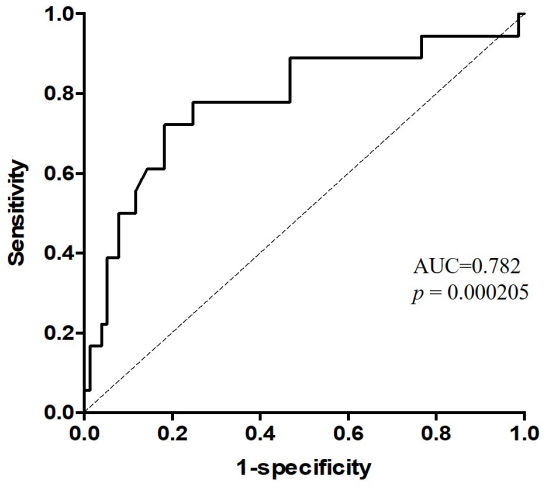

Figure 33. ROC curve obtained from the predictive model of response to treatment with FOLFIRI plus aflibercept. ROC curve classifying responders and non-responders patients with an AUC=0.782, p=0.000205 and with a sensitivity and specificity of 72.2% and 81.8%, respectively, with a cut-off of 0.20.

Additionally, from the predictive model, the relative risks were calculated, with the qualitative variable (dichotomous) and the minimum, third quartile and maximum values for the quantitative variables, to establish the patterns. As shown in **Table 11**, the maximum chance of response to treatment with FOLFIRI plus aflibercept corresponded to a patient with primary tumour not located in the colon (rectum), minimum value of hsa-miR-30a-3p and maximum value of hsa-miR-33b-5p.

Table 11. Relative risk table for the predictive model of response to treatment with FOLFIRI plus aflibercept.

Primary tumour location	**hsa-miR-30a-3p**	Hazard Ratio (Relative Risk)		
		hsa-miR-33b-5p		
		Minimum	Q3	Maximum
No located in colon	Minimum	19.34	43.60	**50.29**
	Q3	6.87	24.52	33.68
	Maximum	3.38	14.40	22.01
Located in colon	Minimum	7.08	25.02	34.20
	Q3	2.12	9.73	15.69
	Maximum	1	4.91	8.40

4.4. Predictive model of progression on FOLFIRI plus aflibercept treatment

Eighty patients progressed on FOLFIRI plus aflibercept treatment or died before the end of the study, whereas eighteen patients still had not progressed. All the clinical characteristics summarised in **Table 8** and the 47 selected miRNAs (**Table 9**) were included in the model. After exclusion of those variables with $p>0.15$ (univariate binary logistic regression), twenty-three variables were finally included in the analysis. Stepwise regression with bidirectional elimination was performed, and primary tumour surgery prior to study inclusion and seven miRNAs, hsa-miR-142-5p, hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328-3p, hsa-miR-33b-5p, hsa-miR-652-3p and hsa-miR-93-5p, were the variables finally included in the optimal model (**Table 12**). In brief, patients without surgery of primary tumour prior to study inclusion presented higher probability to progress or die on FOLFIRI plus aflibercept treatment. Similarly, higher circulating levels of hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328-3p and hsa-miR-652-3p were also associated with higher probability of progression or death, whereas higher circulating levels of hsa-miR-142-5p, hsa-miR-33b-5p and hsa-miR-93-5p were related with lower probability to progress or die on FOLFIRI plus aflibercept treatment.

Additionally, from the predictive model, the relative risks were calculated, with the qualitative variable (dichotomous) and the minimum, median and maximum values for the quantitative variables, to establish the pattern. The maximum risk to progress or die on FOLFIRI plus aflibercept treatment corresponded to a patient without surgery of primary tumour prior to study inclusion, minimum value of hsa-miR-142-5p, hsa-miR-33b-5p and

hsa-miR-93-5p and maximum value of hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328-3p and hsa-miR-652-3p (**Table 13**).

Table 12. Predictive model of progression on FOLFIRI plus aflibercept treatment.

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95% C.I. for Exp (B)
Surgery of primary tumour prior to study inclusion (Yes (ref.) versus No)	0.760	0.270	7.920	1	0.005	2.138	1.259-3.630
hsa-miR-193b-3p	0.079	0.021	13.664	1	<0.001	1.082	1.038-1.129
hsa-miR-29c-3p	0.075	0.037	4.166	1	0.041	1.078	1.003-1.158
hsa-miR-328-3p	0.547	0.182	9.006	1	0.003	1.729	1.209-2.472
hsa-miR-652-3p	0.285	0.132	4.648	1	0.031	1.330	1.026-1.724
hsa-miR-142-5p	-0.051	0.018	7.762	1	0.005	0.951	0.917-0.985
hsa-miR-33b-5p	-0.046	0.023	4.130	1	0.042	0.955	0.914-0.998
hsa-miR-93-5p	-0.566	0.164	11.966	1	<0.001	0.568	0.412-0.782

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

Table 13. Relative risk table for the predictive model of progression on FOLFIRI plus aflibercept treatment.

Risk Pattern	Minimum	Intermediate[a]	Maximum
Variables			
Surgery of primary tumour prior to study inclusion	Yes	Yes	No
hsa-miR-142-5p	Maximum	Minimum	Minimum
hsa-miR-33b-5p	Maximum	Maximum	Minimum
hsa-miR-93-5p	Maximum	Median	Minimum
hsa-miR-193b-3p	Minimum	Maximum	Maximum
hsa-miR-29c-3p	Minimum	Maximum	Maximum
hsa-miR-328-3p	Minimum	Median	Maximum
hsa-miR-652-3p	Minimum	Maximum	Maximum
HR	1	3372.99	256435.4

[a]HR closest to the mean has been considered as intermediate pattern

Remarkably, low levels of hsa-miR-33b-5p were associated with higher risk of disease progression in this model, while higher levels of this miRNA predicted a higher probability of response to treatment with FOLFIRI plus aflibercept. Therefore, we next evaluated the utility of hsa-miR-33b-5p as a single biomarker to differentiate between responders and non-responders patients and also to predict the risk of disease progression. As shown in **figure 34**, patients responding to treatment with FOLFIRI plus aflibercept showed significantly higher hsa-miR-33b-5p circulating levels than non-responder patients (p=0.0026; **Figure 34A**). Furthermore, using the mean value level of hsa-miR-33b-5p as a cut-off, those patients with higher circulating levels of this miRNA had a significant better PFS (9 versus 6 months; p=0.0028; **Figure 34B**).

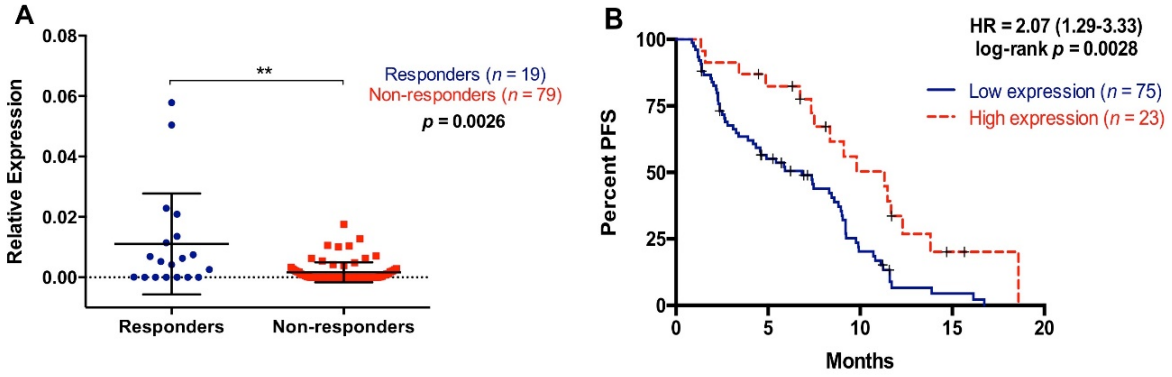

Figure 34. Circulating levels of hsa-miR-33b-5p discriminate between responders and non-responders patients treated with FOLFIRI plus aflibercept and predict the risk of disease progression. (**A**) Circulating levels of hsa-miR-33b-5p according to response to treatment with FOLFIRI plus aflibercept; (**B**) PFS according to circulating levels of hsa-miR-33b-5p (mean value as cut-off). (** p<0.01).

4.5. Predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment

Sixty-two patients died before the end of the study, whereas thirty-six patients still were alive. All the clinical characteristics summarised in **Table**

8 and the 47 selected miRNAs (**Table 9**) were included in the model. After exclusion of those variables with $p > 0.15$ (univariate binary logistic regression) twenty-three variables were finally included in the analysis. Stepwise regression with bidirectional elimination was performed, and sex, primary tumour located in the colon, RAS mutation, primary tumour surgery prior to study inclusion, and four miRNAs (hsa-miR-185-3p, hsa-miR-19b-3p, hsa-miR-425-5p and hsa-miR-432-5p) were the variables finally included in the optimal model (**Table 14**). In brief, the characteristics associated with high risk of death on FOLFIRI plus aflibercept treatment were: male sex, tumour located in the colon, presence of tumour in the colon prior to study inclusion, RAS mutation, and lower circulating levels of hsa-miR-185-3p, hsa-miR-19b-3p, hsa-miR-425-5p and hsa-miR-432-5p.

Table 14. Predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95% C.I. for Exp (B)
Sex (Men (ref.) versus Women)	-0.615	0.310	3.919	1	0.048	0.541	0.294-0.994
Primary tumour located in colon (Yes versus No (ref.))	0.770	0.346	4.964	1	0.026	2.160	1.097-4.251
RAS mutational status (WT (ref.) versus MUT)	0.712	0.307	5.378	1	0.020	2.039	1.117-3.722
Surgery of primary tumour prior to study inclusion (Yes (ref.) versus No)	0.645	0.306	4.448	1	0.035	1.906	1.047-3.472
hsa-miR-185-3p	-0.048	0.021	5.351	1	0.021	0.953	0.914-0.993
hsa-miR-19b-3p	-0.043	0.017	6.275	1	0.012	0.958	0.927-0.991
hsa-miR-425-5p-5p	-0.964	0.240	16.193	1	<0.0001	0.381	0.238-0.610
hsa-miR-432-5p	-0.051	0.024	4.745	1	0.029	0.950	0.907-0.995

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

Additionally, from the predictive model, the relative risks were calculated, with qualitative variables (dichotomous) and the minimum, median and maximum values for the quantitative variables, to establish the patterns. The maximum risk to die on FOLFIRI plus aflibercept treatment (**Table 15**) corresponded to a female patient with primary tumour located in the colon, with primary tumour surgery prior to study inclusion, RAS mutated and minimum value of all the miRNAs included in the final model (hsa-miR-185-3p, hsa-miR-19b-3p, hsa-miR-425-5p and hsa-miR-432-5p).

Table 15. Relative risk table for the predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment.

Risk Pattern Variables	Minimum	Intermediate[a]	Maximum
Surgery of primary tumour prior to study inclusion	No	No	Yes
Primary tumour located in colon	No	No	Yes
Sex	Male	Male	Female
RAS mutational status	Wild type	Mutant	Mutant
hsa-miR-185-3p	Maximum	Median	Minimum
hsa-miR-19b-3p	Maximum	Minimum	Minimum
hsa-miR-425-5p-5p	Maximum	Minimum	Minimum
hsa-miR-432-5p	Maximum	Median	Minimum
HR	1	458.33	13886.80

[a]HR closest to the mean has been considered as intermediate pattern

VI. Discussion

1. Circulating cell-free DNA-based liquid biopsy markers for the non-invasive prognosis and monitoring of metastatic pancreatic cancer

Non-invasive, reliable, and reproducible cfDNA-based liquid biopsy markers may help in the management of cancer patients. This is particularly relevant in the case of PDAC, where the high stromal content makes it difficult to obtain molecular information through cytopathological analysis. However, there is no consensus about the techniques, mutations or type of material in liquid biopsy-based approaches for the prognosis of PDAC patients [97,100–102]. In this study we report the utility of cfDNA RAS mutations analysis using the highly sensitive BEAMing technique as prognostic tool for the management care of metastatic PDAC patients.

In agreement with other reports [103], our study supports the value of cfDNA RAS mutations analysis as a prognostic tool in pancreatic cancer. Hence, our results show that the presence of RAS mutated cfDNA in plasma predicts poor prognosis in metastatic PDAC patients. Moreover, circulating RAS mutational status was an independent negative prognostic factor of both OS and PFS. In fact, the prognosis was more accurately predicted by RAS mutation analysis in cfDNA than in tissue. The allelic ratio and dosage of mutated KRAS may impact on PDAC biology [104], and KRAS MAF in cfDNA has been found to correlate with clinical stage and outcome in PDAC [99,105]. In this regard, our results reveal that circulating RAS MAF in cfDNA predicted survival in metastatic PDAC patients. Importantly, in our study, RAS MAF in cfDNA was an independent negative prognostic factor of OS by multivariate analysis. Recently, KRAS MAF in DNA from circulating exosomes, but not in cfDNA, was found to be an independent

prognostic factor of OS in metastatic PDAC patients [105]. However, our study demonstrates that highly sensitive approaches, such as BEAMing, may also reveal the independent prognostic value of RAS MAF in cfDNA of metastatic PDAC patients. Exhaustive analyses on tissue, including laser capture microdissection, could establish the pure ratio of RAS mutated allele in tumour. However, these types of analyses rely on the availability of biopsy material to be adequately performed, which is not the case for a significant number of PDAC patients, and this is an important issue that liquid biopsy may effectively address.

Although, KRAS mutations are critical for the initiation of pancreatic ductal carcinogenesis, continued mutant KRAS function and oncogenic dosage are still required to maintain the growth of metastatic PDAC [44,106]. On the other hand, gene expression studies revealed that, compared to head localization, body-tail PDAC are more highly proliferative and aggressive [107,108]. Body/tail location is also associated with poor prognosis in advanced disease [38,109,110]. This may explain the reason why, in our cohort, higher values of RAS MAF in cfDNA of metastatic PDAC patients were significantly associated with primary tumours located in the body/tail of the pancreas and liver metastases. Moreover, the higher MAF observed in patients with liver metastases may be explained by the larger volume of hepatic lesions than the isolated lung and peritoneal metastases [105].

Previous studies have reported the potential prognostic value of cfDNA levels and fragmentation in metastatic cancer [111–113], including metastatic PDAC [52]. In our study, higher plasma cfDNA concentrations were significantly associated with poorer OS and shorter PFS. Besides,

patients with hepatic metastases displayed higher cfDNA levels, compared with those patients with other metastatic locations.

Despite the lack of knowledge about the precise mechanisms of cfDNA release into circulation, the role of apoptosis is becoming clearer [114]. A recent study reported that tumour-derived KRAS mutations in pancreatic cancer are predominantly carried by short and ultra-short cfDNA fragments [115]. This may be the biological explanation for our observation that, in parallel with our RAS MAF results, a higher cfDNA fragmentation was found in patients with tumours located in the body/tail of the pancreas or with hepatic metastases than other metastatic lesions, likely due to more aggressive tumours. Thus, recent reports showed that body/tail PDAC may have more aggressive tumour biology and higher metastasis rate compared to PDAC in the head which may explain worse clinical outcomes [38,107,108].

CA19-9, also known as sialyl Lewis A antigen, is the currently used biomarker for pancreatic cancer, and several studies have reported the link between CA19-9 levels and survival in metastatic PDAC patients [116,117]. However, CA19-9 have some important limitations, such as false negative results in subjects with Lewis negative genotype and CA19-9 increases in patients with benign pancreatic-biliary diseases [118]. In our cohort, CA19-9 exhibited some prognostic value with higher baseline levels associated with poorer OS and PFS rates. However, our study demonstrates that the combination of CA19-9 with liquid biopsy markers greatly helped in the prognostic stratification of metastatic PDAC patients.

CA19-9 is also used for monitoring treatment response as the reduction of CA19-9 serum levels during treatment are usually associated with longer survival rates. However, in clinical practice, there is no

consensus on the interpretation of the change in CA19-9 levels and its role in the management of PDAC patients [119]. Therefore, novel reliable biomarkers are required for monitoring the response of PDAC patients to chemotherapy [120]. In our analysis, the change in circulating RAS MAF levels was a suitable surrogate marker for monitoring each patient's response to therapy. Moreover, the rise in MAF levels in some patients was better than CA19-9 in anticipating disease progression, and dynamics of circulating MAF better correlated with patients' outcome compared with CA19-9. Therefore, our results support MAF as a valuable complementary tool for monitoring the response to chemotherapy treatment in metastatic PDAC patients.

In summary, our study supports cfDNA-based liquid biopsy markers as promising clinical tools for the non-invasive prognosis and monitoring of metastatic PDAC patients.

2. The combination of neutrophil–lymphocyte ratio and platelet-lymphocyte ratio with liquid biopsy biomarkers improves prognosis prediction in metastatic pancreatic cancer

Infiltration of immune cells in PDAC tumours is highly abundant, contributing to immune evasion and chemotherapy resistance [121]. In our study, we have described the utility of NLR and PLR along with others circulating tumour-specific markers to evaluate the prognosis in metastatic PDAC patients.

Previous reports have related high NLR and PLR values with poor prognosis in advanced pancreatic cancer [122–125]. However, most of the studies that have related high PLR values with poor OS involved locally advanced patients [125–127], who were not included in the present study. In

this regard our analysis showed that metastatic PDAC patients with higher NLR values had significantly poorer OS and PFS rates, whereas both NLR and PLR were associated with poor-prognosis clinical features. Thus, higher NLR values were related with male gender and higher ECOG status. This is in agreement with other cancer studies, in which higher NLR values were reported in male cancer patients [128,129] and patients with high ECOG status [126]. In addition, those patients younger than 60 years had higher PLR values than older patients, likely because aging is known to be accompanied by a decrease in platelet count [130]. Further, patients with a primary tumour located in the head of the pancreas showed higher PLR values than those patients with a tumour in the body/tail of this organ.

Cancer cells can activate platelets leading to pro-cancerous effects. Activated platelets participate in the regulation of inflammation, releasing proinflammatory cytokines, and in modulating tumour microenvironment by recruiting leukocytes, including neutrophils. Additionally, activated platelets participate in tumour immune evasion by releasing transforming growth factor β (TGF-β), which is a cytokine with a potent immunosuppressive activity. Besides, TGF-β participates in the transition of tumour-associated neutrophils from an anti-tumorigenic (N1) towards a pro-tumorigenic (N2) phenotype [121]. Furthermore, activated platelets have been implicated in the formation of NETs (neutrophil extracellular traps), with a positive feedback loop, because NETs in turn promote platelet activation [131,132]. Therefore, the platelet–neutrophil crosstalk plays an important role in the development and progression of cancer. In this regard, our analysis indicated that NLR and PLR positively correlate and the combination of both factors increased their prognostic value. On the other hand, platelets and neutrophils have been related with the metastatic process [133–135], and our results

confirmed that NLR was associated with liver metastasis in PDAC patients, in agreement with a previous report [136].

There is increasing evidence connecting KRAS mutations with tumour-promoting inflammation in several human cancers, including PDAC [137,138]. KRAS activation in cancer cells induces the expression and secretion of proinflammatory cytokines, stimulating the recruitment of neutrophils to the tumour [139]. On the other hand, gene dosage of mutant KRAS has an important role in PDAC biology [104], and as we have described above, and other studies have recently reported, there is a correlation of KRAS MAF in cfDNA with clinical stage and outcome in PDAC [105,140]. Importantly, in our study, those patients with RAS-mutated cfDNA had higher NLR values and a positive correlation between NLR and RAS mutational load in cfDNA was found. Furthermore, the combination of NLR with RAS mutational status or load (MAF) in cfDNA greatly improved the prognostic classification of metastatic PDAC patients.

The prognostic significance of cfDNA levels and fragmentation has been previously described in metastatic cancer [111–113], including metastatic PDAC [52]. Specifically, as discussed above, our results support that higher cfDNA concentration and smaller cfDNA fragment size are associated with poor outcomes in metastatic PDAC patients. Our study also shows that PLR negatively correlates with cfDNA fragment size, and this may explain why higher PLR values are associated with more aggressive tumours. Moreover, although apoptosis and necrosis seem to provide most of cfDNA, some stimuli can activate neutrophils leading to DNA release and NETosis [14,141]. A previous study demonstrated a relation between NLR and altered values of cfDNA in endometrial cancer [142]. In this regard, our results showed a high positive correlation between NLR and cfDNA

concentration but not cfDNA fragmentation. Moreover, a positive correlation between neutrophil elastase circulating levels and cfDNA concentration was found, suggesting that neutrophil activation significantly contributes to cfDNA content in plasma of metastatic PDAC patients. Additionally, the positive correlation found between elastase and CA19-9 suggests that neutrophil activation and NETosis are related with disease progression in metastatic PDAC patients. In fact, higher elastase circulating levels were related with liver metastasis and poor OS and PFS. These findings are consistent with the reported interaction of neutrophils with circulating tumour cells facilitating their contact with hepatic endothelial cells, thus helping cancer cells dissemination and liver metastasis [143,144]. Also, inhibition of NETs has been shown to reduce liver metastasis in a preclinical model of metastatic colorectal cancer [145], while recent studies have suggested that NETs may also contribute to hepatic metastasis in PDAC [146].

In conclusion, our study supports that the use of NLR and PLR, along with other non-invasive biomarkers in a multi-parameter prognostic model, may constitute a valuable tool for the clinical management of metastatic PDAC patients.

3. Circulating cell-free DNA-based liquid biopsy markers for the non-invasive prognosis of metastatic colorectal cancer

CRC female patients have better prognosis than males in general population [147]. However, in studies with older patients with advanced disease, OS is poorer in females than in males [148,149]. Moreover, older patients have also been related with poor prognosis [150]. This is in agreement with the results of our study, which included metastatic CRC

patients with a median age of 66 years, and in which female patients had worse OS and PFS than male patients, while older (> 60 years) patients were also associated with a worse outcome.

RAS mutation is routinely assessed in tumour tissue from metastatic CRC patients to predict anti-EGFR therapy response. However, new non-invasive techniques have been developed to analyse RAS mutation in plasma cfDNA. In the present study we used BEAMing for the detection of RAS mutation in plasma cfDNA. Importantly, we obtained a high overall concordance (93%) between tissue and plasma RAS mutation, in agreement with other studies [151–154]. The presence of liver metastases has been related with better detection of RAS mutation in ctDNA [152,155]. Accordingly, we found that concordance between tissue and plasma was higher in patients with liver metastases, in comparison to patients without hepatic lesions. The lower plasma ctDNA representation in CRC patients without hepatic lesions is probably due to inherent biological differences in ctDNA shedding [152].

KRAS mutational status in tumour tissue is controversial as biomarker for prognosis in CRC [156,157]. Notably, in our study prognosis was more accurately predicted by RAS mutation status in plasma than in tissue. Other studies have reported similar results [158,159], although they were focused on patients treated with anti-EGFR therapy independently of RAS mutation status. In agreement with our study, Normanno *et al*, showed that PFS of patients without RAS mutations in tumour tissue but RAS mutated in plasma was similar to that of patients with RAS mutated tissue [159]. Therefore, RAS mutation analysis by liquid biopsy may be a useful tool for a more accurate prognostic stratification of metastatic CRC patients.

We also found that higher RAS MAF values were associated with worse outcome, hepatic lesions and more than one metastatic site. Previous reports have reported the association of higher RAS MAF values with poor prognosis [154,160], with the presence of liver metastases [151,152] and with the number of metastatic locations [152]. However, unlike our study, these studies included patients whose primary tumour or metastasis had been resected before blood drawn, patients with recurrent disease at the time of inclusion and/or patients previously treated with other therapies than anti-EGFR. This is an important aspect because these factors have been associated with a reduction of RAS mutation status overall concordance and MAF values [152,153,161].

Higher levels of cfDNA have been previously found in metastatic CRC patients and have been associated with poor prognosis [162,163]. Accordingly, we found that higher levels of cfDNA were associated with a worse OS and PFS. Besides, there was a trend to higher cfDNA levels in plasma RAS mutated patients, while patients with liver metastasis showed higher cfDNA levels than patients without metastasis in this organ, in agreement with other reports [163,164]. Moreover, we also found that patients with more than one metastatic site presented higher cfDNA values than patients with only one metastatic location.

Smaller fragments of cfDNA have been described in CRC patients compared with healthy donors [165]. Moreover, mutant rather than wild type alleles have been associated to shorter cfDNA fragments [166]. Accordingly, we observed a trend towards higher cfDNA fragmentation in RAS mutated patients, while lower cfDNA fragmentation was significantly associated with better outcome. Remarkably, a positive correlation between cfDNA concentration and cfDNA fragmentation was found, pointing to the tumoral

origin of detected cfDNA. Besides, higher cfDNA fragmentation was also related with liver metastases. Therefore, those CRC patients with liver metastases may be best informed by liquid biopsy, while caution must be exercised with negative results in those patients with metastatic lesions only in other organs.

As discussed above, we have shown that liquid biopsy-based biomarkers, such as plasma cfDNA and RAS mutation status, may greatly help in the prognosis stratification of CRC patients. To our knowledge there is one study to date reporting the combination of cfDNA and RAS mutation status in plasma for the prognosis stratification in CRC, but this study only included CRC patients prior to receive anti-EGFR therapy [163]. On the other hand, CEA is widely used as a biomarker for detection of recurrent CRC and for monitoring the response to systemic therapy [167,168], although its prognostic value must be improved with other biomarkers, as around 20% of CRC tumours are CEA negative [169]. Notably, here we report that, at the time of diagnosis, the combination of liquid biopsy-based biomarkers cfDNA and RAS mutation status with the standard biomarker CEA greatly improves prognostic stratification of metastatic CRC patients.

In conclusion, our results support that circulating cell-free DNA-based liquid biopsy markers may greatly help in the non-invasive prognostic stratification of metastatic CRC patients.

4. Circulating microRNAs as liquid biopsy biomarkers to predict response to treatment with FOLFIRI plus aflibercept and prognosis of metastatic colorectal cancer patients

Several miRNAs have been reported to be involved in tumour angiogenesis regulation and they participate both in the promotion or the

inhibition of angiogenic processes [170,171]. In the present study we found a differential circulating miRNA profile between metastatic CRC patients responding or not responding to treatment with chemotherapy plus the anti-angiogenic drug aflibercept. Notably, these miRNAs were not only related with colorectal cancer but most of them were also involved in angiogenesis signalling.

Our study also demonstrates the usefulness of some clinical variables in combination with the circulating levels of some of these miRNAs to predict the response to therapy, the progression of disease and survival in patients treated with FOLFIRI plus aflibercept. Hence, our model of response predicts a very good chance of response to treatment with FOLFIRI plus aflibercept in those patients with primary tumour located in rectum, along with minimum expression of hsa-miR-30a-3p and high expression of hsa-miR-33b-5p. Members of the miR-30 family act either as tumour suppressor miRNAs or oncomiRNAs [172]. However, the miR-30 family has been shown to regulate angiogenesis [173] and particularly, downregulation of hsa-miR-30a-3p impairs endothelial angiogenic activity [174], while this miRNA has been reported to promote the angiogenic potential of melanoma cells [175]. Accordingly, we found a higher probability of response to FOLFIRI plus aflibercept treatment in those patients with lower circulating levels of this angiogenic miRNA. As for hsa-miR-33b-5p, this miRNA has been described as tumour suppressor in several cancers [176,177], including CRC, where high miR-33b expression in tumours was related with better prognosis of patients [178]. Moreover, hsa-miR-33b-5p has been associated with the suppression of HMGA2 (High Mobility Group A 2) gene in several types of cancers leading to the inhibition of cancer cell growth [179] and, specifically in gastric cancer, this

suppression also sensitized cancer cells to chemotherapy drugs [180]. Interestingly, HMGA2 promotes angiogenesis in several tumours [181,182] and chemoresistance to 5-FU therapy in CRC [183]. Correspondingly, we found higher circulating levels of hsa-miR-33b-5p in those patients with better response to FOLFIRI plus aflibercept treatment, raising the possibility that miR-33b-HMGA2 signalling may be involved in the sensitivity of tumours to chemotherapy plus anti-angiogenic therapy.

Remarkably, hsa-miR-33b-5p was also one of the miRNAs identified in our predictive model of disease progression. Furthermore, our results support that this miRNA may constitute a valuable single biomarker to differentiate between responders and non-responders patients and also to predict the risk of disease progression in patients treated with FOLFIRI plus aflibercept.

In summary, distinct basal circulating miRNAs profiles were found in metastatic CRC patients according to their response to FOLFIRI plus aflibercept treatment, their risk of disease progression and survival. Further studies are warranted to validate the value of circulating miRNAs as valuable liquid-biopsy biomarkers for predicting the response to aflibercept and other anti-angiogenic drugs in metastatic CRC patients.

VII. Conclusions

1. Higher circulating RAS MAF, cfDNA levels and fragmentation in metastatic pancreatic adenocarcinoma (PDAC) patients are associated with poor prognosis and their combination with standard CA19-9 marker greatly improves the prognostic stratification of these patients.

2. Dynamics of RAS MAF better correlated with the outcome of metastatic PDAC patients than CA19-9 and constitutes a promising non-invasive clinical tool for monitoring disease progression and response to therapy.

3. The combination of NLR and PLR with cfDNA-based liquid biopsy markers greatly improves the prognostic classification of metastatic PDAC patients.

4. Higher RAS MAF, cfDNA levels and fragmentation in metastatic colorectal cancer (CRC) patients are associated with poor prognosis, and their combination with standard CEA marker greatly improves the prognostic stratification of these patients.

5. The analysis of circulating microRNAs improves the prediction of outcome in metastatic CRC patients treated with FOLFIRI plus aflibercept and may constitute a valuable predictive biomarker of response to anti-angiogenic therapy in these patients.

VIII. References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin*. **2018**, *68*, 394–424, doi:10.3322/caac.21492.

2. Goossens, N.; Nakagawa, S.; Sun, X.; Hoshida, Y. Cancer biomarker discovery and validation. *Transl. Cancer Res.* **2015**, *4*, 256–269.

3. Marrugo-Ramírez, J.; Mir, M.; Samitier, J. Blood-based cancer biomarkers in liquid biopsy: A promising non-invasive alternative to tissue biopsy. *Int. J. Mol. Sci.* **2018**, *19*, doi: 10.3390/ijms19102877.

4. Heitzer, E.; Haque, I.S.; Roberts, C.E.S.; Speicher, M.R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat. Rev. Genet.* **2019**, *20*, 71–88.

5. Mattox, A.K.; Bettegowda, C.; Zhou, S.; Papadopoulos, N.; Kinzler, K.W.; Vogelstein, B. Applications of liquid biopsies for cancer. *Sci. Transl. Med.* **2019**, *11*, doi:10.1126/scitranslmed.aay1984.

6. Lianidou, E.; Pantel, K. Liquid biopsies. *Genes Chromosom. Cancer* **2019**, *58*, 219–232.

7. Rossi, E.; Fabbri, F. CTCs 2020: Great Expectations or Unreasonable Dreams. *Cells* **2019**, *8*, doi: 10.3390/cells8090989.

8. Krebs, M.G.; Hou, J.M.; Ward, T.H.; Blackhall, F.H.; Dive, C. Circulating tumour cells: Their utility in cancer management and predicting outcomes. *Ther. Adv. Med. Oncol.* **2010**, *2*, 351–365.

9. Dongre, A.; Weinberg, R.A. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 69–84.

10. Eslami-S, Z.; Cortés-Hernández, L.E.; Alix-Panabières, C. Circulating tumor cells: moving forward into clinical applications. *Precis. Cancer Med.* **2020**, *3*, 4–4, doi:10.21037/pcm.2019.11.07.

11. Raimondi, C.; Gradilone, A.; Gazzaniga, P. Controversies in circulating tumor cell count during therapy. *Expert Rev. Mol. Diagn.* **2013**, *13*, 499–507.

12. Snyder, M.W.; Kircher, M.; Hill, A.J.; Daza, R.M.; Shendure, J. Cell-free DNA Comprises an in Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell* **2016**, *164*, 57–68, doi:10.1016/j.cell.2015.11.050.

13. Bronkhorst, A.J.; Ungerer, V.; Holdenrieder, S. The emerging role of cell-free DNA as a molecular marker for cancer management. *Biomol. Detect. Quantif.* **2019**, *17*, doi: 10.1016/j.bdq.2019.100087.

14. Kustanovich, A.; Schwartz, R.; Peretz, T.; Grinshpun, A. Life and death of circulating cell-free DNA. *Cancer Biol. Ther.* **2019**, *20*, 1057–1067.

15. Schwarzenbach, H.; Hoon, D.S.B.; Pantel, K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat. Rev. Cancer* **2011**, *11*, 426–437.

16. Mouliere, F.; Chandrananda, D.; Piskorz, A.M.; Moore, E.K.; Morris, J.; Ahlborn, L.B.; Mair, R.; Goranova, T.; Marass, F.; Heider, K.; et al. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci. Transl. Med.* **2018**, *10*, doi:10.1126/scitranslmed.aat4921.

17. Cristiano, S.; Leal, A.; Phallen, J.; Fiksel, J.; Adleff, V.; Bruhm, D.C.; Jensen, S.Ø.; Medina, J.E.; Hruban, C.; White, J.R.; et al. Genome-

wide cell-free DNA fragmentation in patients with cancer. *Nature* **2019**, *570*, 385–389, doi:10.1038/s41586-019-1272-6.

18. Volik, S.; Alcaide, M.; Morin, R.D.; Collins, C. Cell-free DNA (cfDNA): Clinical significance and utility in cancer shaped by emerging technologies. *Mol. Cancer Res.* **2016**, *14*, 898–908.

19. Condrat, C.E.; Thompson, D.C.; Barbu, M.G.; Bugnar, O.L.; Boboc, A.; Cretoiu, D.; Suciu, N.; Cretoiu, S.M.; Voinea, S.C. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells* **2020**, *9*, doi: 10.3390/cells9020276.

20. Filipów, S.; Łaczmański, Ł. Blood circulating miRNAs as cancer biomarkers for diagnosis and surgical treatment response. *Front. Neurosci.* **2019**, *10*, doi: 10.3389/fgene.2019.00169.

21. Wang, H.; Peng, R.; Wang, J.; Qin, Z.; Xue, L. Circulating microRNAs as potential cancer biomarkers: The advantage and disadvantage. *Clin. Epigenetics* **2018**, *10*, doi: 10.1186/s13148-018-0492-1.

22. Cui, M.; Wang, H.; Yao, X.; Zhang, D.; Xie, Y.; Cui, R.; Zhang, X. Circulating MicroRNAs in Cancer: Potential and Challenge. *Front. Genet.* **2019**, *10*, doi:10.3389/fgene.2019.00626.

23. Lane, R.E.; Korbie, D.; Hill, M.M.; Trau, M. Extracellular vesicles as circulating cancer biomarkers: opportunities and challenges. *Clin. Transl. Med.* **2018**, *7*, 14, doi:10.1186/s40169-018-0192-7.

24. Van Niel, G.; D'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 213–228.

25. Kosaka, N.; Kogure, A.; Yamamoto, T.; Urabe, F.; Usuba, W.; Prieto-Vila, M.; Ochiya, T. Exploiting the message from cancer: the diagnostic value of extracellular vesicles for clinical applications. *Exp. Mol. Med.* **2019**, *51*, doi:10.1038/s12276-019-0219-1.

26. Hessvik, N.P.; Llorente, A. Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci.* **2018**, *75*, 193–208.

27. Latifkar, A.; Hur, Y.H.; Sanchez, J.C.; Cerione, R.A.; Antonyak, M.A. New insights into extracellular vesicle biogenesis and function. *J. Cell Sci.* **2019**, *132*, doi: 10.1242/jcs.222406.

28. Ferlay, J.; Colombet, M.; Soerjomataram, I.; Dyba, T.; Randi, G.; Bettio, M.; Gavin, A.; Visser, O.; Bray, F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur. J. Cancer* **2018**, *103*, 356–387.

29. Rahib, L.; Smith, B.D.; Aizenberg, R.; Rosenzweig, A.B.; Fleshman, J.M.; Matrisian, L.M. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the united states. *Cancer Res.* **2014**, *74*, 2913–2921.

30. Ryan, D.P.; Hong, T.S.; Bardeesy, N. Pancreatic Adenocarcinoma. *N. Engl. J. Med.* **2014**, *371*, 1039–1049, doi:10.1056/NEJMra1404198.

31. Rawla, P.; Sunkara, T.; Gaduputi, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J. Oncol.* **2019**, *10*, 10–27, doi:10.14740/wjon1166.

32. McGuigan, A.; Kelly, P.; Turkington, R.C.; Jones, C.; Coleman, H.G.; McCain, R.S. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J. Gastroenterol.* **2018**,

24, 4846–4861.

33. Bartsch, D.K.; Gress, T.M.; Langer, P. Familial pancreatic cancer-current knowledge. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9*, 445–453.

34. Zhang, Q.; Zeng, L.; Chen, Y.; Lian, G.; Qian, C.; Chen, S.; Li, J.; Huang, K. Pancreatic Cancer Epidemiology, Detection, and Management. *Gastroenterol. Res. Pract.* **2016**, *2016*, doi: 10.1155/2016/8962321

35. Chhoda, A.; Lu, L.; Clerkin, B.M.; Risch, H.; Farrell, J.J. Current Approaches to Pancreatic Cancer Screening. *Am. J. Pathol.* **2019**, *189*, 22–35.

36. Cong, L.; Liu, Q.; Zhang, R.; Cui, M.; Zhang, X.; Gao, X.; Guo, J.; Dai, M.; Zhang, T.; Liao, Q.; et al. Tumor size classification of the 8th edition of TNM staging system is superior to that of the 7th edition in predicting the survival outcome of pancreatic cancer patients after radical resection and adjuvant chemotherapy. *Sci. Rep.* **2018**, *8*, 10383–10383, doi:10.1038/s41598-018-28193-4.

37. Ling, Q.; Xu, X.; Zheng, S. Sen; Kalthoff, H. The diversity between pancreatic head and body/tail cancers: Clinical parameters and in vitro models. *Hepatobiliary Pancreat. Dis. Int.* **2013**, *12*, 480–487.

38. van Erning, F.N.; Mackay, T.M.; van der Geest, L.G.M.; Groot Koerkamp, B.; van Laarhoven, H.W.M.; Bonsing, B.A.; Wilmink, J.W.; van Santvoort, H.C.; de Vos-Geelen, J.; van Eijck, C.H.J.; et al. Association of the location of pancreatic ductal adenocarcinoma (head, body, tail) with tumor stage, treatment, and survival: a

population-based analysis. *Acta Oncol. (Madr).* **2018**, *57*, 1655–1662, doi:10.1080/0284186X.2018.1518593.

39. Vladov, N.; Takorov, I.; Lukanova, T. The Role of Vascular Resection in Pancreatic Cancer Treatment. *InTech Open*, **2017**, doi: 10.5772/66910

40. Mackay, T.M.; van Erning, F.N.; van der Geest, L.G.M.; de Groot, J.W.B.; Haj Mohammad, N.; Lemmens, V.E.; van Laarhoven, H.W.; Besselink, M.G.; Wilmink, J.W. Association between primary origin (head, body and tail) of metastasised pancreatic ductal adenocarcinoma and oncologic outcome: A population-based analysis. *Eur. J. Cancer* **2019**, *106*, 99–105, doi:10.1016/j.ejca.2018.10.008.

41. Gupta, R.; Amanam, I.; Chung, V. Current and future therapies for advanced pancreatic cancer. *J. Surg. Oncol.* **2017**, *116*, 25–34.

42. Mizrahi, J.D.; Surana, R.; Valle, J.W.; Shroff, R.T. Pancreatic cancer. *Lancet* **2020**, *395*, 2008–2020.

43. Lanfredini, S.; Thapa, A.; O'Neill, E. RAS in pancreatic cancer. *Biochem. Soc. Trans.* **2019**, *47*, 961–972.

44. Waters, A.M.; Der, C.J. KRAS: The critical driver and therapeutic target for pancreatic cancer. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, doi:10.1101/cshperspect.a031435.

45. Bryant, K.L.; Mancias, J.D.; Kimmelman, A.C.; Der, C.J. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem. Sci.* **2014**, *39*, 91–100, doi:10.1016/j.tibs.2013.12.004.

46. Buscail, L.; Bournet, B.; Cordelier, P. Role of oncogenic KRAS in the diagnosis, prognosis and treatment of pancreatic cancer. *Nat. Rev.*

Gastroenterol. Hepatol. **2020**, *17*, 153–168.

47. Tao, L. yuan; Zhang, L. fu; Xiu, D. rong; Yuan, C. hui; Ma, Z. lai; Jiang, B. Prognostic significance of K-ras mutations in pancreatic cancer: A meta-analysis. *World J. Surg. Oncol.* **2016**, *14*, doi:10.1186/s12957-016-0888-3.

48. Singh, N.; Gupta, S.; Pandey, R.M.; Chauhan, S.S.; Saraya, A. High levels of cell-free circulating nucleic acids in pancreatic cancer are associated with vascular encasement, metastasis and poor survival. *Cancer Invest.* **2015**, *33*, 78–85, doi:10.3109/07357907.2014.1001894.

49. Sikora, K.; Bedin, C.; Vicentini, C.; Malpeli, G.; D'Angelo, E.; Sperandio, N.; Lawlor, R.T.; Bassi, C.; Tortora, G.; Nitti, D.; et al. Evaluation of cell-free DNA as a biomarker for pancreatic malignancies. *Int. J. Biol. Markers* **2015**, *30*, e136–e141, doi:10.5301/jbm.5000088.

50. Takai, E.; Totoki, Y.; Nakamura, H.; Morizane, C.; Nara, S.; Hama, N.; Suzuki, M.; Furukawa, E.; Kato, M.; Hayashi, H.; et al. Clinical utility of circulating tumor DNA for molecular assessment in pancreatic cancer. *Sci. Rep.* **2015**, *5*, doi:10.1038/srep18425.

51. Chen, L.; Zhang, Y.; Cheng, Y.; Zhang, D.; Zhu, S.; Ma, X. Prognostic value of circulating cell-free DNA in patients with pancreatic cancer: A systemic review and meta-analysis. *Gene* **2018**, *679*, 328–334.

52. Lapin, M.; Oltedal, S.; Tjensvoll, K.; Buhl, T.; Smaaland, R.; Garresori, H.; Javle, M.; Glenjen, N.I.; Abelseth, B.K.; Gilje, B.; et al.

Fragment size and level of cell-free DNA provide prognostic information in patients with advanced pancreatic cancer. *J. Transl. Med.* **2018**, *16*, doi:10.1186/s12967-018-1677-2.

53. Grunvald, M.W.; Jacobson, R.A.; Kuzel, T.M.; Pappas, S.G.; Masood, A. Current status of circulating tumor dna liquid biopsy in pancreatic cancer. *Int. J. Mol. Sci.* **2020**, *21*, 1–15.

54. Bettegowda, C.; Sausen, M.; Leary, R.J.; Kinde, I.; Wang, Y.; Agrawal, N.; Bartlett, B.R.; Wang, H.; Luber, B.; Alani, R.M.; et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* **2014**, *6*, doi:10.1126/scitranslmed.3007094.

55. Lee, J.S.; Rhee, T.M.; Pietrasz, D.; Bachet, J.B.; Laurent-Puig, P.; Kong, S.Y.; Takai, E.; Yachida, S.; Shibata, T.; Lee, J.W.; et al. Circulating tumor DNA as a prognostic indicator in resectable pancreatic ductal adenocarcinoma: A systematic review and meta-analysis. *Sci. Rep.* **2019**, *9*, doi:10.1038/s41598-019-53271-6.

56. Loft, M.; Lee, B.; Tie, J.; Gibbs, P. Clinical applications of circulating tumour DNA in pancreatic adenocarcinoma. *J. Pers. Med.* **2019**, *9*, doi: 10.3390/jpm9030037.

57. Sausen, M.; Phallen, J.; Adleff, V.; Jones, S.; Leary, R.J.; Barrett, M.T.; Anagnostou, V.; Parpart-Li, S.; Murphy, D.; Li, Q.K.; et al. Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. *Nat. Commun.* **2015**, *6*, doi:10.1038/ncomms8686.

58. Dekker, E.; Tanis, P.J.; Vleugels, J.L.A.; Kasi, P.M.; Wallace, M.B.

Colorectal cancer. *Lancet* **2019**, *394*, 1467–1480.

59. Rawla, P.; Sunkara, T.; Barsouk, A. Epidemiology of colorectal cancer: Incidence, mortality, survival, and risk factors. *Prz. Gastroenterol.* **2019**, *14*, 89–103.

60. De La Chapelle, A. Genetic predisposition to colorectal cancer. *Nat. Rev. Cancer* **2004**, *4*, 769–780.

61. Armaghany, T.; Wilson, J.D.; Chu, Q.; Mills, G. Genetic alterations in colorectal cancer. *Gastrointest. Cancer Res.* **2012**, *5*, 19–27.

62. Chung, T.P.; Fleshman, J.W. The genetics of sporadic colon cancer. *Semin. Colon Rectal Surg.* **2004**, *15*, 128–135, doi:10.1053/j.scrs.2005.02.001.

63. Mármol, I.; Sánchez-de-Diego, C.; Dieste, A.P.; Cerrada, E.; Yoldi, M.J.R. Colorectal carcinoma: A general overview and future perspectives in colorectal cancer. *Int. J. Mol. Sci.* **2017**, *18*, doi: 10.3390/ijms18010197.

64. Nguyen, H.T.; Duong, H.Q. The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy (review). *Oncol. Lett.* **2018**, *16*, 9–18.

65. Mundade, R.; Imperiale, T.F.; Prabhu, L.; Loehrer, P.J.; Lu, T. Genetic pathways, prevention, and treatment of sporadic colorectal cancer. *Oncoscience* **2014**, *1*, 400–406, doi:10.18632/oncoscience.59.

66. Mattiuzzi, C.; Sanchis-Gomar, F.; Lippi, G. Concise update on colorectal cancer epidemiology. *Ann. Transl. Med.* **2019**, *7*, 609–609, doi:10.21037/atm.2019.07.91.

67. Rosen, R.D.; Sapra, A. TNM Classification. *StatPearls Publishing*

2021.

68. Baran, B.; Mert Ozupek, N.; Yerli Tetik, N.; Acar, E.; Bekcioglu, O.; Baskin, Y. Difference Between Left-Sided and Right-Sided Colorectal Cancer: A Focused Review of Literature. *Gastroenterol. Res.* **2018**, *11*, 264–273, doi:10.14740/gr1062w.

69. Ghidini, M.; Petrelli, F.; Tomasello, G. Right Versus Left Colon Cancer: Resectable and Metastatic Disease. *Curr. Treat. Options Oncol.* **2018**, *19*, doi: 10.1007/s11864-018-0544-y.

70. Goldstein, J.; Tran, B.; Ensor, J.; Gibbs, P.; Wong, H.L.; Wong, S.F.; Vilar, E.; Tie, J.; Broaddus, R.; Kopetz, S.; et al. Multicenter retrospective analysis of metastatic colorectal cancer (CRC) with high-level microsatellite instability (MSI-H). *Ann. Oncol.* **2014**, *25*, 1032–1038, doi:10.1093/annonc/mdu100.

71. Holch, J.W.; Demmer, M.; Lamersdorf, C.; Michl, M.; Schulz, C.; von Einem, J.C.; Modest, D.P.; Heinemann, V. Pattern and Dynamics of Distant Metastases in Metastatic Colorectal Cancer. *Visc. Med.* **2017**, *33*, 70–75, doi:10.1159/000454687.

72. Riihimaki, M.; Hemminki, A.; Sundquist, J.; Hemminki, K. Patterns of metastasis in colon and rectal cancer. *Sci. Rep.* **2016**, *6*, doi:10.1038/srep29765.

73. Wang, J.; Li, S.; Liu, Y.; Zhang, C.; Li, H.; Lai, B. Metastatic patterns and survival outcomes in patients with stage IV colon cancer: A population-based analysis. *Cancer Med.* **2020**, *9*, 361–373, doi:10.1002/cam4.2673.

74. Prasanna, T.; Karapetis, C.S.; Roder, D.; Tie, J.; Padbury, R.; Price,

T.; Wong, R.; Shapiro, J.; Nott, L.; Lee, M.; et al. The survival outcome of patients with metastatic colorectal cancer based on the site of metastases and the impact of molecular markers and site of primary cancer on metastatic pattern. *Acta Oncol. (Madr).* **2018**, *57*, 1438–1444, doi:10.1080/0284186X.2018.1487581.

75. Brown, K.G.M.; Solomon, M.J.; Mahon, K.; O'Shannassy, S. Management of colorectal cancer. *BMJ* **2019**, *366*, doi: 10.1136/bmj.l4561.

76. Ganesh, K.; Stadler, Z.K.; Cercek, A.; Mendelsohn, R.B.; Shia, J.; Segal, N.H.; Diaz, L.A. Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 361–375.

77. Gong, J.; Cho, M.; Fakih, M. RAS and BRAF in metastatic colorectal cancer management. *J. Gastrointest. Oncol.* **2016**, *7*, 687–704.

78. Zhao, B.; Wang, L.; Qiu, H.; Zhang, M.; Sun, L.; Peng, P.; Yu, Q.; Yuan, X. Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. *Oncotarget* **2017**, *8*, 3980–4000, doi:10.18632/oncotarget.14012.

79. Li, Z.N.; Zhao, L.; Yu, L.F.; Wei, M.J. BRAF and KRAS mutations in metastatic colorectal cancer: Future perspectives for personalized therapy. *Gastroenterol. Rep.* **2020**, *8*, 192–205.

80. Sorich, M.J.; Wiese, M.D.; Rowland, A.; Kichenadasse, G.; McKinnon, R.A.; Karapetis, C.S. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: A meta-analysis of randomized, controlled trials. *Ann. Oncol.*

2015, *26*, 13–21.

81. Soulières, D.; Greer, W.; Magliocco, A.M.; Huntsman, D.; Young, S.; Tsao, M.-S.; Kamel-Reid, S. KRAS Mutation Testing in the Treatment of Metastatic Colorectal Cancer with Anti-EGFR Therapies. *Curr. Oncol.* **2010**, *17*, 31–40, doi:10.3747/co.v17is1.614.

82. Yamada, T.; Matsuda, A.; Koizumi, M.; Shinji, S.; Takahashi, G.; Iwai, T.; Takeda, K.; Ueda, K.; Yokoyama, Y.; Hara, K.; et al. Liquid biopsy for the management of patients with colorectal cancer. *Digestion* **2019**, 99, 39–45.

83. Vymetalkova, V.; Cervena, K.; Bartu, L.; Vodicka, P. Circulating cell-free dna and colorectal cancer: A systematic review. *Int. J. Mol. Sci.* **2018**, *19*, doi: 10.3390/ijms19113356.

84. Spindler, K.L.G. Methodological, biological and clinical aspects of circulating free DNA in metastatic colorectal cancer. *Acta Oncol. (Madr).* **2017**, *56*, 7–16.

85. Petit, J.; Carroll, G.; Gould, T.; Pockney, P.; Dun, M.; Scott, R.J. Cell-free DNA as a Diagnostic Blood-Based Biomarker for Colorectal Cancer: A Systematic Review. *J. Surg. Res.* **2019**, *236*, 184–197.

86. De Rubis, G.; Rajeev Krishnan, S.; Bebawy, M. Liquid Biopsies in Cancer Diagnosis, Monitoring, and Prognosis. *Trends Pharmacol. Sci.* **2019**, *40*, 172–186.

87. Berger, A.W.; Schwerdel, D.; Welz, H.; Marienfeld, R.; Schmidt, S.A.; Kleger, A.; Ettrich, T.J.; Seufferlein, T. Treatment monitoring in metastatic colorectal cancer patients by quantification and KRAS genotyping of circulating cell-free DNA. *PLoS One* **2017**, *12*,

doi:10.1371/journal.pone.0174308.

88. Rapado-González, Ó.; Álvarez-Castro, A.; López-López, R.; Iglesias-Canle, J.; Suárez-Cunqueiro, M.M.; Muinelo-Romay, L. Circulating microRNAs as promising biomarkers in colorectal cancer. *Cancers (Basel)*. **2019**, *11*, doi:10.3390/cancers11070898.

89. Marcuello, M.; Vymetalkova, V.; Neves, R.P.L.; Duran-Sanchon, S.; Vedeld, H.M.; Tham, E.; van Dalum, G.; Flügen, G.; Garcia-Barberan, V.; Fijneman, R.J.; et al. Circulating biomarkers for early detection and clinical management of colorectal cancer. *Mol. Aspects Med.* **2019**, *69*, 107–122.

90. Ferracin, M.; Lupini, L.; Mangolini, A.; Negrini, M. Circulating non-coding RNA as biomarkers in colorectal cancer. *Adv. Exp. Med. Biol.* **2016**, *937*, 171-81, doi: 10.1007/978-3-319-42059-2_9.

91. Vivancos, A.; Aranda, E.; Benavides, M.; Élez, E.; Gómez-España, M.A.; Toledano, M.; Alvarez, M.; Parrado, M.R.C.; García-Barberán, V.; Diaz-Rubio, E. Comparison of the Clinical Sensitivity of the Idylla Platform and the OncoBEAM RAS CRC Assay for KRAS Mutation Detection in Liquid Biopsy Samples. *Sci. Rep.* **2019**, *9*, doi:10.1038/s41598-019-45616-y.

92. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **2004**, *64*, 5245–5250, doi:10.1158/0008-5472.CAN-04-0496.

93. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression

data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **2001**, *25*, 402–408, doi:10.1006/meth.2001.1262.

94. Li, J.; Han, X.; Wan, Y.; Zhang, S.; Zhao, Y.; Fan, R.; Cui, Q.; Zhou, Y. TAM 2.0: Tool for MicroRNA set analysis. *Nucleic Acids Res.* **2018**, *46*, W180–W185, doi:10.1093/nar/gky509.

95. Xia, J.; Psychogios, N.; Young, N.; Wishart, D.S. MetaboAnalyst: A web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* **2009**, *37*, doi:10.1093/nar/gkp356.

96. Heagerty, P.J.; Lumley, T.; Pepe, M.S. Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics* **2000**, *56*, 337–344, doi:10.1111/j.0006-341X.2000.00337.x.

97. Kinugasa, H.; Nouso, K.; Miyahara, K.; Morimoto, Y.; Dohi, C.; Tsutsumi, K.; Kato, H.; Matsubara, T.; Okada, H.; Yamamoto, K. Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. *Cancer* **2015**, *121*, 2271–2280, doi:10.1002/cncr.29364.

98. Park, G.; Park, J.K.; Son, D.S.; Shin, S.H.; Kim, Y.J.; Jeon, H.J.; Lee, J.; Park, W.Y.; Lee, K.H.; Park, D. Utility of targeted deep sequencing for detecting circulating tumor DNA in pancreatic cancer patients. *Sci. Rep.* **2018**, *8*, doi:10.1038/s41598-018-30100-w.

99. Wang, Z.Y.; Ding, X.Q.; Zhu, H.; Wang, R.X.; Pan, X.R.; Tong, J.H. KRAS Mutant Allele Fraction in Circulating Cell-Free DNA Correlates With Clinical Stage in Pancreatic Cancer Patients. *Front. Oncol.* **2019**, *9*, doi:10.3389/fonc.2019.01295.

100. Chen, I.; Raymond, V.M.; Geis, J.A.; Collisson, E.A.; Jensen, B. V;

Hermann, K.L.; Erlander, M.G.; Tempero, M.; Johansen, J.S. Ultrasensitive plasma ctDNA KRAS assay for detection, prognosis, and assessment of therapeutic response in patients with unresectable pancreatic ductal adenocarcinoma. *Oncotarget* **2017**, *8*, 97769–97786, doi:10.18632/oncotarget.22080.

101. Perets, R.; Greenberg, O.; Shentzer, T.; Semenisty, V.; Epelbaum, R.; Bick, T.; Sarji, S.; Ben-Izhak, O.; Sabo, E.; Hershkovitz, D. Mutant KRAS Circulating Tumor DNA Is an Accurate Tool for Pancreatic Cancer Monitoring. *Oncologist* **2018**, *23*, 566–572, doi:10.1634/theoncologist.2017-0467.

102. Hadano, N.; Murakami, Y.; Uemura, K.; Hashimoto, Y.; Kondo, N.; Nakagawa, N.; Sueda, T.; Hiyama, E. Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. *Br J Cancer* **2016**, *115*, 59–65, doi:10.1038/bjc.2016.175.

103. Gall, T.M.H.; Belete, S.; Khanderia, E.; Frampton, A.E.; Jiao, L.R. Circulating Tumor Cells and Cell-Free DNA in Pancreatic Ductal Adenocarcinoma. *Am J Pathol* **2019**, *189*, 71–81, doi:10.1016/j.ajpath.2018.03.020.

104. Lennerz, J.K.; Stenzinger, A. Allelic ratio of KRAS mutations in pancreatic cancer. *Oncologist* **2015**, *20*, e8-9, doi:10.1634/theoncologist.2014-0408.

105. Bernard, V.; Kim, D.U.; San Lucas, F.A.; Castillo, J.; Allenson, K.; Mulu, F.C.; Stephens, B.M.; Huang, J.; Semaan, A.; Guerrero, P.A.; et al. Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* **2019**, *156*, 108-118 e4, doi:10.1053/j.gastro.2018.09.022.

106. Mueller, S.; Engleitner, T.; Maresch, R.; Zukowska, M.; Lange, S.; Kaltenbacher, T.; Konukiewitz, B.; Ollinger, R.; Zwiebel, M.; Strong, A.; et al. Evolutionary routes and KRAS dosage define pancreatic cancer phenotypes. *Nature* **2018**, *554*, 62–68, doi:10.1038/nature25459.

107. Birnbaum, D.J.; Bertucci, F.; Finetti, P.; Birnbaum, D.; Mamessier, E. Head and Body/Tail Pancreatic Carcinomas Are Not the Same Tumors. *Cancers (Basel)* **2019**, *11*, doi:10.3390/cancers11040497.

108. Dreyer, S.B.; Jamieson, N.B.; Upstill-Goddard, R.; Bailey, P.J.; McKay, C.J.; Biankin, A. V.; Chang, D.K. Defining the molecular pathology of pancreatic body and tail adenocarcinoma. *Br. J. Surg.* **2018**, doi:10.1002/bjs.10772.

109. Tomasello, G.; Ghidini, M.; Costanzo, A.; Ghidini, A.; Russo, A.; Barni, S.; Passalacqua, R.; Petrelli, F. Outcome of head compared to body and tail pancreatic cancer: a systematic review and meta-analysis of 93 studies. *J Gastrointest Oncol* **2019**, *10*, 259–269, doi:10.21037/jgo.2018.12.08.

110. Artinyan, A.; Soriano, P.A.; Prendergast, C.; Low, T.; Ellenhorn, J.D.; Kim, J. The anatomic location of pancreatic cancer is a prognostic factor for survival. *HPB* **2008**, *10*, 371–376, doi:10.1080/13651820802291233.

111. El Messaoudi, S.; Mouliere, F.; Du Manoir, S.; Bascoul-Mollevi, C.; Gillet, B.; Nouaille, M.; Fiess, C.; Crapez, E.; Bibeau, F.; Theillet, C.; et al. Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. *Clin Cancer Res* **2016**, *22*, 3067–3077, doi:10.1158/1078-0432.CCR-15-0297.

112. Tabernero, J.; Lenz, H.J.; Siena, S.; Sobrero, A.; Falcone, A.; Ychou, M.; Humblet, Y.; Bouche, O.; Mineur, L.; Barone, C.; et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol* **2015**, *16*, 937–948, doi:10.1016/S1470-2045(15)00138-2.

113. Valpione, S.; Gremel, G.; Mundra, P.; Middlehurst, P.; Galvani, E.; Girotti, M.R.; Lee, R.J.; Garner, G.; Dhomen, N.; Lorigan, P.C.; et al. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *Eur J Cancer* **2018**, *88*, 1–9, doi:10.1016/j.ejca.2017.10.029.

114. Heitzer, E.; Auinger, L.; Speicher, M.R. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends Mol Med* **2020**, *26*, 519–528, doi:10.1016/j.molmed.2020.01.012.

115. Zvereva, M.; Roberti, G.; Durand, G.; Voegele, C.; Nguyen, M.D.; Delhomme, T.M.; Chopard, P.; Fabianova, E.; Adamcakova, Z.; Holcatova, I.; et al. Circulating tumour-derived KRAS mutations in pancreatic cancer cases are predominantly carried by very short fragments of cell-free DNA. *EBioMedicine* **2020**, 102462, doi:10.1016/j.ebiom.2019.09.042.

116. Saad, E.D.; Machado, M.C.; Wajsbrot, D.; Abramoff, R.; Hoff, P.M.; Tabacof, J.; Katz, A.; Simon, S.D.; Gansl, R.C. Pretreatment CA 19-9 level as a prognostic factor in patients with advanced pancreatic cancer treated with gemcitabine. *Int J Gastrointest Cancer* **2002**, *32*,

35–41, doi:10.1385/IJGC:32:1:35.

117. Maisey, N.R.; Norman, A.R.; Hill, A.; Massey, A.; Oates, J.; Cunningham, D. CA19-9 as a prognostic factor in inoperable pancreatic cancer: the implication for clinical trials. *Br J Cancer* **2005**, *93*, 740–743, doi:10.1038/sj.bjc.6602760.

118. Scara, S.; Bottoni, P.; Scatena, R. CA 19-9: Biochemical and Clinical Aspects. *Adv Exp Med Biol* **2015**, *867*, 247–260, doi:10.1007/978-94-017-7215-0_15.

119. Bauer, T.M.; El-Rayes, B.F.; Li, X.; Hammad, N.; Philip, P.A.; Shields, A.F.; Zalupski, M.M.; Bekaii-Saab, T. Carbohydrate antigen 19-9 is a prognostic and predictive biomarker in patients with advanced pancreatic cancer who receive gemcitabine-containing chemotherapy: a pooled analysis of 6 prospective trials. *Cancer* **2013**, *119*, 285–292, doi:10.1002/cncr.27734.

120. van der Sijde, F.; Vietsch, E.E.; Mustafa, D.A.M.; Besselink, M.G.; Groot Koerkamp, B.; van Eijck, C.H.J. Circulating Biomarkers for Prediction of Objective Response to Chemotherapy in Pancreatic Cancer Patients. *Cancers (Basel)* **2019**, *11*, doi:10.3390/cancers11010093.

121. Shi, J.; Xue, J. Inflammation and development of pancreatic ductal adenocarcinoma. *Chinese Clin. Oncol.* **2019**, *8*, 19, doi:10.21037/cco.2019.04.02.

122. Luo, G.; Guo, M.; Liu, Z.; Xiao, Z.; Jin, K.; Long, J.; Liu, L.; Liu, C.; Xu, J.; Ni, Q.; et al. Blood Neutrophil–Lymphocyte Ratio Predicts Survival in Patients with Advanced Pancreatic Cancer Treated with

Chemotherapy. *Ann. Surg. Oncol.* **2015**, *22*, 670–6, doi:10.1245/s10434-014-4021-y.

123. An, X.; Ding, P.R.; Li, Y.H.; Wang, F.H.; Shi, Y.X.; Wang, Z.Q.; He, Y.J.; Xu, R.H.; Jiang, W.Q. Elevated neutrophil to lymphocyte ratio predicts survival in advanced pancreatic cancer. *Biomarkers* **2010**, *15*, 516–22, doi:10.3109/1354750X.2010.491557.

124. Chen, Y.; Yan, H.; Wang, Y.; Shi, Y.; Dai, G. Significance of baseline and change in neutrophil-to-lymphocyte ratio in predicting prognosis: A retrospective analysis in advanced pancreatic ductal adenocarcinoma. *Sci. Rep.* **2017**, *7*, 753, doi:10.1038/s41598-017-00859-5.

125. Lee, B.M.; Chung, S.Y.; Chang, J.S.; Lee, K.J.; Seong, J. The neutrophil-lymphocyte ratio and platelet-lymphocyte ratio are prognostic factors in patients with locally advanced pancreatic cancer treated with chemoradiotherapy. *Gut Liver* **2018**, *12*, 342–352, doi:10.5009/gnl17216.

126. Iwai, N.; Okuda, T.; Sakagami, J.; Harada, T.; Ohara, T.; Taniguchi, M.; Sakai, H.; Oka, K.; Hara, T.; Tsuji, T.; et al. Neutrophil to lymphocyte ratio predicts prognosis in unresectable pancreatic cancer. *Sci. Rep.* **2020**, *10*, 18758, doi:10.1038/s41598-020-75745-8.

127. Qi, Q.; Geng, Y.; Sun, M.; Wang, P.; Chen, Z. Clinical implications of systemic inflammatory response markers as independent prognostic factors for advanced pancreatic cancer. *Pancreatology* **2015**, *15*, 145–50, doi:10.1016/j.pan.2014.12.004.

128. Howard, R.; Kanetsky, P.A.; Egan, K.M. Exploring the prognostic

value of the neutrophil-to-lymphocyte ratio in cancer. *Sci. Rep.* **2019**, *9*, 19673, doi:10.1038/s41598-019-56218-z.

129. Piciucchi, M.; Stigliano, S.; Archibugi, L.; Zerboni, G.; Signoretti, M.; Barucca, V.; Valente, R.; Fave, G.D.; Capurso, G. The neutrophil/lymphocyte ratio at diagnosis is significantly associated with survival in metastatic pancreatic cancer patients. *Int. J. Mol. Sci.* **2017**, *18*, doi:10.3390/ijms18040730.

130. Jones, C.I. Platelet function and ageing. *Mamm. Genome* **2016**, *27*, 358–66, doi:10.1007/s00335-016-9629-8.

131. Palacios-Acedo, A.L.; Mège, D.; Crescence, L.; Dignat-George, F.; Dubois, C.; Panicot-Dubois, L. Platelets, Thrombo-Inflammation, and Cancer: Collaborating With the Enemy. *Front. Immunol.* **2019**, *10*, 1805, doi:10.3389/fimmu.2019.01805.

132. Olsson, A.K.; Cedervall, J. The pro-inflammatory role of platelets in cancer. *Platelets* **2018**, *29*, 569–573, doi:10.1080/09537104.2018.1453059.

133. Gay, L.J.; Felding-Habermann, B. Contribution of platelets to tumour metastasis. *Nat. Rev. Cancer* **2011**, *11*, 123–34, doi:10.1038/nrc3004.

134. Cools-Lartigue, J.; Spicer, J.; McDonald, B.; Gowing, S.; Chow, S.; Giannias, B.; Bourdeau, F.; Kubes, P.; Ferri, L. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J. Clin. Invest.* **2013**, *8*, 3446–3458, doi:10.1172/JCI67484.

135. Yang, J.J.; Hu, Z.G.; Shi, W.X.; Deng, T.; He, S.Q.; Yuan, S.G. Prognostic significance of neutrophil to lymphocyte ratio in pancreatic

cancer: A meta-analysis. *World J. Gastroenterol.* **2015**, *21*, 2807–15, doi:10.3748/wjg.v21.i9.2807.

136. Gao, Y.; Wang, W.J.; Zhi, Q.; Shen, M.; Jiang, M.; Bian, X.; Gong, F.R.; Zhou, C.; Lian, L.; Wu, M.Y.; et al. Neutrophil/lymphocyte ratio is a more sensitive systemic inflammatory response biomarker than platelet/lymphocyte ratio in the prognosis evaluation of unresectable pancreatic cancer. *Oncotarget* **2017**, *8*, 88835–88844, doi:10.18632/oncotarget.21340.

137. Kitajima, S.; Thummalapalli, R.; Barbie, D.A. Inflammation as a driver and vulnerability of KRAS mediated oncogenesis. *Semin. Cell Dev. Biol.* **2016**, *58*, 127–35, doi:10.1016/j.semcdb.2016.06.009.

138. Hamarsheh, S.; Groß, O.; Brummer, T.; Zeiser, R. Immune modulatory effects of oncogenic KRAS in cancer. *Nat. Commun.* **2020**, *11*, 5439, doi:10.1038/s41467-020-19288-6.

139. Dias Carvalho, P.; Guimaraes, C.F.; Cardoso, A.P.; Mendonca, S.; Costa, A.M.; Oliveira, M.J.; Velho, S. KRAS Oncogenic Signaling Extends beyond Cancer Cells to Orchestrate the Microenvironment. *Cancer Res* **2018**, *78*, 7–14, doi:10.1158/0008-5472.CAN-17-2084.

140. Wang, Y.; Fang, T.; Huang, L.; Wang, H.; Zhang, L.; Wang, Z.; Cui, Y. Neutrophils infiltrating pancreatic ductal adenocarcinoma indicate higher malignancy and worse prognosis. *Biochem. Biophys. Res. Commun.* **2018**, *501*, 313–319, doi:10.1016/j.bbrc.2018.05.024.

141. Goggs, R.; Jeffery, U.; LeVine, D.N.; Li, R.H.L. Neutrophil-Extracellular Traps, Cell-Free DNA, and Immunothrombosis in Companion Animals: A Review. *Vet. Pathol.* **2020**, *57*, 6–23,

doi:10.1177/0300985819861721.

142. Cicchillitti, L.; Corrado, G.; de Angeli, M.; Mancini, E.; Baiocco, E.; Patrizi, L.; Zampa, A.; Merola, R.; Martayan, A.; Conti, L.; et al. Circulating cell-free DNA content as blood based biomarker in endometrial cancer. *Oncotarget* **2017**, *8*, 115230–115243, doi:10.18632/oncotarget.23247.

143. Heeke, S.; Mograbi, B.; Alix-Panabières, C.; Hofman, P. Never Travel Alone: The Crosstalk of Circulating Tumor Cells and the Blood Microenvironment. *Cells* **2019**, *8*, doi:10.3390/cells8070714.

144. Spicer, J.D.; McDonald, B.; Cools-Lartigue, J.J.; Chow, S.C.; Giannias, B.; Kubes, P.; Ferri, L.E. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. *Cancer Res.* **2012**, *72*, 3919–27, doi:10.1158/0008-5472.CAN-11-2393.

145. Miller-Ocuin, J.L.; Liang, X.; Boone, B.A.; Doerfler, W.R.; Singhi, A.D.; Tang, D.; Kang, R.; Lotze, M.T.; Zeh, H.J. DNA released from neutrophil extracellular traps (NETs) activates pancreatic stellate cells and enhances pancreatic tumor growth. *Oncoimmunology* **2019**, *8*, e1605822, doi:10.1080/2162402X.2019.1605822.

146. Huber, M.; Brehm, C.U.; Gress, T.M.; Buchholz, M.; Alhamwe, B.A.; von Strandmann, E.P.; Slater, E.P.; Bartsch, J.W.; Bauer, C.; Lauth, M. The immune microenvironment in pancreatic cancer. *Int. J. Mol. Sci.* **2020**, *21*, doi:10.3390/ijms21197307.

147. Yang, Y.; Wang, G.; He, J.; Ren, S.; Wu, F.; Zhang, J.; Wang, F. Gender differences in colorectal cancer survival: A meta-analysis. *Int.*

J. Cancer **2017**, *141*, 1942–1949.

148. Hendifar, A.; Yang, D.; Lenz, F.; Lurje, G.; Pohl, A.; Lenz, C.; Yan, N.; Wu, Z.; Lenz, H.J. Gender disparities in metastatic colorectal cancer survival. *Clin. Cancer Res.* **2009**, *15*, 6391–6397, doi:10.1158/1078-0432.CCR-09-0877.

149. Majek, O.; Gondos, A.; Jansen, L.; Emrich, K.; Holleczek, B.; Katalinic, A.; Nennecke, A.; Eberle, A.; Brenner, H. Sex Differences in Colorectal Cancer Survival: Population-Based Analysis of 164,996 Colorectal Cancer Patients in Germany. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0068077.

150. Yang, L.; Yang, X.; He, W.; Liu, S.; Jiang, C.; Xie, K.; Peng, K.; You, Y.; Zhang, B.; Xia, L. Comparisons of metastatic patterns of colorectal cancer among patients by age group: A population-based study. *Aging (Albany. NY).* **2018**, *10*, 4107–4119, doi:10.18632/aging.101700.

151. Vidal, J.; Muinelo, L.; Dalmases, A.; Jones, F.; Edelstein, D.; Iglesias, M.; Orrillo, M.; Abalo, A.; Rodríguez, C.; Brozos, E.; et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. *Ann. Oncol.* **2017**, *28*, 1325–1332, doi:10.1093/annonc/mdx125.

152. García-Foncillas, J.; Tabernero, J.; Élez, E.; Aranda, E.; Benavides, M.; Camps, C.; Jantus-Lewintre, E.; López, R.; Muinelo-Romay, L.; Montagut, C.; et al. Prospective multicenter real-world RAS mutation comparison between OncoBEAM-based liquid biopsy and tissue analysis in metastatic colorectal cancer. *Br. J. Cancer* **2018**, *119*, 1464–1470, doi:10.1038/s41416-018-0293-5.

153. Germetaki, T.; Nicholls, C.; Adams, R.A.; Braun, M.; Rogan, J.; Moghadam, S.; Lenfert, E.; Lukas, A.; Edelstein, D.L.; Jones, F.S.; et al. Blood-based mutation Testing: Concordance with tissue-based testing and mutational changes on progression. *Futur. Oncol.* **2020**, *16*, 2177–2189, doi:10.2217/fon-2020-0523.

154. Grasselli, J.; Elez, E.; Caratù, G.; Matito, J.; Santos, C.; Macarulla, T.; Vidal, J.; Garcia, M.; Viéitez, J.M.; Paéz, D.; et al. Concordance of blood- and tumor-based detection of RAS mutations to guide anti-EGFR therapy in metastatic colorectal cancer. *Ann. Oncol.* **2017**, *28*, 1294–1301, doi:10.1093/annonc/mdx112.

155. Bachet, J.B.; Bouché, O.; Taieb, J.; Dubreuil, O.; Garcia, M.L.; Meurisse, A.; Normand, C.; Gornet, J.M.; Artru, P.; Louafi, S.; et al. RAS mutation analysis in circulating tumor DNA from patients with metastatic colorectal cancer: The AGEO RASANC prospective multicenter study. *Ann. Oncol.* **2018**, *29*, 1211–1219, doi:10.1093/annonc/mdy061.

156. Castagnola, P.; Giaretti, W. Mutant KRAS, chromosomal instability and prognosis in colorectal cancer. *Biochim. Biophys. Acta* **2005**, *1756*, 115–125.

157. Yokota, T. Are KRAS/BRAF Mutations Potent Prognostic and/or Predictive Biomarkers in Colorectal Cancers? *Anticancer. Agents Med. Chem.* **2012**, *12*, 163–171, doi:10.2174/187152012799014968.

158. Spindler, K.L.G.; Pallisgaard, N.; Appelt, A.L.; Andersen, R.F.; Schou, J. V.; Nielsen, D.; Pfeiffer, P.; Yilmaz, M.; Johansen, J.S.; Hoegdall, E. V.; et al. Clinical utility of KRAS status in circulating plasma DNA compared to archival tumour tissue from patients with

metastatic colorectal cancer treated with anti-epidermal growth factor receptor therapy. *Eur. J. Cancer* **2015**, *51*, 2678–2685, doi:10.1016/j.ejca.2015.06.118.

159. Normanno, N.; Esposito Abate, R.; Lambiase, M.; Forgione, L.; Cardone, C.; Iannaccone, A.; Sacco, A.; Rachiglio, A.M.; Martinelli, E.; Rizzi, D.; et al. RAS testing of liquid biopsy correlates with the outcome of metastatic colorectal cancer patients treated with first-line FOLFIRI plus cetuximab in the CAPRI-GOIM trial. *Ann. Oncol.* **2018**, *29*, 112–118, doi:10.1093/annonc/mdx417.

160. Elez, E.; Chianese, C.; Sanz-García, E.; Martinelli, E.; Noguerido, A.; Mancuso, F.M.; Caratù, G.; Matito, J.; Grasselli, J.; Cardone, C.; et al. Impact of circulating tumor DNA mutant allele fraction on prognosis in RAS-mutant metastatic colorectal cancer. *Mol. Oncol.* **2019**, *13*, 1827–1835, doi:10.1002/1878-0261.12547.

161. Schmiegel, W.; Scott, R.J.; Dooley, S.; Lewis, W.; Meldrum, C.J.; Pockney, P.; Draganic, B.; Smith, S.; Hewitt, C.; Philimore, H.; et al. Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: concordance of results from circulating tumor DNA and tissue-based RAS testing. *Mol. Oncol.* **2017**, *11*, 208–219, doi:10.1002/1878-0261.12023.

162. Spindler, K.G.; Boysen, A.K.; Pallisgård, N.; Johansen, J.S.; Tabernero, J.; Sørensen, M.M.; Jensen, B. V.; Hansen, T.F.; Sefrioui, D.; Andersen, R.F.; et al. Cell□Free DNA in Metastatic Colorectal Cancer: A Systematic Review and Meta□Analysis. *Oncologist* **2017**, *22*, 1049–1055, doi:10.1634/theoncologist.2016-0178.

163. Spindler, K.L.G.; Appelt, A.L.; Pallisgaard, N.; Andersen, R.F.;

Brandslund, I.; Jakobsen, A. Cell-free DNA in healthy individuals, noncancerous disease and strong prognostic value in colorectal cancer. *Int. J. Cancer* **2014**, *135*, 2984–2991, doi:10.1002/ijc.28946.

164. Hamfjord, J.; Guren, T.K.; Dajani, O.; Johansen, J.S.; Glimelius, B.; Sorbye, H.; Pfeiffer, P.; Lingjærde, O.C.; Tveit, K.M.; Kure, E.H.; et al. Total circulating cell-free DNA as a prognostic biomarker in metastatic colorectal cancer before first-line oxaliplatin-based chemotherapy. *Ann. Oncol.* **2019**, *30*, 1088–1095, doi:10.1093/annonc/mdz139.

165. Mouliere, F.; El Messaoudi, S.; Pang, D.; Dritschilo, A.; Thierry, A.R. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. *Mol. Oncol.* **2014**, *8*, 927–941, doi:10.1016/j.molonc.2014.02.005.

166. Hellwig, S.; Nix, D.A.; Gligorich, K.M.; O'Shea, J.M.; Thomas, A.; Fuertes, C.L.; Bhetariya, P.J.; Marth, G.T.; Bronner, M.P.; Underhill, H.R. Automated size selection for short cell-free DNA fragments enriches for circulating tumor DNA and improves error correction during next generation sequencing. *PLoS One* **2018**, *13*, doi:10.1371/journal.pone.0197333.

167. Locker, G.Y.; Hamilton, S.; Harris, J.; Jessup, J.M.; Kemeny, N.; Macdonald, J.S.; Somerfield, M.R.; Hayes, D.F.; Bast, R.C. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J. Clin. Oncol.* **2006**, *24*, 5313–5327.

168. Tampellini, M.; Ottone, A.; Alabiso, I.; Baratelli, C.; Forti, L.; Berruti, A.; Aroasio, E.; Scagliotti, G. V. The prognostic role of baseline CEA and CA 19-9 values and their time-dependent variations

in advanced colorectal cancer patients submitted to first-line therapy. *Tumor Biol.* **2014**, *36*, 1519–1527, doi:10.1007/s13277-014-2693-3.

169. Nicholson, B.D.; Shinkins, B.; Pathiraja, I.; Roberts, N.W.; James, T.J.; Mallett, S.; Perera, R.; Primrose, J.N.; Mant, D. Blood CEA levels for detecting recurrent colorectal cancer. *Cochrane Database Syst. Rev.* **2015**, *2015*, doi: 10.1002/14651858.

170. Salinas-Vera, Y.M.; Marchat, L.A.; Gallardo-Rincón, D.; Ruiz-García, E.; Astudillo-De la Vega, H.; Echavarría-Zepeda, R.; López-Camarillo, C. AngiomiRs: MicroRNAs driving angiogenesis in cancer (Review). *Int. J. Mol. Med.* **2019**, *43*, 657–670, doi:10.3892/ijmm.2018.4003.

171. Annese, T.; Tamma, R.; De Giorgis, M.; Ribatti, D. microRNAs Biogenesis, Functions and Role in Tumor Angiogenesis. *Front. Oncol.* **2020**, *10*, doi:10.3389/fonc.2020.581007.

172. Mao, L.; Liu, S.; Hu, L.; Jia, L.; Wang, H.; Guo, M.; Chen, C.; Liu, Y.; Xu, L. MiR-30 Family: A Promising Regulator in Development and Disease. *Biomed Res. Int.* **2018**, *2018*, doi:10.1155/2018/9623412.

173. Bridge, G.; Monteiro, R.; Henderson, S.; Emuss, V.; Lagos, D.; Georgopoulou, D.; Patient, R.; Boshoff, C. The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis. *Blood* **2012**, *120*, 5063–5072, doi:10.1182/blood-2012-04-423004.

174. Volkmann, I.; Kumarswamy, R.; Pfaff, N.; Fiedler, J.; Dangwal, S.; Holzmann, A.; Batkai, S.; Geffers, R.; Lother, A.; Hein, L.; et al. MicroRNA-mediated epigenetic silencing of sirtuin1 contributes to

impaired angiogenic responses. *Circ. Res.* **2013**, *113*, 997–1003, doi:10.1161/CIRCRESAHA.113.301702.

175. Park, D.; Kim, H.; Kim, Y.; Jeoung, D. miR-30a regulates the expression of CAGE and p53 and regulates the response to anti-cancer drugs. *Mol. Cells* **2016**, *39*, 299–309, doi:10.14348/molcells.2016.2242.

176. Huang, G.; Lai, Y.; Pan, X.; Zhou, L.; Quan, J.; Zhao, L.; Li, Z.; Lin, C.; Wang, J.; Li, H.; et al. Tumor suppressor miR-33b-5p regulates cellular function and acts a prognostic biomarker in RCC. *Am. J. Transl. Res.* **2020**, *12*, 3346–3360.

177. Tian, Q.; Xiao, Y.; Wu, Y.; Liu, Y.; Song, Z.; Gao, W.; Zhang, J.; Yang, J.; Zhang, Y.; Guo, T.; et al. MicroRNA-33b suppresses the proliferation and metastasis of hepatocellular carcinoma cells through the inhibition of Sal-like protein 4 expression. *Int. J. Mol. Med.* **2016**, *38*, 1587–1595, doi:10.3892/ijmm.2016.2754.

178. Liao, W.; Gu, C.; Huang, A.; Yao, J.; Sun, R. MicroRNA-33b inhibits tumor cell growth and is associated with prognosis in colorectal cancer patients. *Clin. Transl. Oncol.* **2016**, *18*, 449–456, doi:10.1007/s12094-015-1388-6.

179. Sgarra, R.; Pegoraro, S.; D'angelo, D.; Ros, G.; Zanin, R.; Sgubin, M.; Petrosino, S.; Battista, S.; Manfioletti, G. High mobility group a (HMGA): Chromatin nodes controlled by a knotty miRNA network. *Int. J. Mol. Sci.* **2020**, *21*, doi:10.3390/ijms21030717.

180. Yang, X.; Zhao, Q.; Yin, H.; Lei, X.; Gan, R. MiR-33b-5p sensitizes gastric cancer cells to chemotherapy drugs via inhibiting HMGA2

expression. *J. Drug Target.* **2017**, *25*, 653–660, doi:10.1080/1061186X.2017.1323220.

181. Sakata, J.; Hirosue, A.; Yoshida, R.; Kawahara, K.; Matsuoka, Y.; Yamamoto, T.; Nakamoto, M.; Hirayama, M.; Takahashi, N.; Nakamura, T.; et al. HMGA2 Contributes to Distant Metastasis and Poor Prognosis by Promoting Angiogenesis in Oral Squamous Cell Carcinoma. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20102473.

182. Li, Y.; Qiang, W.; Griffin, B.B.; Gao, T.; Chakravarti, D.; Bulun, S.; Kim, J.J.; Wei, J.J. HMGA2-mediated tumorigenesis through angiogenesis in leiomyoma. *Fertil. Steril.* **2020**, *114*, 1085–1096, doi:10.1016/j.fertnstert.2020.05.036.

183. Wang, X.; Wang, J.; Wu, J. Emerging roles for HMGA2 in colorectal cancer. *Transl. Oncol.* **2021**, *14*, doi: 10.1016/j.tranon.2020.100894.

IX. Abbreviations

5-FU	5-fluorouracil
Ago2	Argonaute 2
APC	Adenomatous polyposis coli
CA19-9	Carbohydrate antigen
CEA	Carcinoembryogenic antigen
cfDNA	Circulating cell-free DNA
CIN	Chromosomic instability
CRC	Colorectal cancer
CTCs	Circulating tumour cells
ctDNA	Circulating tumour DNA
ECOG	Eastern cooperative oncology group
EGFR	Epidermal growth factor receptor
EVs	Extracellular vesicles
FAP	Familial adenomatous polyposis
FOLFIRI	Folinic acid, fluorouracil and irinotecan
FOLFIRINOX	Folinic acid, fluorouracil, irinotecan and oxaliplatin
HMGA2	High Mobility Group A 2
HR	Hazard ratio
IPA	Ingenuity pathway analysis
MAF	Mutant allelic fraction
miRNAs	microRNAs
MSI	Microsatellite instability
NETs	Neutrophil extracellular traps
NLR	Neutrophil-lymphocyte ratio
OncomiRs	Oncogenic miRNAs
OS	Overall Survival
PanIN	Intraepithelial neoplasm

PDAC	Pancreatic ductal adenocarcinoma
PFS	Progression-free survival
PLR	Platelet-lymphocyte ratio
PR	Partial response
RISC	RNA induced silencing complex
ROC	Receiver operating curve
SD	Stable disease
TGF-β	Transforming growth factor β
TTF	Time to treatment failure
VEGF	Vascular endothelial growth factor
WT	Wild type

X. Annex I

cancers

MDPI

Article

Circulating Cell-Free DNA-Based Liquid Biopsy Markers for the Non-Invasive Prognosis and Monitoring of Metastatic Pancreatic Cancer

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Abstract: Liquid biopsy may assist in the management of cancer patients, which can be particularly applicable in pancreatic ductal adenocarcinoma (PDAC). In this study, we investigated the utility of circulating cell-free DNA (cfDNA)-based markers as prognostic tools in metastatic PDAC. Plasma was obtained from 61 metastatic PDAC patients, and cfDNA levels and fragmentation were determined. BEAMing technique was used for quantitative determination of RAS mutation allele fraction (MAF) in cfDNA. We found that the prognosis was more accurately predicted by RAS mutation detection in plasma than in tissue. RAS mutation status in plasma was a strong independent prognostic factor for both overall survival (OS) and progression-free survival (PFS). Moreover, RAS MAF in cfDNA was also an independent risk factor for poor OS, and was strongly associated with primary tumours in the body/tail of the pancreas and liver metastases. Higher cfDNA levels and fragmentation were also associated with poorer OS and shorter PFS, body/tail tumors, and hepatic metastases, whereas cfDNA fragmentation positively correlated with RAS MAF. Remarkably, the combination of CA19-9 with MAF, cfDNA levels and fragmentation improved the prognostic stratification of patients. Furthermore, dynamics of RAS MAF better correlated with patients' outcome than standard CA19-9 marker. In conclusion, our study supports the use of cfDNA-based liquid biopsy markers as clinical tools for the non-invasive prognosis and monitoring of metastatic PDAC patients.

Keywords: cell-free DNA; liquid biopsy; MAF; pancreatic cancer; RAS mutation

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death in Europe in both males and females, with the lowest survival rate of all cancers and responsible for over 95,000 deaths every year [1,2]. While, the death rates of the most common cancers have mostly declined over the past decades, the mortality rate of pancreatic cancer remains flat or slightly increases over time [3]. Poor prognosis is associated with diagnosis at advanced stage, due to a lack of detection methods, as well as resistance to therapy. Pancreatic ductal adenocarcinoma (PDAC) represents more than 90% of all

pancreatic cancer and the vast majority of deaths are associated with this tumor type. Approximately 60–70% of patients have a primary tumor, located in the head of pancreas, while 20% and 25% are located in the body, and tail, respectively. Moreover, PDAC metastasizes mainly to liver, abdomen and lungs [4].

KRAS mutation is the initiating genetic event for PDAC, and this oncogene is mutationally activated in 94% of pancreatic ductal tumors [5]. KRAS mutational status has been usually analyzed in tumor tissue, but obtaining biopsy specimens from pancreatic lesions may be difficult and requires invasive procedures, such as endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). However, EUS-FNA provides a limited number of cells for molecular profiling and the high stromal content of pancreatic tumors impairs its efficacy for PDAC diagnosis [6]. Moreover, tumor tissue is only available at diagnosis, but not at different time points of the disease to monitor tumor burden during treatment. In this scenario, circulating cell-free DNA (cfDNA)-based liquid biopsy represents a promising non-invasive tool for the diagnosis, prognosis and management of PDAC patients [7]. Hence, the analysis of KRAS mutations in cfDNA has been proposed as non-invasive surrogate for tissue biopsies in patients with pancreatic cancer [8]. On the other hand, cfDNA levels have been shown to be prognostic for clinical outcome in metastatic cancer [9,10].

Therefore, the present study aimed to evaluate the different cfDNA-based liquid biopsy markers as prognostic tools for the management care of metastatic PDAC patients.

2. Results

2.1. Clinicopathological Characteristics and RAS Mutation Analysis from Plasma and Tissue

Sixty-one patients were included in the study between May 2017 and December 2019. Thirty-four patients were men and 27 were women, and they ranged in age from 40 to 84 years, with a median of 65 years of age (baseline characteristics are summarized in Table 1). All patients had pancreatic ductal adenocarcinoma (PDAC) with distant metastases at diagnosis, and the most frequent site of metastasis was the liver (78.7%). Primary tumor was localized in tail, body and head of pancreas in 27.9%, 41%, and 29.5% of patients, respectively. Most patients (78.7%) had a good baseline ECOG performance status (PS), and a majority (75.4%) received first line gemcitabine-based regimes. For the analysis of RAS mutational status, primary tumor tissue was available in 70.5% (43/61) of patients. Whereas, basal blood samples were obtained from all patients before any treatment. RAS mutation was detected in 76.7% (33/43) of tissue samples and in 77% (47/61) of basal plasma samples. The percentage of patients with RAS mutation was comparable to other cohort studies [11–13]. Mutation in codon 12 of the KRAS gene was found in 93.6% (44/47), and 93.9% (31/33) of plasma and tissue samples, respectively (Table S1). The overall concordance between plasma and tissue RAS analysis was 79.1%.

Table 1. Baseline characteristics of patients.

Patient Characteristics		**Number of Cases (*n* = 61)**
Age (median, range)		65 (40–84)
Sex	Male	34 (55.7%)
	Female	27 (44.3%)
ECOG	0	17 (27.9%)
	1	31 (50.8%)
	2	10 (16.4%)
	3	3 (4.9%)
1st line treatment	Gemcitabine	3 (4.9%)
	Gemcitabine/nab-paclitaxel	39 (63.9%)
	Gemcitabine/nab-paclitaxel/FOLFOX	4 (6.6%)
	FOLFIRINOX	11 (18%)
	No treatment	4 (6.6%)
Survival	Alive	19 (31.1%)
	Dead	42 (68.9%)

Disease progression	Yes	45 (73.8%)
	No	11 (18%)
	Not valuable (No treatment or surgery)	5 (8.2%)
Tissue availability	Yes	43 (70.5%)
	No (Cytology)	18 (29.5%)
Primary tumor location	Tail	17 (27.9%)
	Body	25 (41%)
	Head	18 (29.5%)
	Body-Tail	1 (1.6%)
Number of metastatic lesions	One location	26 (42.6%)
	More than one location	35 (57.4%)
Metastatic lesions location	Hepatic lesions	48 (78.7%)
	Non-hepatic lesions	13 (21.3%)
Tissue Biopsy RAS status [1]	RAS mutated	33 (76.7%)
	RAS wild-type	10 (23.3%)
Liquid Biopsy RAS status	RAS mutated	47 (77%)
	RAS wild-type	14 (23%)

[1] For the analysis of RAS mutational status, primary tumor tissue was available in 70.5% (43/61) of patients.

2.2. Detection of RAS Mutations in cfDNA Predicts Poor Prognosis in Metastatic PDAC Patients

The presence of RAS mutations in plasma cfDNA was analysed in 61 metastatic PDAC patients. Detection of RAS mutation in plasma was associated with shorter patient overall survival (OS) (169 versus 372.5 days; p = 0.0004; Table 2, Figure 1A). Besides, prognosis was more accurately predicted by RAS mutation analysis in cfDNA than by tissue analysis (43 patients, RAS mutation in tissue: log-rank p = 0.0730; RAS mutation in cfDNA: p = 0.0068; Table 2, Figure 1B,C). RAS mutation detection in cfDNA was also a predictive factor of poor progression-free survival (PFS) in metastatic PDAC patients (93.5 versus 313.5 days; p < 0.0001; Table 3, Figure 2A). Likewise, tissue analysis was a worse predictive factor of PFS than cfDNA (RAS mutation in tissue: p = 0.0172; RAS mutation in cfDNA: p = 0.0019; Table 3, Figure 2B,C). Finally, multivariate analysis revealed that KRAS mutation status in plasma was a strong independent prognostic factor for both OS (HR 5.692, 95% CI 1.497–21.636; p = 0.011) and PFS (HR 8.631, 95% CI 2.311–32.236; p = 0.001) (Table 4).

Table 2. Overall survival analysis.

Variables	Death Occurrence	Median OS (Days)	HR (95%CI)	*p*
Primary Tumor Location				
Body/Tail	28/42	187	0.818	$p = 0.5802$
Head	12/17	173.5	(0.402–1.667)	
Metastatic Location				
Hepatic	35/48	157	2.403	$p = 0.0114$
Non-hepatic	6/13	339	(1.218–4.738)	
Number of Metastasis				
1	16/26	197.5	0.739	$p = 0.3380$
≥2	25/35	176	(0.398–1.372)	
KRAS mutation status plasma				
MUT	37/47	169	3.455	$p = 0.0004$
WT	4/14	372.5	(1.736–6.876)	
KRAS mutation status tissue				
MUT	24/33	197	2.102	$p = 0.0730$
WT	5/10	440	(0.933–4.734)	
KRAS mutation status plasma (with tissue paired sample)				
MUT	25/32	216.5	3.09	$p = 0.0068$

WT	4/11	537	(1.364–6.997)	
CA19-9				
<45,500 U/mL	32/50	202.5	2.272	$p = 0.0408$
>45,500 U/mL	8/10	125	(1.407–4.930)	
cfDNA concentration				
<26.46 ng/mL	8/21	285	2.468	$p = 0.0057$
>26.46 ng/mL	31/38	149.5	(1.302–4.681)	
MAF				
<0.351%	6/10	310	2.212	$p = 0.0261$
>0.351%	31/37	142	(1.099–4.452)	
cfDNA fragmentation				
<38.08%	28/45	197	2.637	$p = 0.0297$
>38.08%	11/13	116	(1.1–6.321)	

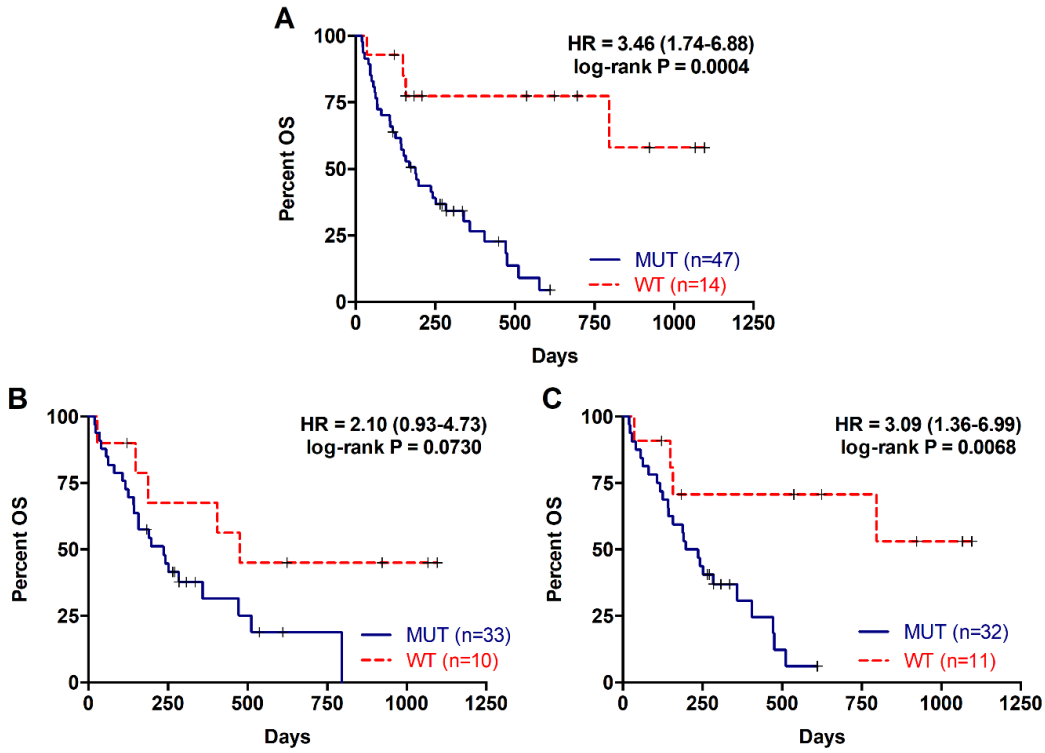

Figure 1. Overall survival rates for patients with metastatic PDAC according to RAS mutation status. (**A**) OS according to RAS mutation status in cfDNA; (**B**) OS according to RAS mutation status in tissue; (**C**) OS according to RAS mutation status in cfDNA of those patients with RAS mutations analyzed in tissue.

Table 3. Progression-free survival analysis.

Variables	Disease Progression	Median PFS (Days)	HR (95%CI)	p
Primary Tumor Location				
Body/Tail	33/40	152	0.783	p = 0.5318
Head	10/14	81.5	(0.364–1.685)	
Metastatic Location				
Hepatic	35/43	86	2.565	p = 0.0048
Non-hepatic	10/13	272	(1.333–4.937)	
Number of Metastasis				
1	19/23	127	0.86	p = 0.6304
≥2	26/33	139	(0.465–1.591)	

KRAS mutation status plasma				
MUT	38/42	93.5	3.84	p < 0.0001
WT	7/14	313.5	(1.974–7.469)	
KRAS mutation status tissue				
MUT	27/32	122.5	2.495	p = 0.0172
WT	7/9	382	(1.176–5.294)	
KRAS mutation status plasma (with tissue paired sample)				
MUT	27/30	142.5	3.41	p = 0.0019
WT	7/11	472	(1.572–7.395)	
CA19-9				
<45,500 U/mL	35/45	143	3.013	p = 0.0289
>45,500 U/mL	9/10	72	(1.12–8.103)	
cfDNA concentration				
<26.46 ng/mL	11/20	149.5	2.190	p = 0.0107
>26.46 ng/mL	34/36	86.5	(1.199–4.001)	
MAF				
<0.351%	8/9	175	2.015	p = 0.0556
>0.351%	30/33	85	(0.9834–4.129)	
cfDNA fragmentation				
<38.08%	33/42	145	3.137	p = 0.0101
>38.08%	12/13	81	(1.313–7.494)	

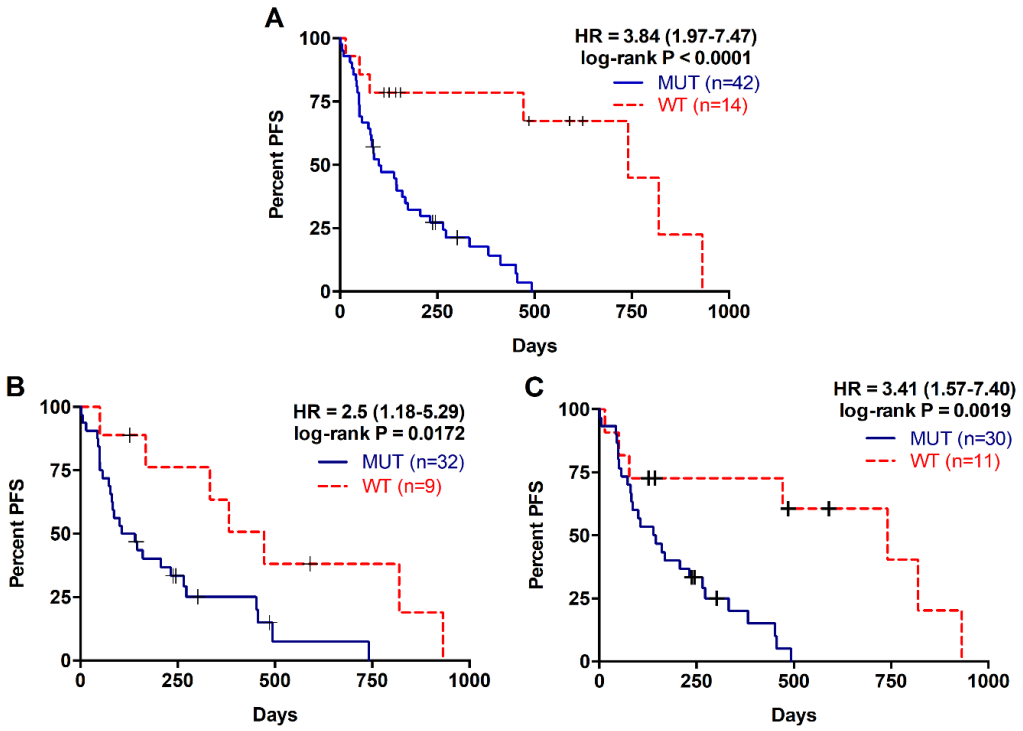

Figure 2. Progression free survival rates for patients with metastatic PDAC according to RAS mutation status. (**A**) PFS according to RAS mutation status in cfDNA; (**B**) PFS according to RAS mutation status in tissue; (**C**) PFS according to RAS mutation status in cfDNA of those patients with RAS mutations analyzed in tissue.

Table 4. Multivariate Analysis.

Variables	OS		PFS	
	p Value	HR (95%CI)	p Value	HR (95%CI)
KRAS mutation status plasma	0.011	5.692 (1.497–21.636)	0.001	8.631 (2.311–32.236)
MAF	0.047	1.070 (1.001–1.143)	0.280	1.035 (0.972–1.103)

2.3. Higher RAS Mutational Load in cfDNA is Associated with Poor Prognosis in Metastatic PDAC

For the 47 patients with detectable plasma RAS mutations, the median mutation allele fraction (MAF) was 2.92% (range 0.02–29.33%). As shown in Figure 3 a higher RAS mutational load in cfDNA was associated with poor OS (142 versus 310 days; p = 0.0261; cut-off value: 0.351%, with 82.5% sensitivity and 100% specificity; Table 2, Figure 3A) and poor PFS (85 versus 175 days; p = 0.0556; cut-off-value: 0.351%, with 83% sensitivity and 48% specificity; Table 3, Figure 3B). Moreover, multivariate analysis identified MAF in cfDNA as an independent risk factor for poor OS (HR 1.070, 95% CI 1.001–1.143; p = 0.047) (Table 4). Although, no differences were observed in the MAF values according to the number of metastatic lesions, higher MAF values were strongly associated with primary tumors located in the body/tail of the pancreas (p = 0.0281, Figure 4A) and liver metastases (p = 0.0072, Figure 4B). In this regard, the primary tumor location (OS p = 0.5802; PFS p = 0.5318) or the number of metastatic lesions (OS p = 0.3380; PFS p = 0.6304) were not related to OS or PFS. Whereas, significant poorer OS and PFS were observed in patients with hepatic lesions compared to patients with metastasis affecting other organs (OS 157 versus 339 days; p = 0.0114; PFS 86 versus 272; p = 0.0048) (Table 2; Table 3).

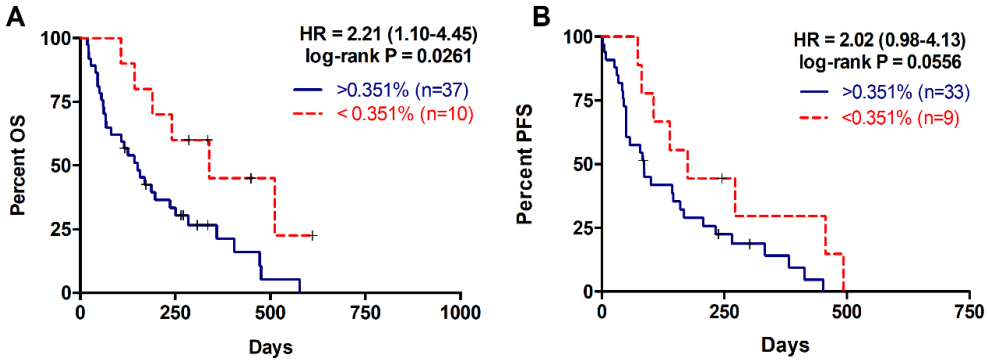

Figure 3. Overall and progression free survival rates according to circulating RAS mutation allele fraction (MAF). (**A**) OS according to circulating MAF (cut-off: 0.351%); (**B**) PFS according to circulating MAF (cut-off: 0.351%).

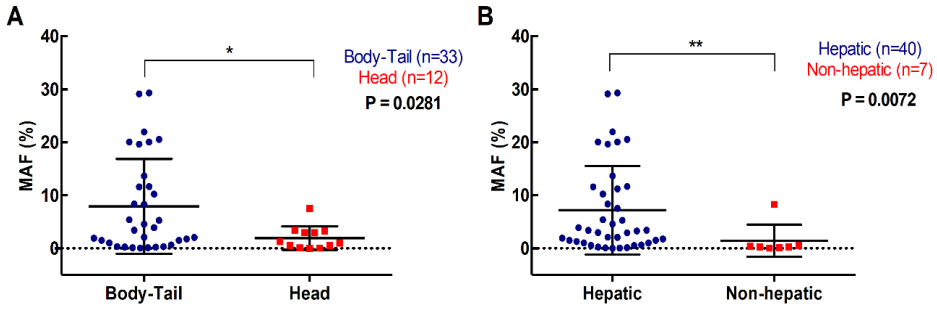

Figure 4. Association of circulating RAS mutation allele fraction (MAF) with primary tumor and metastases location. (**A**) circulating MAF levels in patients with tumor located in the body-tail or the

head of the pancreas; (**B**) circulating MAF levels in patients with metastatic lesions in the liver or elsewhere (* $p < 0.05$, ** $p < 0.01$).

2.4. Higher cfDNA Concentration and Fragmentation Levels are Associated with Poorer Survival in Metastatic PDAC Patients

The median cfDNA concentration in plasma of PDAC patients was 33 ng/mL (range 10–700), while the fragment size of plasma cfDNA ranged between 100–1100 bp, with a prominent mode at 135 pb for the shortest fragments detected. In this study cfDNA fragmentation was defined as the percentage of shortest fragments to total cfDNA. As shown in Figure 5, cfDNA concentration was significantly higher in those patients in whom plasma RAS mutations were detected (42.65 versus 24.71 ng/mL, p = 0.0057; Figure 5A). Although not significant, higher cfDNA fragmentation was observed in RAS mutated patients (Figure 5B), and a significant positive correlation between cfDNA fragmentation and KRAS MAF was found (r = 0.31, p = 0.0189).

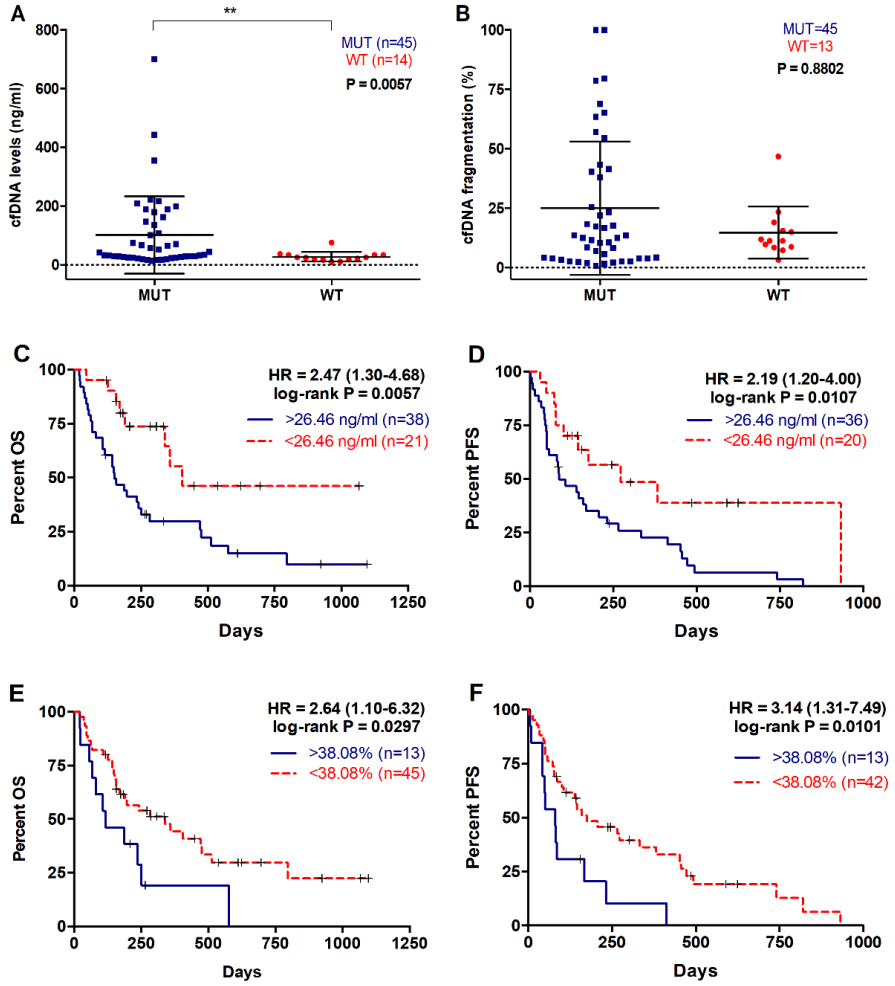

Figure 5. cfDNA concentration and fragmentation in metastatic PDAC patients. (**A**) cfDNA levels and (**B**) fragmentation according to RAS mutational status; (**C**) OS according to cfDNA levels (cut-off: 26.46ng/mL); (**D**) PFS according to cfDNA levels (cut-off: 26.46ng/mL); (**E**) OS according to cfDNA fragmentation (cut-off: 38.08%); (**F**) PFS according to cfDNA fragmentation (cut-off: 38.08%). (** $p < 0.01$).

When metastatic PDAC patients were stratified according to plasma cfDNA concentration, those with higher values (>26.46ng/mL) had a poorer OS rate (149.5 versus 285 days, p = 0.0057; cut-off value: 26.46 ng/mL, with 70.6% sensitivity and 64.1% specificity; Figure 5C, Table 2). Also, higher

plasma cfDNA concentration was associated with shorter PFS (86.5 versus 149.5 days, p = 0.0107, cut-off value: 26.46 ng/mL, with 67.7% sensitivity and 100% specificity; Figure 5D, Table 3). Similarly, a higher percentage of plasma cfDNA fragmentation in metastatic PDAC patients was significantly associated with a poorer OS (116 versus 197 days, p = 0.0297; cut-off value: 38.08%, with 27.2% sensitivity and 100% specificity; Figure 5E, Table 2) and PFS rates (145 versus 81 days, p = 0.0101; cut-off value: 38.08%, with 27.2% sensitivity and 100% specificity; Figure 5F, Table 3).

Plasma cfDNA concentration or fragmentation were not associated with number of metastatic lesions ($p = 0.4928$; $p = 0.7735$). However, there was an association between cfDNA fragmentation and primary tumor located in body/tail compared to head of the pancreas (15.27 versus 9.27%, $p = 0.0401$) (Figure 6A) and a trend towards higher cfDNA concentration in the plasma of metastatic PDAC patients with body/tail tumors (36.17ng/mL) compared with those with tumors in the head of the pancreas (26.23ng/mL, $p = 0.0691$) (Figure 6B). Also, patients with hepatic metastasis displayed higher cfDNA levels in plasma (38.10ng/mL), when compared with those patients with other metastatic locations (28.93ng/mL, $p = 0.0547$) (Figure 6D). Similarly, a trend towards higher cfDNA fragmentation was observed in patients with metastatic lesions in the liver (12.165%), compared with those with metastases elsewhere (10.655%, $p = 0.3257$) (Figure 6C).

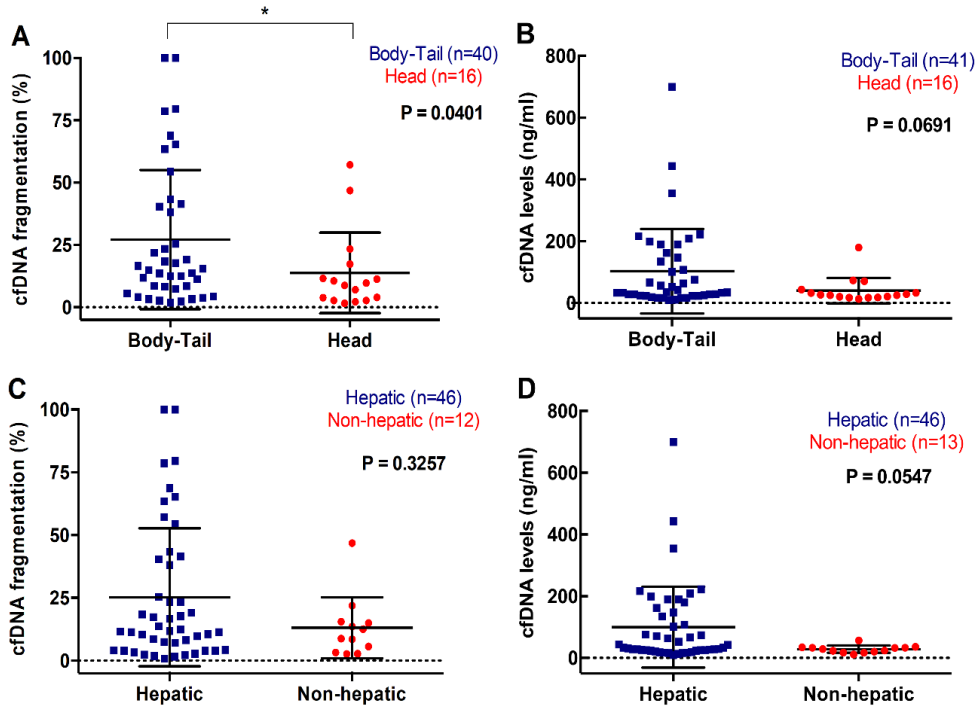

Figure 6. Association between cfDNA concentration and fragmentation and primary tumor and metastasis location. (**A**) cfDNA fragmentation; and (**B**) cfDNA levels according to primary tumor location; (**C**) cfDNA fragmentation; and (**D**) cfDNA levels according to metastatic location (* $p < 0.05$).

2.5. Multiparameter Liquid Biopsy Refines Prognostic Stratification of Metastatic PDAC Patients

In our cohort, CA19-9 demonstrated some prognostic value, with higher baseline levels associated with poorer OS and PFS rates (OS 125 versus 202.5 days, p = 0.0408; cut-off value: 45,500 U/mL, with 16.2% sensitivity and 80.9% specificity; PFS 72 versus 143 days, p = 0.0289; cut-off value: 45,500 U/mL, with 23% sensitivity and 93.7% specificity; Table 2; Table 3). No association was found between CA19-9 levels and RAS mutation status ($p = 0.2909$), primary tumor location ($p = 0.5053$), number of metastasis ($p = 0.4723$), location of metastatic lesions ($p = 0.4908$), MAF ($p = 0.1642$), cfDNA levels ($p = 0.7692$) or cfDNA fragmentation ($p = 0.2769$).

Remarkably, the combination of CA19-9 with liquid biopsy improved the prognostic stratification of metastatic PDAC patients. A scoring system was applied by combining CA19-9 with MAF, cfDNA concentration and cfDNA fragmentation. Positive or negative values were assigned depending on whether the corresponding marker was above (positive) or below (negative) the cut-off with prognostic value in OS. Thus, score 0 was defined as negative for all markers; score 1 was defined as positive for 1 marker; and score 2 was defined as positive for 2, 3 or 4 markers. As shown in Figure 7, those patients with score 2 displayed poorer survival outcomes in comparison with those patients with score 0 and score 1 in Kaplan-Meier analysis ($p = 0.0002$, and $p = 0.0072$, respectively).

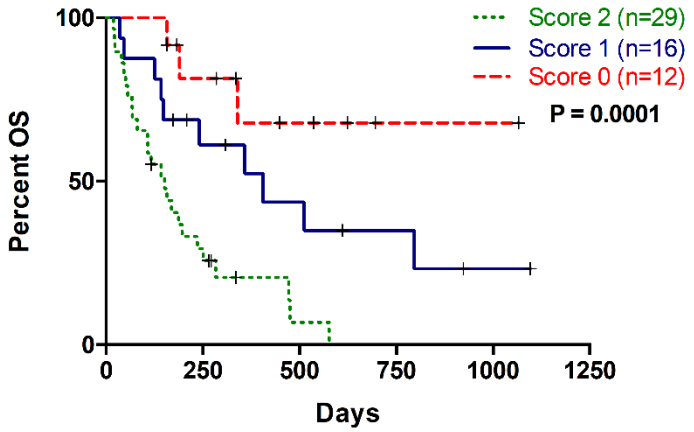

Figure 7. Multi-parametric analysis combining CA19-9 and cfDNA based liquid biopsy markers (MAF, cfDNA concentration and cfDNA fragmentation). Score 0: no positive markers; Score 1: One positive marker; Score 2: More than one positive markers.

2.6. RAS Mutational Load in cfDNA Enables Monitoring of Disease Progression and Response to Therapy in Metastatic PDAC Patients

Due to the limitations in CA19-9 as a reliable marker of pancreatic cancer, the utility of circulating MAF was compared to CA19-9 in monitoring disease progression and response to therapy in metastatic PDAC patients. No RAS mutation was detected in blood at baseline in two of the seven monitored patients, but it was detected in disease progression. In patient 1, KRAS codon 12 mutation was found in tissue but not in blood at baseline. Eventually, a novel NRAS mutation was detected during stable disease and a circulating KRAS codon 12 mutation was detected later in blood, along with both elevation of CA19-9 levels and disease progression revealed by radiological criteria and followed by rapid deterioration and death (Figure 8A). In patient 2, no RAS mutation was detected at baseline in either tissue or blood, but a KRAS codon 12 mutation was detected later in blood at progression of the disease (Figure 8B).

In the three patients (3, 4 and 5) in whom RAS mutation was detected at baseline in blood, circulating MAF dropped following treatment and concurring with lower CA19-9 levels and partial response (PR) to therapy (Figure 8C–E). In patient 3, circulating KRAS mutation level markedly declined at PR and rose again at disease progression, along with the detection of a novel circulating NRAS mutation (Figure 8C). In patient 4, KRAS mutation remained undetectable in blood, while CA9-19 levels were low and the disease was stable (SD), but unlike CA19-9, MAF was augmented again at the progression of disease (Figure 8D). In patient 5, circulating KRAS mutation dropped to undetectable levels in the stable disease. Despite standard criteria and CA19-9 levels in the following monitoring suggested stable disease, KRAS mutation was detected again in plasma anticipating disease progression (Figure 8E).

Finally, in patients 6 and 7, circulating RAS mutation levels increased during treatment, compared to baseline levels (Figure 8F,G). Notably, the increase in circulating MAF was associated with a very short survival period (5 months since diagnosis) in these patients.

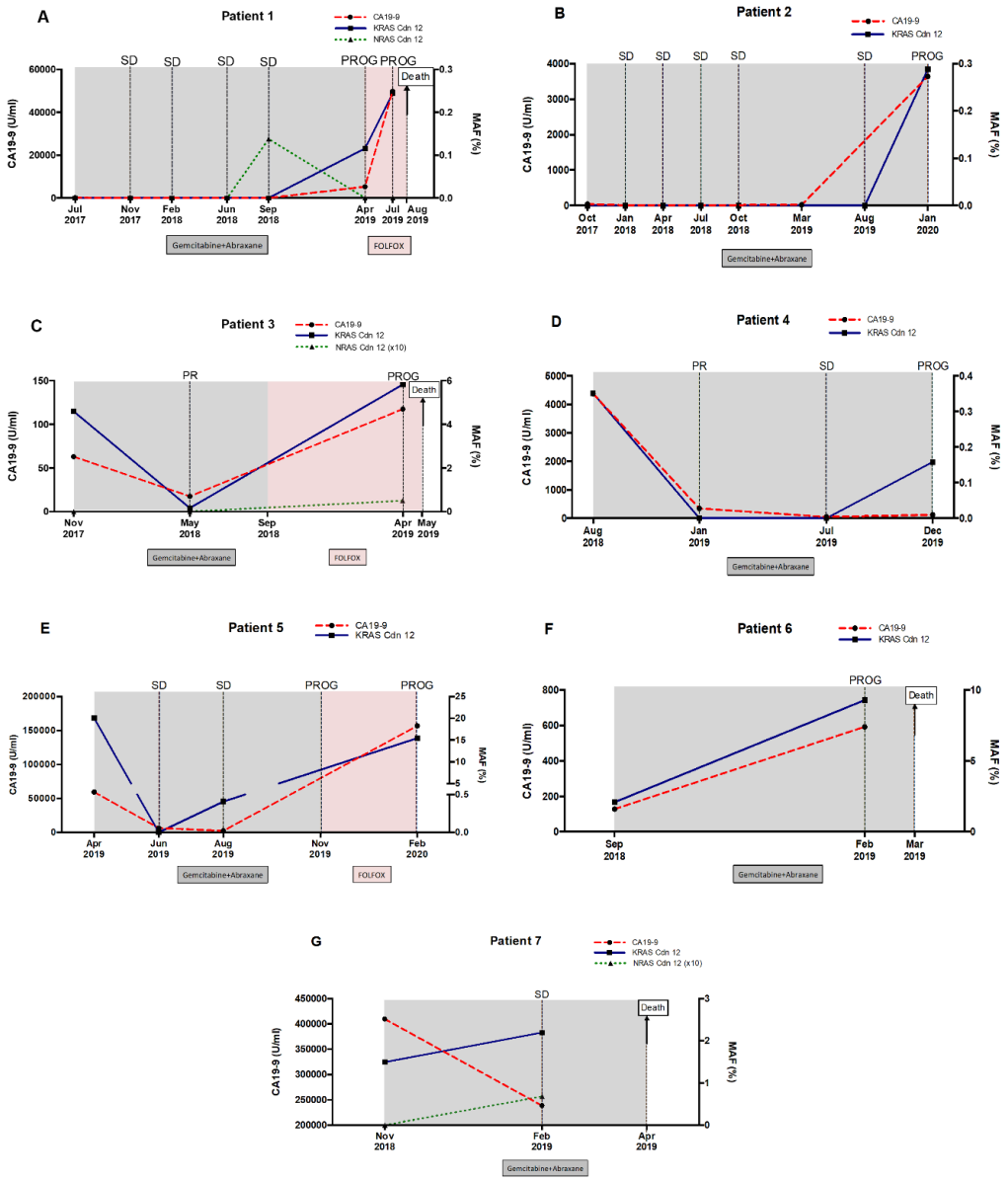

Figure 8. Circulating RAS mutation allele fraction (MAF) enables monitoring of disease progression and response to therapy in metastatic PDAC patients. Circulating MAF (%) was compared to CA19-9 (U/mL), in monitoring response to therapy and disease progression in 8 (**A**–**G**) metastatic PDAC patients.

As a whole, the above results suggest that the dynamics of circulating RAS mutation may better correlate with patients' outcome and survival compared with standard CA19-9 marker. Accordingly, a significant correlation was found between the increase in MAF ($r = -0.65$, $p = 0.02$), but not in CA19-9 ($r = 0.09$, $p = 0.78$) and survival time (Figure 9). Hence, higher increases in circulating RAS mutation during patient monitoring predicted a shorter survival time.

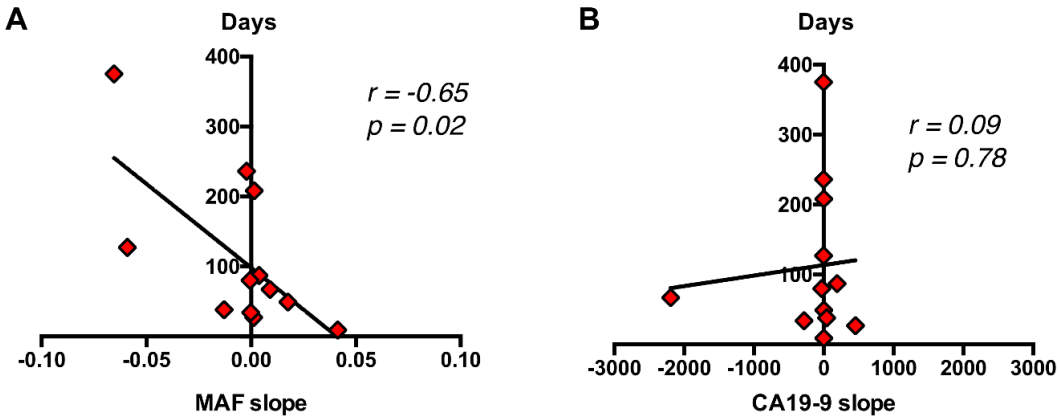

Figure 9. Correlation of dynamics of circulating RAS MAF and CA19-9 with patient's outcome and survival. (**A**) A significant correlation was found between the increase in MAF (**B**), but not in CA19-9 and survival time.

3. Discussion

Non-invasive, reliable, and reproducible cfDNA-based liquid biopsy markers may help in the management of cancer patients. This is particularly relevant in the case of PDAC, where the high stromal content makes it difficult to obtain molecular information through cytopathological analysis. However, there is no consensus about the techniques, mutations or type of material in liquid biopsy-based approaches for the prognosis of PDAC patients [11,14–16]. In this study we report the utility of cfDNA RAS mutations analysis using the highly sensitive BEAMing technique as prognostic tool for the management care of metastatic PDAC patients.

In agreement with other reports [17], our study supports the value of cfDNA RAS mutations analysis as a prognostic tool in pancreatic cancer. Therefore, our results show that the presence of RAS mutated cfDNA in plasma predicts poor prognosis in metastatic PDAC patients. Moreover, circulating KRAS mutational status was an independent negative prognostic factor of both OS and PFS. In fact, the prognosis was more accurately predicted by RAS mutation analysis in cfDNA than in tissue. The allelic ratio and dosage of mutated KRAS may impact on PDAC biology [18], and KRAS MAF in cfDNA has been found to correlate with clinical stage and outcome in PDAC [13,19]. In this regard, our results reveal that circulating KRAS MAF in cfDNA predicted survival in metastatic PDAC patients. Importantly, in our study, KRAS MAF in cfDNA was an independent negative prognostic factor of OS by multivariate analysis. Recently, KRAS MAF in DNA from circulating exosomes, but not in cfDNA, was found to be an independent prognostic factor of OS in metastatic PDAC patients [19]. However, our study demonstrates that highly sensitive approaches, such as BEAMing, may also reveal the independent prognostic value of KRAS MAF in cfDNA of metastatic PDAC patients. Exhaustive analyses on tissue, including laser capture microdissection, could establish the pure ratio of RAS mutated allele in tumor. However, these types of analyses rely on the availability of biopsy material to be adequately performed, which is not the case for a significant number of PDAC patients, and is identifiably the issue by which a liquid biopsy may effectively address.

Although, KRAS mutations are critical for the initiation of pancreatic ductal carcinogenesis, continued mutant KRAS function and oncogenic dosage are still required to maintain the growth of metastatic PDAC [5,20]. On the other hand, gene expression studies revealed that, compared to head localization, body-tail PDAC are more highly proliferative and aggressive [21,22]. Body/tail location is also associated with poor prognosis in advanced disease [23–25]. This may explain the reason why, in our cohort, higher values of KRAS MAF in cfDNA of metastatic PDAC patients were significantly associated with primary tumors located in the body/tail of the pancreas and liver metastases. Moreover, the higher MAF observed in patients with liver metastases may be explained by the larger volume of hepatic lesions than the isolated lung and peritoneal metastases [19].

Previous studies have reported the potential prognostic value of cfDNA levels and fragmentation in metastatic cancer [9,10,26], including metastatic PDAC [27]. In our study, higher plasma cfDNA concentrations were significantly associated with poorer OS and shorter PFS. Patients with hepatic metastasis displayed higher cfDNA levels, compared with those patients with other metastatic locations.

Despite the lack of knowledge about the precise mechanisms of cfDNA release into circulation, the role of apoptosis is becoming clearer [28]. A recent study reported that tumor-derived KRAS mutations in pancreatic cancer are predominantly carried by short and ultra-short cfDNA fragments [29]. This may be the biological explanation for our observation that, in parallel with our KRAS MAF results, a higher cfDNA fragmentation was found in patients with tumors located in the body/tail of the pancreas or with hepatic metastases than other metastatic lesions, likely due to more aggressive tumors. Thus, recent reports showed that body/tail PDAC may have more aggressive tumor biology and higher metastasis rate compared to PDAC in the head which may explain worse clinical outcomes [21–23].

CA19-9, also known as sialyl Lewis A antigen, is the currently used biomarker for pancreatic cancer, and several studies have reported the link between CA19-9 levels and survival in metastatic PDAC patients [30,31]. However, CA19-9 have some important limitations, such as false negative results in subjects with Lewis negative genotype and CA19-9 increases in patients with benign pancreatic-biliary diseases [32]. In our cohort, CA19-9 exhibited some prognostic value with higher baseline levels associated with poorer OS and PFS rates. However, our study demonstrates that the combination of CA19-9 with liquid biopsy markers greatly helped in the prognostic stratification of metastatic PDAC patients.

CA19-9 is also used for monitoring treatment response as the reduction of CA19-9 serum levels during treatment are usually associated with longer survival rates. However, in clinical practice, there is no consensus on the interpretation of the change in CA19-9 levels and its role in the management of PDAC patients [33]. Therefore, novel reliable biomarkers are required for monitoring the response of PDAC patients to chemotherapy [34]. In our analysis, the change in circulating KRAS MAF levels was a suitable surrogate marker for monitoring each patient's response to therapy. Moreover, the rise in MAF levels in some patients was better than CA19-9 in anticipating disease progression, and dynamics of circulating MAF better correlated with patients' outcome compared with CA19-9. Therefore, our results support MAF as a valuable complementary tool for monitoring the response to chemotherapy treatment in metastatic PDAC patients.

In summary, our study supports cfDNA-based liquid biopsy markers as promising clinical tools for the non-invasive prognosis and monitoring of metastatic PDAC patients.

4. Materials and Methods

4.1. Patients

Sixty-one patients diagnosed with metastatic PDAC in the Reina Sofía Hospital (Córdoba, Spain) were enrolled in this study from 2017 to 2019. Eligible patients were 18 years or older with histologically confirmed metastatic PDAC and were not treated by chemotherapy or radiotherapy before the enrollment. Metastatic PDAC pathology was confirmed in all patients included in our study by pathological analysis of tumor tissue (n = 43) or by cytological analysis (n = 18), and by computed tomography. All subjects gave their informed consent for their inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Córdoba (Comité de Ética de la Investigación de Córdoba, CEI Córdoba, PANCREAS-BIOPSIA-LIQ protocol, approved on April 26, 2017, Act nº263, ref, 3490). The baseline characteristics of the patients included in the study are listed in Table 1.

4.2. Procedures for Sample Analyses

Plasma was obtained from 10 mL of blood collected in Streck cell- free DNA BCT™ tubes before any therapeutic intervention. In seven patients, the plasma was also obtained at specified intervals

after the onset of treatment. Blood samples were centrifuged at 1600× *g* during 10 min at room temperature (RT) to separate plasma, followed by centrifugation at 6000× *g* during 10 min at RT to remove any possible remaining cells. Plasma samples were then aliquoted, transferred to cryotubes and stored at −80 °C. QIAamp Circulating Nucleic Acid Kit and the vacuum system QIAvac 24 Plus (Qiagen) were used for cfDNA extraction from 3 mL of plasma and extracted cfDNA was quantified using the Quantus fluorometer (Promega). The High Sensitivity D1000 ScreenTape Assay was used in an Agilent 2200 TapeStation System (Agilent) to analyse cfDNA fragmentation.

OncoBEAM™ RAS assay (Sysmex Inostics GmbH, Baltimore, MD, USA), which detects 34 mutations in KRAS/NRAS codons 12, 13, 59, 61, 117, and 146 was used to analyze RAS mutations in cfDNA and determine MAF in plasma. In brief, OncoBEAM™ RAS Assay started with a conventional PCR to amplify a locus of interest, which included 7 amplicons covering 12 codons and 34 mutations in KRAS/NRAS genes. For each codon a digital PCR was then performed and cfDNA was hybridized with fluorescent probes to quantify by flow cytometry KRAS/NRAS mutant and wild type molecules. This approach allows reliable detection of MAF < 0.1% in cfDNA [35].

In 43 patients, FFPE primary tumor tissue was available for RAS mutation analysis by standard-of-care procedures validated in our hospital. Specifically, the Idylla™ plattform (Biocartis), that utilizes microfluidics processing with specific cartridges and all reagents on board, was employed for RAS mutation analysis in tissue. The process is fully automated, including nucleic acid extraction and, if the results indicate WT KRAS, testing for NRAS mutations is mandatory using another specific cartridge. Serum CA19-9 levels were measured using a standard radioimmunoassay test in the Clinical Laboratory Department of our hospital.

4.3. Statistical Analyses

Statistical analyses were performed using SPSS Statistic 20.0.0, GraphPad Prism 7.0 Software and R Software 4.0.0. Overall survival (OS) was calculated from the date of diagnosis to the date of death. Progression-free survival (PFS) was calculated from the start date of therapy until disease progression. The survival rates were estimated using the Kaplan–Meier method and the Log-Rank test was used to identify the prognostic variables. SurvivalROC package in the R software was used to find optimal cut-off values in OS analyzing time-dependent ROC curve. The optimal cut-off value was chosen by minimizing the sum of false negative rate and false positive rate. In each case, the cut-off with prognostic value for OS was also tested for prognosis of PFS. When the optimal cut-off chosen with ROC curves was not able to separate statistically the groups according to the Kaplan-Meier analysis, the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used to find a cut-off value, using the Kaplan Scan (KaplanScan) feature, based on statistical testing. The Kaplan scanner separates the samples of a dataset into two groups based on values of variable of interest. In the order of values, it uses every increasing value as a cutoff to create 2 groups and test the p-value in a Log-Rank test. Mann-Whitney test was used to compare two groups and ANOVA test for analysis with more than two groups. Multivariate analysis was performed to establish independent prognostic factors using Cox proportional hazards modeling. Graph data are represented as mean ± standard deviation. Correlation analyses were performed using Pearson's correlation coefficient. All statistical tests were considered significant when $p < 0.05$.

5. Conclusions

We evaluated different cfDNA-based liquid biopsy markers as prognostic tools for the management care of metastatic PDAC patients. Our study shows that prognosis was more accurately predicted by RAS mutation analysis in cfDNA than by tissue analysis. Hence, both RAS mutation status and mutational load in cfDNA were independent risk factors for OS. Whereas, a higher cfDNA concentration and fragmentation levels were also associated with poorer survival. Notably, our data support the theory that multi-parameter liquid biopsy may significantly assist in the prognostic stratification of metastatic PDAC patients, while RAS MAF in cfDNA may facilitate with the monitoring of disease progression and response to therapy. Future larger studies with independent

cohorts are warranted to validate cfDNA-based liquid biopsy markers for the non-invasive prognosis and monitoring of metastatic PDAC patients.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6694/12/7/1754/s1, Table S1. Results of baseline RAS mutation analysis in tissue and plasma

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References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424, doi:10.3322/caac.21492.
2. Ferlay, J.; Colombet, M.; Soerjomataram, I.; Dyba, T.; Randi, G.; Bettio, M.; Gavin, A.; Visser, O.; Bray, F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J. Cancer* **2018**, *103*, 356–387, doi:10.1016/j.ejca.2018.07.005.
3. Rahib, L.; Smith, B.D.; Aizenberg, R.; Rosenzweig, A.B.; Fleshman, J.M.; Matrisian, L.M. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* **2014**, *74*, 2913–2921, doi:10.1158/0008-5472.CAN-14-0155.
4. Ryan, D.P.; Hong, T.S.; Bardeesy, N. Pancreatic adenocarcinoma. *N. Engl. J. Med.* **2014**, *371*, 1039–1049, doi:10.1056/NEJMra1404198.
5. Waters, A.M.; Der, C.J. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a031435, doi:10.1101/cshperspect.a031435.
6. Baek, H.W.; Park, M.J.; Rhee, Y.Y.; Lee, K.B.; Kim, M.A.; Park, I.A. Diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration cytology of pancreatic lesions. *J. Pathol. Transl. Med.* **2015**, *49*, 52–60, doi:10.4132/jptm.2014.10.26.
7. Luchini, C.; Veronese, N.; Nottegar, A.; Cappelletti, V.; Daidone, M.G.; Smith, L.; Parris, C.; Brosens, L.A.A.; Caruso, M.G.; Cheng, L.; et al. Liquid Biopsy as Surrogate for Tissue for Molecular Profiling in Pancreatic Cancer: A Meta-Analysis Towards Precision Medicine. *Cancers* **2019**, *11*, 1152, doi:10.3390/cancers11081152.
8. Tjensvoll, K.; Lapin, M.; Buhl, T.; Oltedal, S.; Steen-Ottosen Berry, K.; Gilje, B.; Soreide, J.A.; Javle, M.; Nordgard, O.; Smaaland, R. Clinical relevance of circulating KRAS mutated DNA in plasma from patients with advanced pancreatic cancer. *Mol. Oncol.* **2016**, *10*, 635–643, doi:10.1016/j.molonc.2015.11.012.
9. El Messaoudi, S.; Mouliere, F.; Du Manoir, S.; Bascoul-Mollevi, C.; Gillet, B.; Nouaille, M.; Fiess, C.; Crapez, E.; Bibeau, F.; Theillet, C.; et al. Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. *Clin. Cancer Res.* **2016**, *22*, 3067–3077, doi:10.1158/1078-0432.CCR-15-0297.
10. Tabernero, J.; Lenz, H.J.; Siena, S.; Sobrero, A.; Falcone, A.; Ychou, M.; Humblet, Y.; Bouche, O.; Mineur, L.; Barone, C.; et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: A retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol.* **2015**, *16*, 937–948, doi:10.1016/S1470-2045(15)00138-2.
11. Kinugasa, H.; Nouso, K.; Miyahara, K.; Morimoto, Y.; Dohi, C.; Tsutsumi, K.; Kato, H.; Matsubara, T.; Okada, H.; Yamamoto, K. Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. *Cancer* **2015**, *121*, 2271–2280, doi:10.1002/cncr.29364.
12. Park, G.; Park, J.K.; Son, D.S.; Shin, S.H.; Kim, Y.J.; Jeon, H.J.; Lee, J.; Park, W.Y.; Lee, K.H.; Park, D. Utility

of targeted deep sequencing for detecting circulating tumor DNA in pancreatic cancer patients. *Sci. Rep.* **2018**, *8*, 11631, doi:10.1038/s41598-018-30100-w.

13. Wang, Z.Y.; Ding, X.Q.; Zhu, H.; Wang, R.X.; Pan, X.R.; Tong, J.H. KRAS Mutant Allele Fraction in Circulating Cell-Free DNA Correlates With Clinical Stage in Pancreatic Cancer Patients. *Front. Oncol.* **2019**, *9*, 1295, doi:10.3389/fonc.2019.01295.
14. Chen, I.; Raymond, V.M.; Geis, J.A.; Collisson, E.A.; Jensen, B.V.; Hermann, K.L.; Erlander, M.G.; Tempero, M.; Johansen, J.S. Ultrasensitive plasma ctDNA KRAS assay for detection, prognosis, and assessment of therapeutic response in patients with unresectable pancreatic ductal adenocarcinoma. *Oncotarget* **2017**, *8*, 97769–97786, doi:10.18632/oncotarget.22080.
15. Perets, R.; Greenberg, O.; Shentzer, T.; Semenisty, V.; Epelbaum, R.; Bick, T.; Sarji, S.; Ben-Izhak, O.; Sabo, E.; Hershkovitz, D. Mutant KRAS Circulating Tumor DNA Is an Accurate Tool for Pancreatic Cancer Monitoring. *Oncologist* **2018**, *23*, 566–572, doi:10.1634/theoncologist.2017-0467.
16. Hadano, N.; Murakami, Y.; Uemura, K.; Hashimoto, Y.; Kondo, N.; Nakagawa, N.; Sueda, T.; Hiyama, E. Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. *Br. J. Cancer* **2016**, *115*, 59–65, doi:10.1038/bjc.2016.175.
17. Gall, T.M.H.; Belete, S.; Khanderia, E.; Frampton, A.E.; Jiao, L.R. Circulating Tumor Cells and Cell-Free DNA in Pancreatic Ductal Adenocarcinoma. *Am. J. Pathol.* **2019**, *189*, 71–81, doi:10.1016/j.ajpath.2018.03.020.
18. Lennerz, J.K.; Stenzinger, A. Allelic ratio of KRAS mutations in pancreatic cancer. *Oncologist* **2015**, *20*, e8, doi:10.1634/theoncologist.2014-0408.
19. Bernard, V.; Kim, D.U.; San Lucas, F.A.; Castillo, J.; Allenson, K.; Mulu, F.C.; Stephens, B.M.; Huang, J.; Semaan, A.; Guerrero, P.A.; et al. Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* **2019**, *156*, 108–118, doi:10.1053/j.gastro.2018.09.022.
20. Mueller, S.; Engleitner, T.; Maresch, R.; Zukowska, M.; Lange, S.; Kaltenbacher, T.; Konukiewitz, B.; Ollinger, R.; Zwiebel, M.; Strong, A.; et al. Evolutionary routes and KRAS dosage define pancreatic cancer phenotypes. *Nature* **2018**, *554*, 62–68, doi:10.1038/nature25459.
21. Birnbaum, D.J.; Bertucci, F.; Finetti, P.; Birnbaum, D.; Mamessier, E. Head and Body/Tail Pancreatic Carcinomas Are Not the Same Tumors. *Cancers* **2019**, *11*, 497, doi:10.3390/cancers11040497.
22. Dreyer, S.B.; Jamieson, N.B.; Upstill-Goddard, R.; Bailey, P.J.; McKay, C.J.; Biankin, A.V.; Chang, D.K. Defining the molecular pathology of pancreatic body and tail adenocarcinoma. *Br. J. Surg.* **2018**, doi:10.1002/bjs.10772.
23. van Erning, F.N.; Mackay, T.M.; van der Geest, L.G.M.; Groot Koerkamp, B.; van Laarhoven, H.W.M.; Bonsing, B.A.; Wilmink, J.W.; van Santvoort, H.C.; de Vos-Geelen, J.; van Eijck, C.H.J.; et al. Association of the location of pancreatic ductal adenocarcinoma (head, body, tail) with tumor stage, treatment, and survival: A population-based analysis. *Acta Oncol.* **2018**, *57*, 1655–1662, doi:10.1080/0284186X.2018.1518593.
24. Tomasello, G.; Ghidini, M.; Costanzo, A.; Ghidini, A.; Russo, A.; Barni, S.; Passalacqua, R.; Petrelli, F. Outcome of head compared to body and tail pancreatic cancer: A systematic review and meta-analysis of 93 studies. *J. Gastrointest. Oncol.* **2019**, *10*, 259–269, doi:10.21037/jgo.2018.12.08.
25. Artinyan, A.; Soriano, P.A.; Prendergast, C.; Low, T.; Ellenhorn, J.D.; Kim, J. The anatomic location of pancreatic cancer is a prognostic factor for survival. *HPB* **2008**, *10*, 371–376, doi:10.1080/13651820802291233.
26. Valpione, S.; Gremel, G.; Mundra, P.; Middlehurst, P.; Galvani, E.; Girotti, M.R.; Lee, R.J.; Garner, G.; Dhomen, N.; Lorigan, P.C.; et al. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *Eur. J. Cancer* **2018**, *88*, 1–9, doi:10.1016/j.ejca.2017.10.029.
27. Lapin, M.; Oltedal, S.; Tjensvoll, K.; Buhl, T.; Smaaland, R.; Garresori, H.; Javle, M.; Glenjen, N.I.; Abelseth, B.K.; Gilje, B.; et al. Fragment size and level of cell-free DNA provide prognostic information in patients with advanced pancreatic cancer. *J. Transl. Med.* **2018**, *16*, 300, doi:10.1186/s12967-018-1677-2.
28. Heitzer, E.; Auinger, L.; Speicher, M.R. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends Mol. Med.* **2020**, *26*, 519–528, doi:10.1016/j.molmed.2020.01.012.
29. Zvereva, M.; Roberti, G.; Durand, G.; Voegele, C.; Nguyen, M.D.; Delhomme, T.M.; Chopard, P.; Fabianova, E.; Adamcakova, Z.; Holcatova, I.; et al. Circulating tumour-derived KRAS mutations in pancreatic cancer cases are predominantly carried by very short fragments of cell-free DNA. *EBioMedicine* **2020**, 102462, doi:10.1016/j.ebiom.2019.09.042.
30. Saad, E.D.; Machado, M.C.; Wajsbrot, D.; Abramoff, R.; Hoff, P.M.; Tabacof, J.; Katz, A.; Simon, S.D.; Gansl, R.C. Pretreatment CA 19-9 level as a prognostic factor in patients with advanced pancreatic cancer treated

with gemcitabine. *Int. J. Gastrointest. Cancer* **2002**, *32*, 35–41, doi:10.1385/IJGC:32:1:35.
31. Maisey, N.R.; Norman, A.R.; Hill, A.; Massey, A.; Oates, J.; Cunningham, D. CA19-9 as a prognostic factor in inoperable pancreatic cancer: The implication for clinical trials. *Br. J. Cancer* **2005**, *93*, 740–743, doi:10.1038/sj.bjc.6602760.
32. Scara, S.; Bottoni, P.; Scatena, R. CA 19-9: Biochemical and Clinical Aspects. *Adv. Exp. Med. Biol.* **2015**, *867*, 247–260, doi:10.1007/978-94-017-7215-0_15.
33. Bauer, T.M.; El-Rayes, B.F.; Li, X.; Hammad, N.; Philip, P.A.; Shields, A.F.; Zalupski, M.M.; Bekaii-Saab, T. Carbohydrate antigen 19-9 is a prognostic and predictive biomarker in patients with advanced pancreatic cancer who receive gemcitabine-containing chemotherapy: A pooled analysis of 6 prospective trials. *Cancer* **2013**, *119*, 285–292, doi:10.1002/cncr.27734.
34. van der Sijde, F.; Vietsch, E.E.; Mustafa, D.A.M.; Besselink, M.G.; Groot Koerkamp, B.; van Eijck, C.H.J. Circulating Biomarkers for Prediction of Objective Response to Chemotherapy in Pancreatic Cancer Patients. *Cancers* **2019**, *11*, 93, doi:10.3390/cancers11010093.
35. Vivancos, A.; Aranda, E.; Benavides, M.; Elez, E.; Gomez-Espana, M.A.; Toledano, M.; Alvarez, M.; Parrado, M.R.C.; Garcia-Barberan, V.; Diaz-Rubio, E. Comparison of the Clinical Sensitivity of the Idylla Platform and the OncoBEAM RAS CRC Assay for KRAS Mutation Detection in Liquid Biopsy Samples. *Sci. Rep.* **2019**, *9*, 8976, doi:10.1038/s41598-019-45616-y.

XI. Annex II

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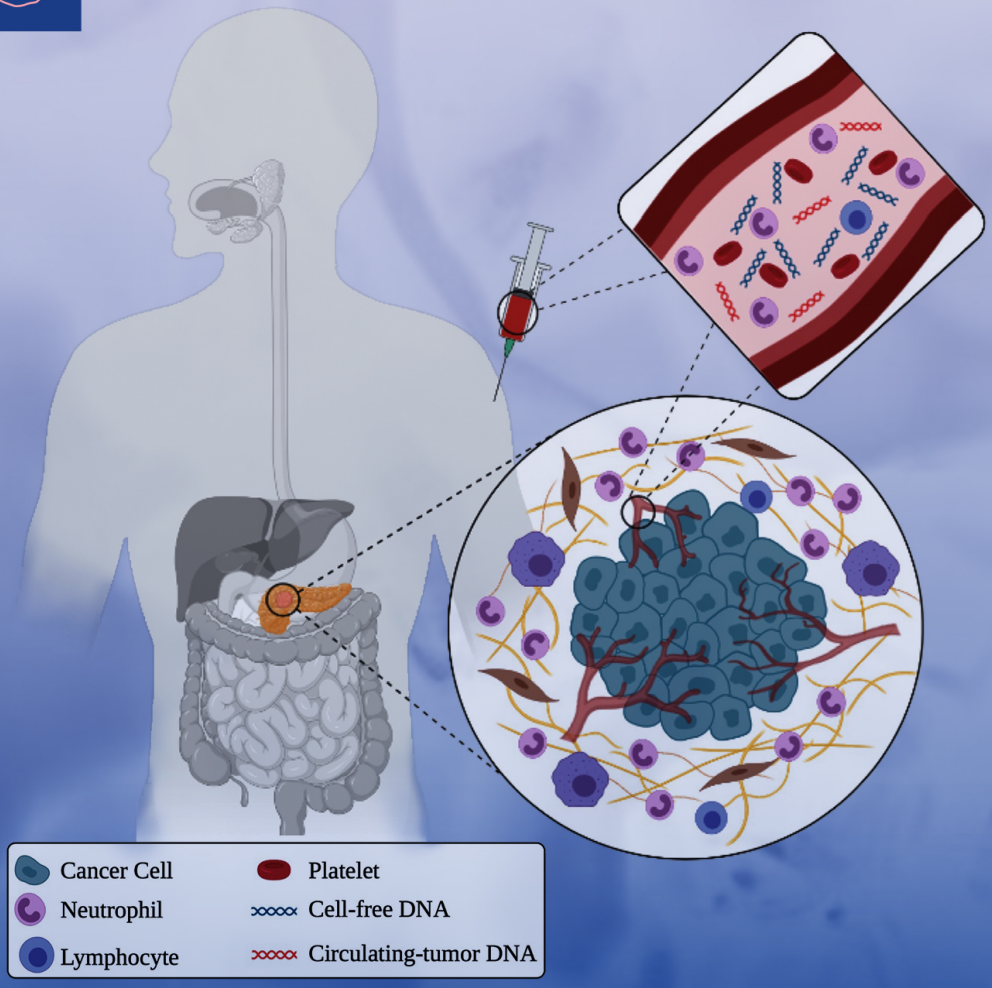
IMPACT FACTOR 6.126



Combining Inflammatory and Liquid Biopsy Biomarkers Improves Prognosis Prediction in Metastatic Pancreatic Cancer

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Article

The Combination of Neutrophil–Lymphocyte Ratio and Platelet–Lymphocyte Ratio with Liquid Biopsy Biomarkers Improves Prognosis Prediction in Metastatic Pancreatic Cancer

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Simple Summary: Liquid biopsy is a noninvasive approach that provides tumor molecular profiling. On the other hand, the vast majority of pancreatic tumors are pancreatic ductal adenocarcinomas (PDAC), which are characterized by pronounced inflammation. Therefore, we hypothesized that the combination of biomarkers of systemic inflammation, such as the neutrophil-to-lymphocyte-ratio (NLR) and platelet-to-lymphocyte ratio (PLR), with liquid biopsy-based biomarkers may increase their clinical usefulness. Our study shows that combining NLR, PLR, and the standard PDAC marker CA19-9 with circulating cell-free DNA and circulating RAS-mutated DNA outperforms traditional clinical tools for the clinical management of metastatic PDAC patients.

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a highly inflammatory microenvironment and liquid biopsy has emerged as a promising tool for the noninvasive analysis of this tumor. In this study, plasma was obtained from 58 metastatic PDAC patients, and neutrophil–lymphocyte ratio (NLR), platelet–lymphocyte ratio (PLR), circulating cell-free DNA (cfDNA) concentration, and circulating RAS mutation were determined. We found that NLR was significantly associated with both overall survival (OS) and progression-free survival. Remarkably, NLR was an independent risk factor for poor OS. Moreover, NLR and PLR positively correlated, and combination of both inflammatory markers significantly improved the prognostic stratification of metastatic PDAC patients. NLR also showed a positive correlation with cfDNA levels and RAS mutant allelic fraction (MAF). Besides, we found that neutrophil activation contributed to cfDNA content in the plasma of metastatic PDAC patients. Finally, a multi-parameter prognosis model was designed by combining NLR, PLR, cfDNA levels, RAS mutation, RAS MAF, and CA19-9, which performs as a promising tool to predict the prognosis of metastatic PDAC patients. In conclusion, our study supports the idea that the use of systemic inflammatory markers along with circulating tumor-specific markers may constitute a valuable tool for the clinical management of metastatic PDAC patients.

Keywords: NLR; PLR; circulating tumor DNA; pancreatic ductal adenocarcinoma; RAS mutation; neutrophil elastase

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1. Introduction

Pancreatic cancer is among the major lethal cancers, with five-year survival of around 5%, and it is the fourth highest cause of cancer mortality in Europe, with more than 95,000 deaths annually [1,2]. Improved survival rates have been achieved in the most common cancers, but pancreatic cancer death rate is increasing [3]. This dismal prognosis is mainly due to advanced stage diagnosis and resistance to therapy. More than 90% of pancreatic tumors are pancreatic ductal adenocarcinomas (PDAC) and the vast majority of deaths are associated with this rapidly progressive and highly aggressive tumor type.

Inflammation plays a critical role in the development and progression of many types of cancer. PDAC is characterized by pronounced inflammation and desmoplasia, leading to hypoxia, metabolic reprogramming, and immune suppression that ultimately promotes tumor growth and metastasis [4]. The neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have been proposed as markers of systemic inflammatory response in several solid tumors [5]. Platelets are mainly protumorigenic and neutrophils acquire protumorigenic properties upon recruitment to the tumor microenvironment [6]. Accordingly, levels of circulating neutrophils are elevated in PDAC patients and increased levels of neutrophils infiltrating pancreatic tumors have been reported to correlate with a poor clinical outcome [7,8]. These studies support the theory that neutrophils play a role in inflammation-driven pancreatic tumorigenesis. Besides, low NLR and PLR have been associated with longer overall survival (OS) and progression-free survival (PFS) in pancreatic cancer [9,10].

KRAS oncogene, which is mutationally activated in the vast majority of pancreatic ductal tumors [11], is involved in the release by tumor cells of inflammatory cytokines; the recruitment of immune cells with protumoral activity, thereby promoting an inflamed tumor stroma; and the progression and invasion of PDAC [4,12–14]. We have previously reported that RAS mutation status and mutational load in circulating cell-free DNA are independent risk factors for OS in metastatic PDAC patients. Moreover, higher cell-free DNA (cfDNA) concentration and fragmentation levels were also associated with poorer survival [15].

Although NLR and PLR have been shown to be of prognostic value in PDAC, neutrophils, platelets, and lymphocyte counts are affected by other factors, including chemotherapy toxicity [16]. It is necessary to strengthen the clinical usefulness of these biomarkers of systemic inflammation with the information provided by noninvasive tumor biomarkers. Therefore, the present study was aimed at evaluating the prognostic value of combining NLR and PLR with circulating liquid biopsy markers associated to poor survival outcome in metastatic PDAC patients.

2. Materials and Methods

2.1. Patients

A total of 58 patients with a diagnosis of metastatic PDAC were included in this study. Patients were enrolled from the Reina Sofía Hospital (Córdoba, Spain) from 2017 to 2019 with the following inclusion criteria: older than 18 years with histologically confirmed metastatic PDAC and without previous chemotherapy or radiotherapy. The study was approved by the ethics committee of our hospital and written informed consent was obtained from all patients before enrollment. Patients' characteristics are summarized in Table 1.

Table 1. Baseline characteristics of patients.

Patient Characteristics		*n* (%)
Age	<60 years	20 (34.5)
	>60 years	38 (65.5)
Sex	Male	32 (55.2)
	Female	26 (44.8)
ECOG	0	17 (29.3)
	1	30 (51.7)
	2	8 (13.8)
	3	3 (5.2)
1st line treatment	Gemcitabine	2 (3.4)
	Gemcitabine/nab-paclitaxel	38 (65.5)
	Gemcitabine/nab-paclitaxel/FOLFOX	4 (6.9)
	FOLFIRINOX	11 (19)
	No treatment	3 (5.2)
Survival	Alive	17 (29.3)
	Dead	41 (70.7)
Disease progression	Yes	50 (86.2)
	No	3 (5.2)
	Not valuable (No treatment or surgery)	5 (8.6)
Primary tumor location	Tail	16 (27.6)
	Body	24 (41.4)
	Head	16 (27.6)
	No data	2 (3.4)
Number of metastatic lesions	One location	25 (43.1)
	More than one location	33 (56.9)
Metastatic lesions location	Hepatic lesions	45 (77.6)
	Non-hepatic lesions	13 (22.4)
Liquid Biopsy RAS status	RAS mutated	44 (75.9)
	RAS wild-type	14 (24.1)

2.2. Procedures for Sample Analyses

Platelets, neutrophils, and lymphocyte were measured routinely in the clinical laboratory department of our hospital. NLR and PLR were calculated by dividing absolute neutrophil count and platelets count by the absolute lymphocyte count, respectively. Tumor biomarker CA19-9 was also measured in the clinical laboratory department of our hospital and a cut-off value of 45,500 U/mL, previously determined in our research [15], was used.

The analysis of cell-free DNA (cfDNA) was performed as previously described [15]. In brief, plasma was obtained from 10 mL of peripheral blood before treatment and cfDNA was extracted from 3 mL of plasma with the QIAamp Circulating Nucleic Acid Kit and the vacuum system QIAvac 24 Plus (Qiagen, Hilden, Germany). The Quantus fluorometer (Promega, Madison, WI, USA) and the Agilent 2200 TapeStation system (Agilent, Santa Clara, CA, USA) with the High Sensitivity D1000 ScreenTape assay were used for measuring cfDNA concentration and fragmentation, respectively. The OncoBEAM™ RAS assay (Sysmex Inostics GmbH, Hamburg, Germany) was used for the analysis of RAS mutations in cfDNA and the determination of RAS mutant allelic fraction (MAF) in plasma.

For the quantification of elastase circulating levels, the Human PMN Elastase ELISA Kit (Abcam, Cambridge, UK) was used following the manufacturer's instructions.

2.3. Statistical Analyses

SPSS Statistic 20.0.0, GraphPad Prism 7.0 Software and R Software 4.0.0 were used for data analysis. OS was computed from the time of diagnosis until the date of death and PFS was determined as the time from the start of therapy until documented disease progression. Estimation of survival rates and the identification of prognostic variables were performed with the Kaplan–Meier method and the log-rank test, respectively. The optimal cut-off values were selected with the SurvivalROC package based on the time-dependent ROC curve and were selected by minimalizing the sum of false negative and false positive rates [17]. The cut-off value with prognostic relevance for OS was also tested for prognosis of PFS. Mann–Whitney test was used to compare differences between two groups and ANOVA test was performed when comparing more than two groups. Multivariate analysis with Cox proportional hazards regression was used to determine independent prognostic factors. Correlation analyses were performed using Pearson's correlation coefficient. Data in graphs are represented as mean ± standard deviation. All results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Clinicopathological Characteristics of Patients

Fifty-eight patients were included in the study between 2017 and 2019 (baseline characteristics are summarized in Table 1). Most patients (81%) had a good baseline ECOG (ECOG 0–1) and the majority of them (75.8%) received first-line gemcitabine-based regimes. As shown in Figure 1A,B, there was a trend towards females ($n = 26$) having better OS than males ($n = 32$) (193.5 versus 310 days; $p = 0.0574$) and also better PFS, although not statistically significant (125 versus 265 days; $p = 0.1044$). Patients included in the study ranged in age from 40 to 84 years, with a median of 65 years of age. When patients were stratified according to age (60 years), no differences in OS and PFS were found (Table 2). ECOG was related with better OS ($p = 0.0030$), whereas there were no significant association between ECOG and PFS ($p = 0.1869$) (Figure 1C,D).

All PDAC patients had distant metastases at diagnosis, the liver being the most frequent site of metastasis (77.6%, Table 1). Patients with metastatic lesions located in the liver had significantly poorer OS and PFS rates ($p= 0.0262$ and $p = 0.0006$, respectively) compared with patients with metastasis affecting other organs (Figure 1E,F). On the contrary, there was no significant association between number of metastatic lesions and OS or PFS (Table 2). Primary tumor sites were tail, body, and head of pancreas in 27.6%, 41.4%, and 29.3% of patients, respectively (Table 1), but primary tumor location was not significant related with OS or PFS (Table 2).

3.2. NLR and PLR Are Prognostic Markers in Metastatic PDAC Patients

NLR and PLR were analyzed in 58 metastatic PDAC patients. One patient with an ultra-high platelets count was excluded from the PLR analysis. The median NLR in plasma of metastatic PDAC patients was 3.94 (range 0.38–18.8) and the median PLR was 176.07 (range 43.59–492.86). There was a significant association of high NLR with male gender ($p = 0.0294$), while no relation was found between PLR and gender ($p = 0.2591$) (Figure 2A,B). On the other hand, although no relation was found between NLR and age ($p = 0.4891$), patients older than 60 years showed a significantly lower PLR than those younger (153.39 versus 236.47; $p = 0.0076$) (Figure 2C,D). Besides, as shown in Figure 2E,F, higher NLR, but not PLR, was associated with worse ECOG (ECOG 2–3) (NLR: $p = 0.0018$; PLR: $p = 0.6318$).

NLR was not significantly associated with primary tumor site ($p = 0.7859$) or number of metastasis ($p = 0.2859$), although NLR showed a trend towards higher values in patients with metastatic lesions located in the liver compared with patients with metastasis affecting other organs ($p = 0.1551$) (Figure 3A). Contrarily, PLR was not associated with metastatic location ($p = 0.7558$) or the number of metastasis ($p = 0.7653$), but patients with the primary tumor in the head of the pancreas showed higher PLR compared with tumors in the

body/tail location (266.2 versus 149.16; $p = 0.0245$) (Figure 3B). Patients with higher NLR (>5.52) had significantly poorer OS (108 versus 335 days; $p < 0.0001$) and PFS (85 versus 232 days; $p = 0.0101$) rates (Figure 3C,D). Moreover, multivariate analysis revealed that NLR was an independent prognostic factor for OS (HR 2.466, 95% CI 1.246–4.880; $p = 0.010$), along with ECOG and RAS mutation status (Table 3). Also, although not significant, patients with higher PLR (>90.48) showed a trend towards poorer OS (236 versus 399 days; $p = 0.1430$) and PFS (145 versus 337 days; $p = 0.2960$) (Figure 3E,F).

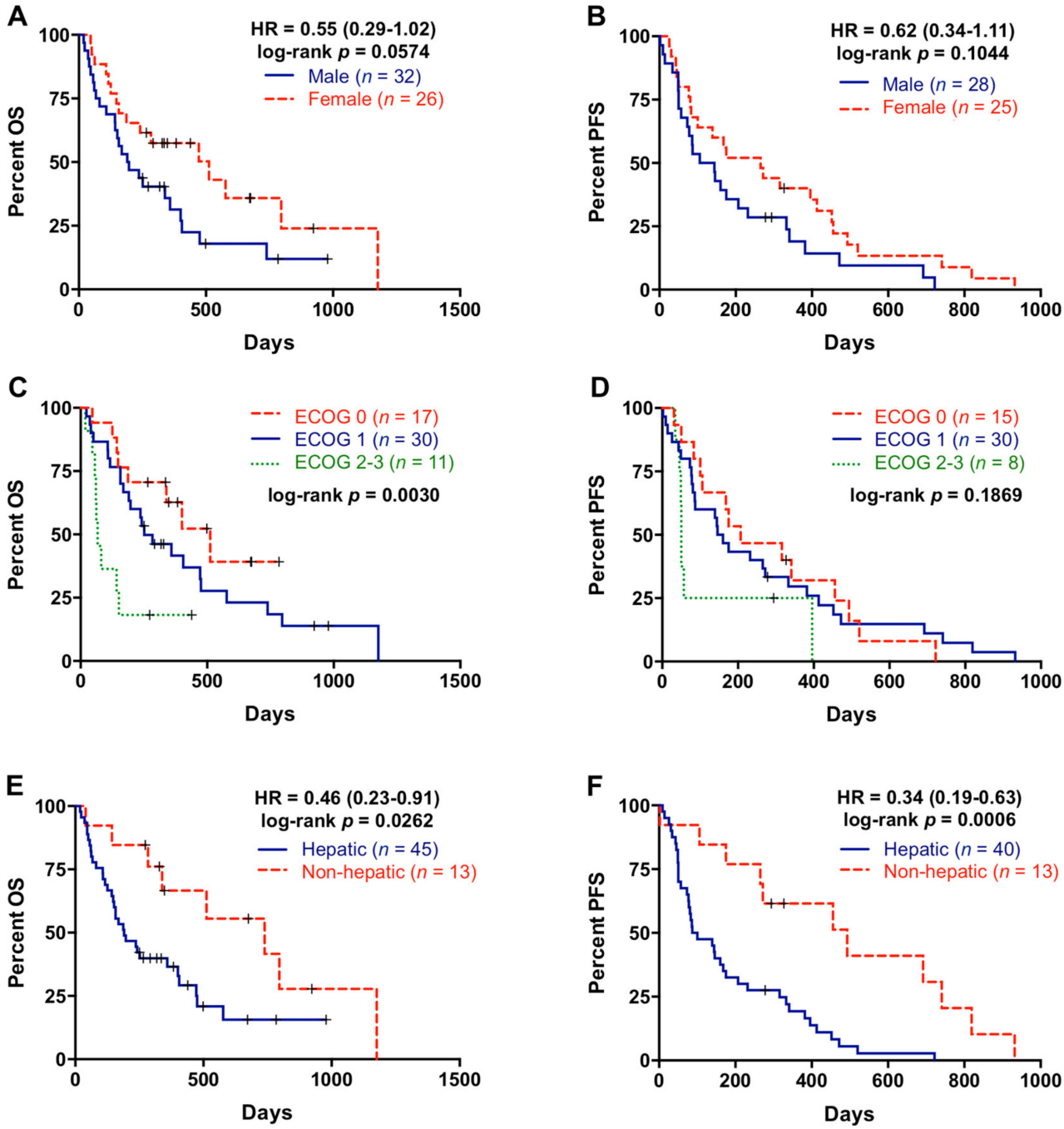

Figure 1. Overall survival and progression-free survival rates according to the clinical characteristics of the patients. (**A**) Overall survival (OS) according to gender; (**B**) progression-free survival (PFS) according to gender; (**C**) OS according to EGOG; (**D**) PFS according to ECOG; (**E**) OS according to metastatic location; (**F**) PFS according to metastatic location.

Table 2. Overall survival analysis.

Variables	OS		PFS	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***
Age ≤60 years >60 years	1.113 (0.584–2.119)	0.7452	0.925 (0.505–1.694)	0.8015
Gender Male Female	0.545 (0.291–1.019)	0.0574	0.615 (0.343–1.106)	0.1044
ECOG 0 1 2–3	1.653 (0.789–3.465) 5.967 (1.86–19.16)	0.0030	1.083 (0.568–2.065) 3.166 (0.978–10.25)	0.1869
Primary Tumor Location Body/Tail Head	1.48 (0.72–3.04)	0.2884	1.52 (0.74–3.10)	0.2500
Number of Metastasis 1 ≥2	1.5 (0.803–2.801)	0.2035	1.449 (0.811–2.588)	0.2100
Metastatic Location Hepatic Nonhepatic	0.462 (0.234–0.913)	0.0262	0.344 (0.187–0.634)	0.0006
RAS mutation status plasma MUT WT	0.283 (0.141–0.565)	0.0003	0.240 (0.126–0.458)	<0.0001
NLR ≤5.52 >5.52	5.881 (2.552–13.55)	<0.0001	2.754 (1.272–5.962)	0.0101
PLR ≤90.48 >90.48	1.816 (0.817–4.035)	0.1430	1.460 (0.718–2.972)	0.2960
cfDNA concentration ≤26.46 ng/mL >26.46 ng/mL	2.173 (1.149–4.107)	0.0169	1.708 (0.964–3.025)	0.0664
MAF ≤0.351% >0.351%	2.151 (1.049–4.409)	0.0365	1.859 (0.925–3.737)	0.0818
CA19-9 ≤45,500 U/mL >45,500 U/mL	3.514 (1.196–10.32)	0.0233	3.508 (1.334–9.227)	0.0110

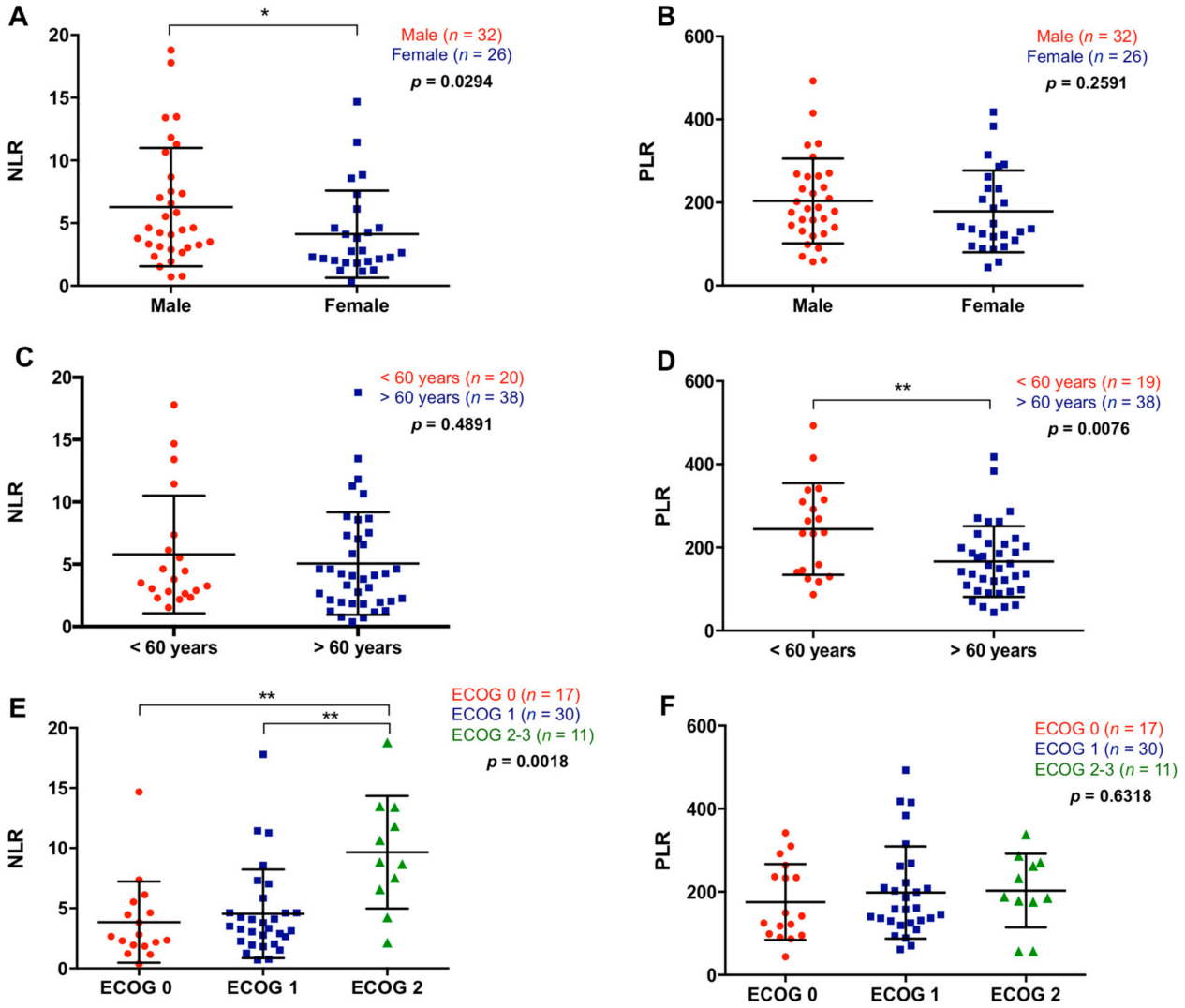

Figure 2. Association between neutrophil–lymphocyte ratio and platelet–lymphocyte ratio with the clinical characteristics of the patients. (**A**) Neutrophil–lymphocyte ratio (NLR) and (**B**) platelet–lymphocyte ratio (PLR) in male or females patients, (**C**) NLR and (**D**) PLR in patients younger or older than 60 years, (**E**) NLR and (**F**) PLR according to the ECOG (* $p < 0.05$, ** $p < 0.01$).

Table 3. Multivariate analysis.

Variables	OS		PFS	
	HR (95%CI)	*p*	HR (95%CI)	*p*
ECOG	2.024 (1.207–3.393)	0.008	-	-
Metastatic Location	-	ns	3.150 (1.359–7.305)	0.007
RAS mutation status plasma	6.944 (2.033–23.73)	0.002	7.908 (2.482–25.20)	0.0001
NLR	2.466 (1.246–4.880)	0.010	-	ns

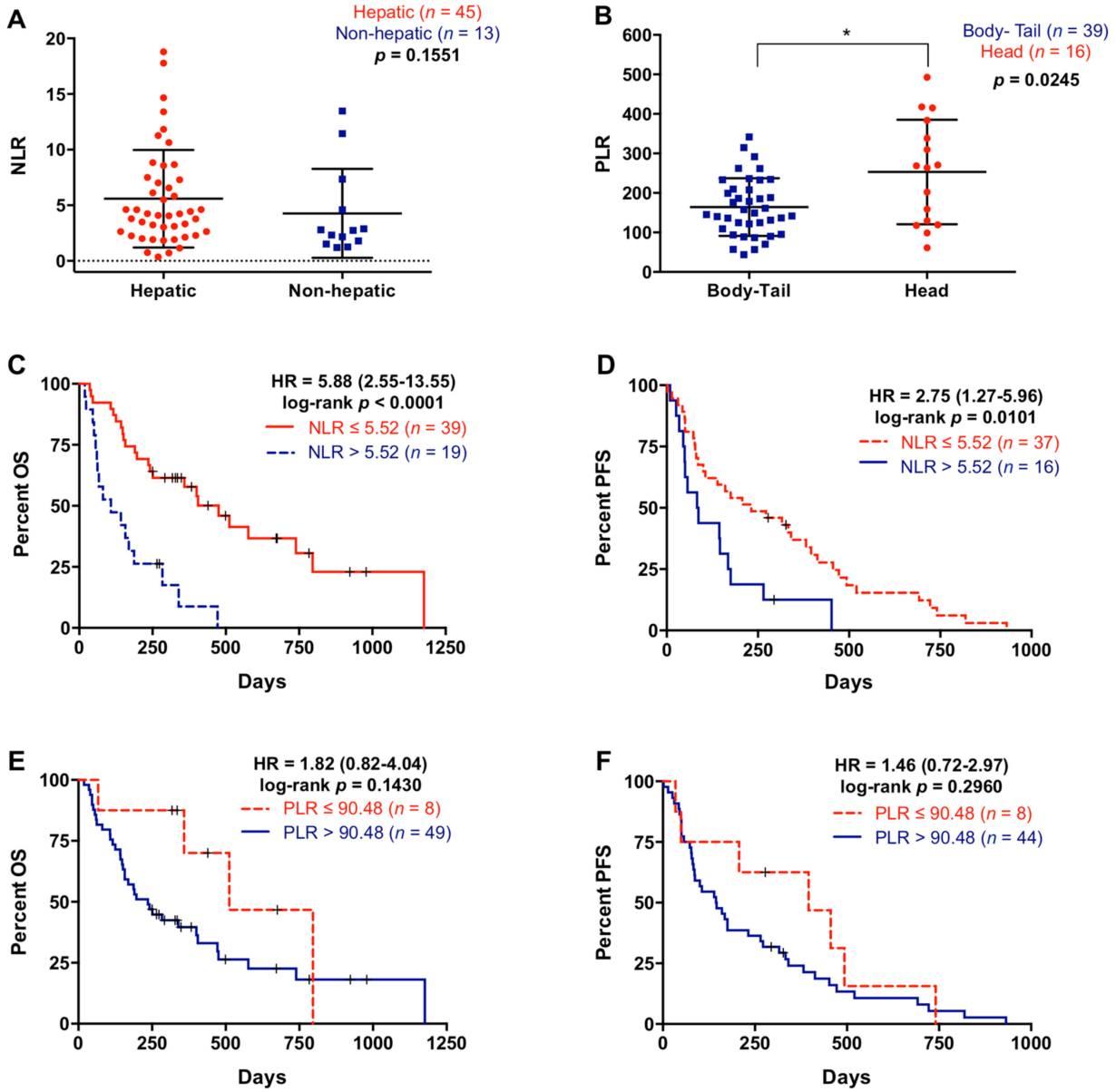

Figure 3. Neutrophil–lymphocyte ratio and platelet–lymphocyte ratio in metastatic pancreatic ductal adenocarcinoma patients. (**A**) Neutrophil–lymphocyte ratio (NLR) in patients with liver metastasis or metastases elsewhere, (**B**) platelet–lymphocyte ratio (PLR) in patients with primary tumor located in the body-tail or the head of the pancreas, (**C**) overall survival (OS) according to NLR (cut-off: 5.52), (**D**) progression-free survival (PFS) according to NLR (cut-off: 5.52), (**E**) OS according to PLR (cut-off: 90.48), (**F**) PFS according to PLR (cut-off: 90.48) (* $p < 0.05$).

3.3. Combined Analysis of NLR and PLR Improves the Prognostic Accuracy in Patients with Metastatic PDAC

A significant positive correlation between NLR and PLR was observed (r = 0.35; $p = 0.0085$) (Figure 4A). Notably, the combination of NLR and PLR improved the prognostic classification of metastatic PDAC patients. For this combined analysis, positive or negative values were assigned when NLR or PLR values were above (positive) or below (negative) the cut-off for prognostic value in OS, and scores 0, 1, and 2 were defined as negative for both markers, positive for one of them, and positive for both markers, respectively. As shown in Figure 4B, those patients with score 2 showed a highly significant poorer survival than those patients with score 0 or 1 in the Kaplan–Meier analysis ($p = 0.0004$ and

$p = 0.0040$, respectively). In contrast, the combination of NLR and PLR did not significantly improve prognosis accuracy for PFS ($p = 0.0856$) (Figure 4C).

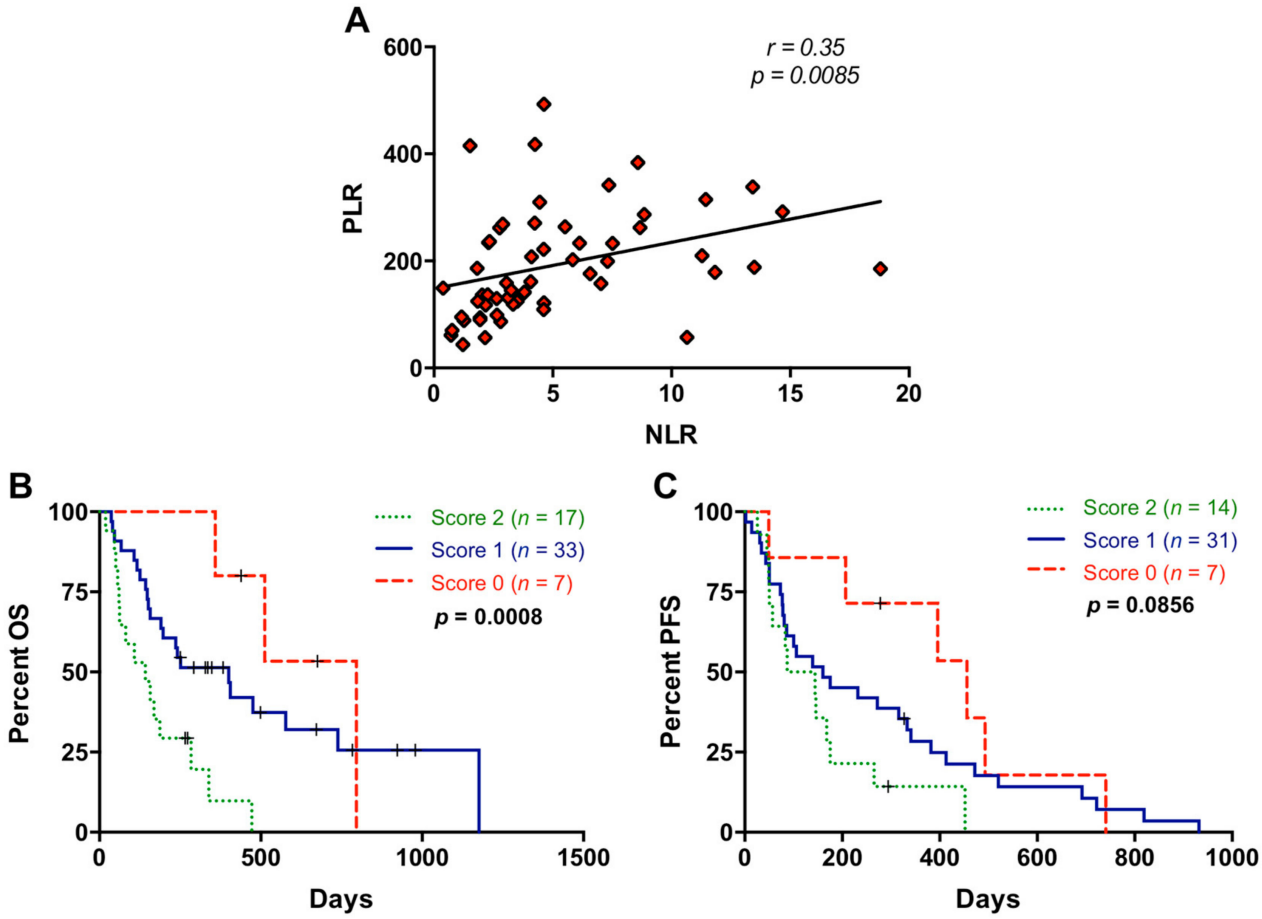

Figure 4. Association between neutrophil–lymphocyte ratio and platelet–lymphocyte ratio. (**A**) Correlation between neutrophil–lymphocyte ratio (NLR) and platelet–lymphocyte ratio (PLR) values; (**B**) overall survival (OS) according to the NLR and PLR combination (score 2 compared to score 0: $p = 0.0004$, score 2 compared to score 1: $p = 0.0040$); (**C**) progression-free survival (PFS) according to the NLR and PLR combination (score 2 compared to score 0: $p = 0.0097$; score 2 compared to score 1: $p = 0.1463$) (score 2, positive for both markers; score 1, positive for one of them; score 0: negative for both markers).

3.4. The Combination of NLR and cfDNA Values Significantly Improves Prognostic Stratification of Metastatic PDAC Patients

cfDNA concentration was measured in our patient cohort (median: 32.6 ng/mL; range: 10–700 ng/mL). A high positive correlation (r = 0.71; $p < 0.0001$) was found between NLR and cfDNA concentration (Figure 5A). No correlation was found between PLR and cfDNA concentration ($p = 0.8205$). However, a negative correlation (r = −0.30; $p = 0.0244$) was found between PLR and cfDNA size (Figure S1). No significant association was found between NLR and cfDNA fragmentation ($p = 0.4381$).

We previously reported that higher levels of cfDNA were significantly associated with shorter OS and PFS in metastatic PDAC patients and a cut-off for cfDNA concentration (26.46 ng/mL) was determined [15]. Using this cut-off, patients included in the present study also showed differences in OS (172 versus 339 days; $p = 0.0169$) and a trend for PFS (122.5 versus 278 days; $p = 0.0664$) according to cfDNA concentration. When NLR and cfDNA levels were combined according to the scoring system described above, patients with score 2 showed significantly shorter OS than patients with score 0 or score 1 ($p = 0.0001$ and $p = 0.0008$, respectively) (Figure 5B). We also found an improvement in the prognostic

stratification of patients according to PFS when NLR and cfDNA levels were combined (score 2 versus score 0: $p = 0.0037$ and score 2 versus score 1: $p = 0.0119$) (Figure 5C).

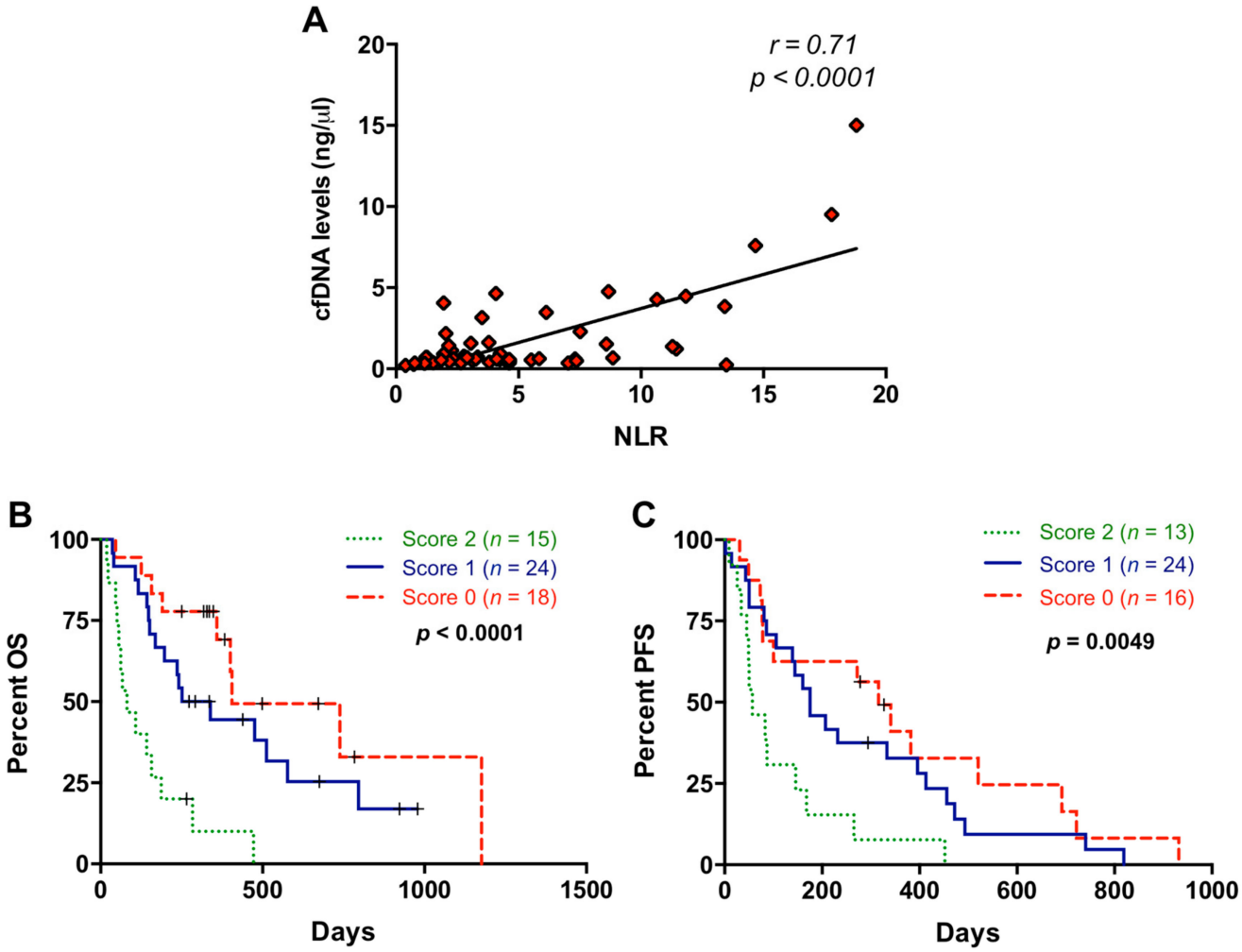

Figure 5. Association between neutrophil–lymphocyte ratio and cell-free DNA concentration. (**A**) Correlation between neutrophil–lymphocyte ratio (NLR) values and circulating cell-free DNA (cfDNA) levels; (**B**) overall survival (OS) according to the NLR and cfDNA combination (score 2 compared to score 0: $p = 0.0001$, score 2 compared to score 1: $p = 0.0008$); (**C**) progression-free survival (PFS) according to the NLR and cfDNA combination (score 2 compared to score 0: $p = 0.0037$; score 2 compared to score 1: $p = 0.0119$) (score 2, positive for both markers; score 1, positive for one of them; score 0: negative for both markers).

Since NLR and cfDNA concentration were highly correlated, we next measured neutrophil elastase in plasma as a marker of NETosis, which is a process involving the formation of neutrophil extracellular traps (NETs), to determine whether neutrophil activation contributes to cfDNA content in plasma of metastatic PDAC patients. As shown in Figure 6A–C, elastase concentration in plasma positively correlated with NLR (r = 0.5618; $p < 0.0001$), cfDNA concentration (r = 0.5246; $p < 0.0001$), and CA19-9 (r = 0.4995; $p < 0.0001$). Elastase concentration was higher in patients with liver metastasis ($p = 0.0423$, Figure 6D), in agreement with higher (although not statistically significant) NLR values in patients with hepatic metastases (Figure 3A), and also with our previously reported trend towards higher cfDNA levels in patients with hepatic lesions [15]. However, there was no relation between elastase levels and primary tumor location ($p = 0.9890$) or number of metastasis ($p = 0.7515$). Moreover, high elastase concentration in plasma (>23.15 ng/mL) was a prognostic factor of worse OS ($p = 0.0110$) and PFS ($p = 0.0241$) (Figure 6E,F).

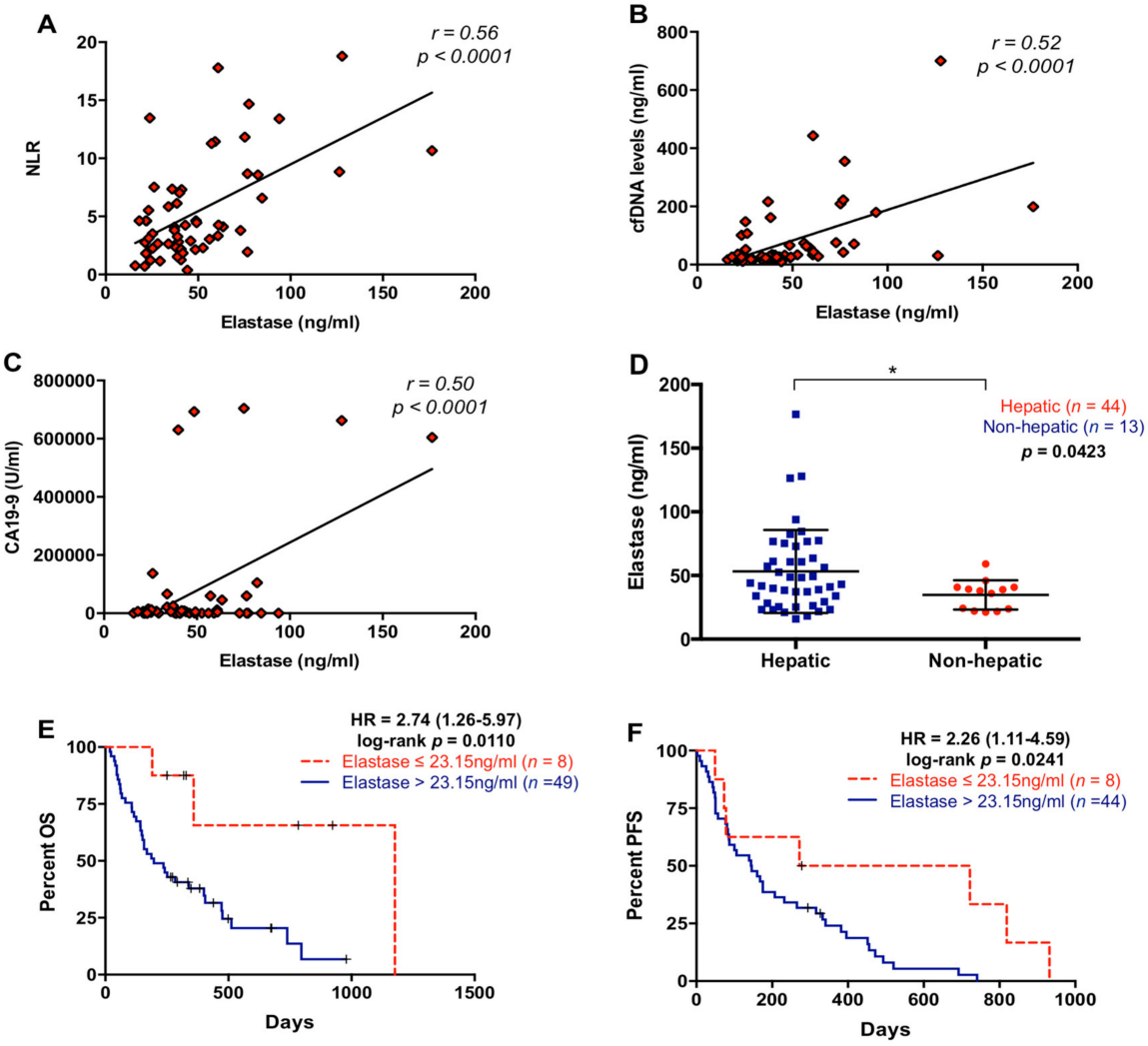

Figure 6. Circulating levels of neutrophil elastase in metastatic pancreatic ductal adenocarcinoma patients. (**A**) Correlation between plasma levels of neutrophil elastase and neutrophil–lymphocyte ratio (NLR); (**B**) correlation between plasma levels of neutrophil elastase and cell-free DNA (cfDNA); (**C**) correlation between plasma levels of neutrophil elastase and CA19-9; (**D**) plasma levels of neutrophil elastase in pancreatic ductal adenocarcinoma patients with metastatic lesions in the liver or elsewhere; (**E**) overall survival (OS) according to circulating levels of neutrophil elastase (cut-off: 23.15 ng/mL); (**F**) progression-free survival (PFS) according to circulating levels of neutrophil elastase (cut-off: 23.15 ng/mL) (* $p < 0.05$).

3.5. NLR Is Related to RAS Mutational Status in cfDNA of Metastatic PDAC Patients

Analysis of RAS mutation status in cfDNA was performed in 58 metastatic PDAC patients included in this study. RAS mutation was detected in 75.9% (44/58) of plasma samples (Table 1). As shown in Figure 7A, NLR was significantly higher in those patients in which plasma RAS mutations were detected (4.53 versus 2.24; $p = 0.0024$). Moreover, a positive correlation between NLR and the RAS MAF was found (r = 0.4481; $p = 0.0023$) (Figure 7B). We have previously reported [15] that the detections of RAS mutations and RAS mutational load in cfDNA were related to shorter OS and PFS in metastatic PDAC patients and a cut-off for MAF was determined (RAS MAF cut-off: 0.351%). Patients included in the present study also showed poor OS (193.5 versus 510 days; $p = 0.0003$) and PFS (122.5 versus 472 days; $p < 0.0001$) when RAS mutation was detected in plasma. Moreover, patients with higher RAS MAF in cfDNA had worse OS (163 versus 333.5 days; $p = 0.0365$) and a trend towards poorer PFS (87 versus 175 days; $p = 0.0818$). As shown in Figure 7C, when NLR and RAS mutation status in plasma were combined, patients with score 2 showed

poorer OS compared with patients with score 0 and score 1 ($p < 0.0001$ and $p = 0.0003$, respectively). Similarly, this combination of markers also better stratified patients for PFS (score 2 versus score 0: $p = 0.0003$ and score 2 versus score 1: $p = 0.0533$) (Figure 7D). When NLR was combined with RAS MAF (Figure 7E), an improved stratification of RAS-mutated patients for OS (score 2 versus 0, $p = 0.0029$; score 2 versus 1, $p = 0.0037$) but not for PFS ($p = 0.0869$) was observed (Figure 7F). No significant association was found between PLR and RAS mutation ($p = 0.5071$) or RAS mutational load ($p = 0.6854$) in plasma.

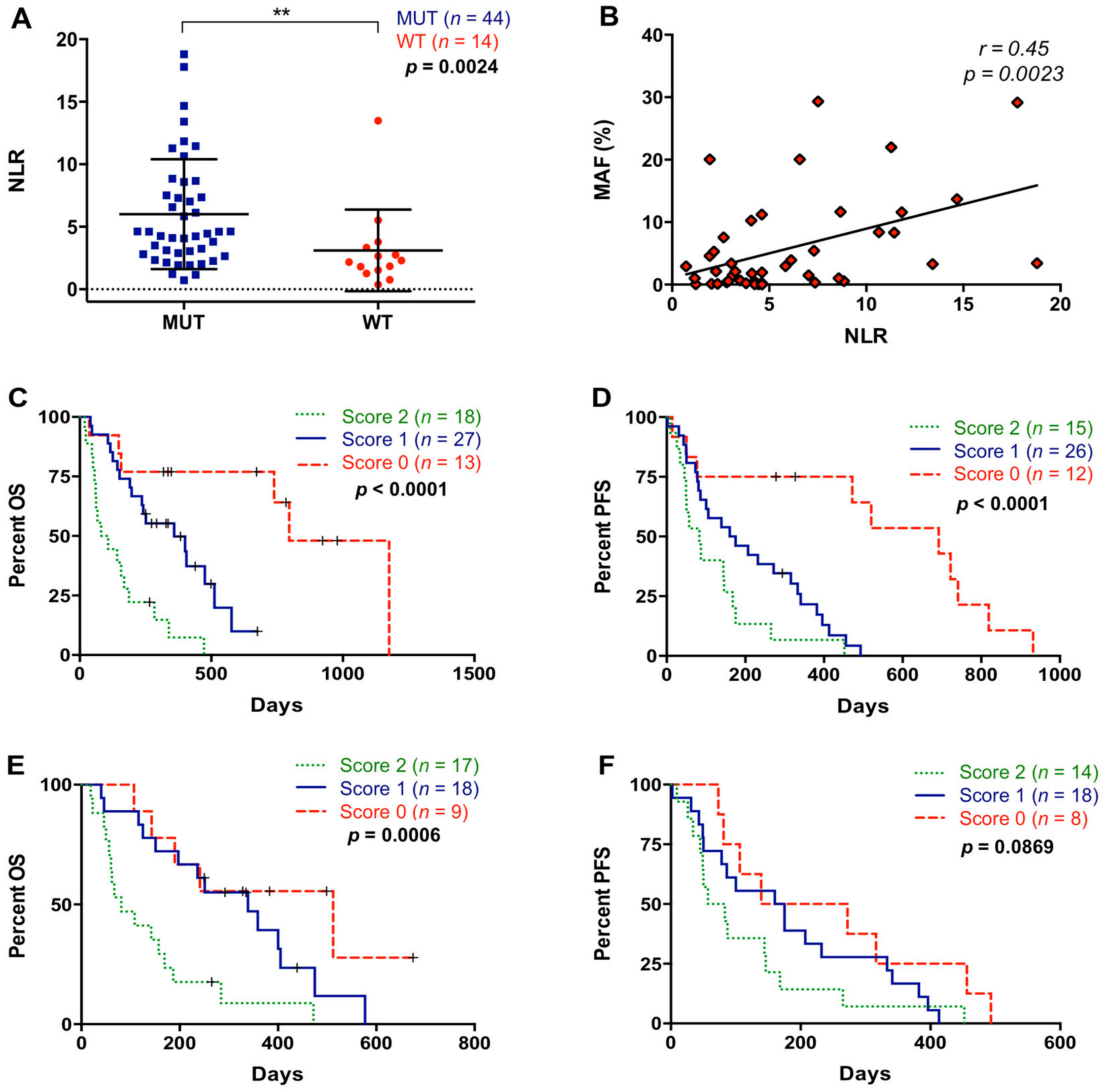

Figure 7. Association between neutrophil–lymphocyte ratio and plasma RAS mutation. (**A**) Neutrophil–lymphocyte ratio (NLR) according to RAS mutational status in plasma; (**B**) correlation between NLR values and RAS mutant allelic fraction (MAF) in plasma; (**C**) overall survival (OS) according to the NLR and RAS mutational status combination (score 2 compared to score 0: $p < 0.0001$; score 2 compared to score 1: $p = 0.0003$); (**D**) progression-free survival (PFS) according to the NLR and RAS mutational status combination (score 2 compared to score 0: $p = 0.0003$; score 2 compared to score 1: $p = 0.0533$); (**E**) OS according to the NLR and MAF combination (score 2 compared to score 0: $p = 0.0029$; score 2 compared to score 1: $p = 0.0037$); (**F**) PFS according to the NLR and MAF combination (score 2 compared to score 0: $p = 0.0420$; score 2 compared to score 1: $p = 0.3008$) (score 2, positive for both markers; score 1, positive for one of them; score 0, negative for both markers) (** $p < 0.01$).

3.6. Multiple Blood-Based Biomarkers Improve the Prognostic Stratification of Metastatic PDAC Patients

Patients included in this analysis showed differences in OS (125 versus 284 days; $p = 0.0223$) and in PFS (72 versus 203.5 days; $p = 0.0110$) according to CA19-9 levels. CA19-9 levels and NLR showed a positive correlation (r = 0.3684; $p = 0.0048$; Figure S2) and the combination of both showed an improvement in patient stratification for OS (score 2 versus score 0: $p < 0.0001$ and score 2 versus score 1: $p = 0.0226$) and PFS (score 2 versus score 0: $p = 0.0016$ and score 2 versus score 1: $p = 0.0021$) (Figure 8A,B).

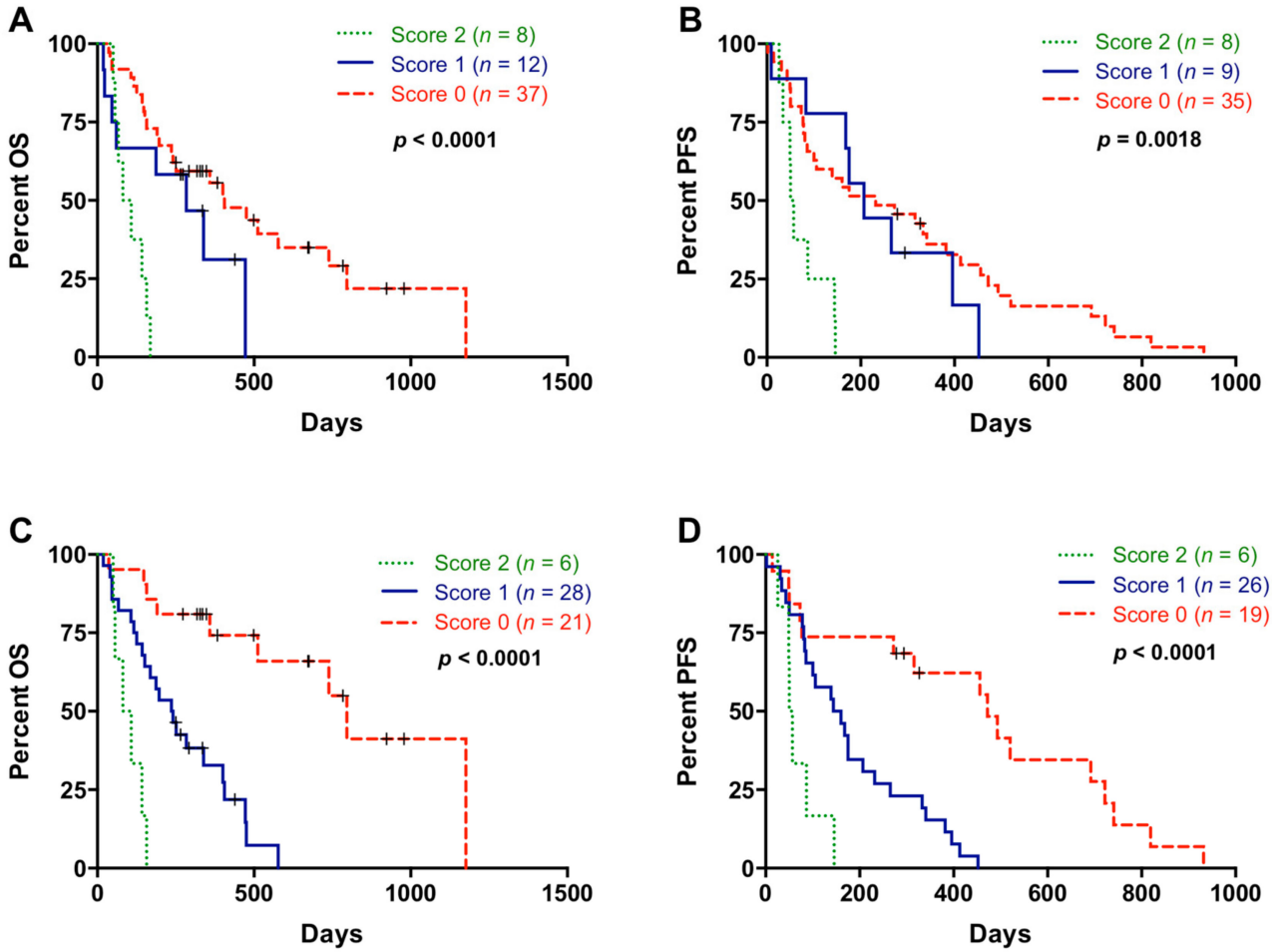

Figure 8. Multiple blood-based biomarkers for the prognosis of metastatic pancreatic ductal adenocarcinoma patients. (**A**) Overall survival (OS) according to neutrophil–lymphocyte ratio (NLR) and CA19-9 combination (score 2 compared to score 0: $p < 0.0001$; score 2 compared to score 1: $p = 0.0226$); (**B**) progression-free survival (PFS) according to NLR and CA19-9 combination (score 2 compared to score 0: $p = 0.0016$; score 2 compared to score 1: $p = 0.0021$) (score 2, positive for both markers; score 1, positive for one of them; score 0, negative for both markers); (**C**) OS according to the combination of multiple blood-based biomarkers (NLR, platelet–lymphocyte ratio (PLR), cell-free DNA (cfDNA) concentration, RAS status, RAS mutant allelic fraction (MAF) and CA19-9) (score 2 compared to score 0: $p < 0.0001$; score 2 compared to score 1: $p = 0.0026$); (**D**) PFS according to the combination of multiple blood-based biomarkers (NLR, PLR, cfDNA concentration, RAS status, RAS MAF, and CA19-9) (score 2 compared to score 0: $p = 0.0008$; score 2 compared to score 1: $p = 0.0086$) (score 2, positive for all markers; score 1, positive for 3, 4 or 5 markers; score 0, positive for 1 or 2 markers or negative for all of them).

Next, a combination of multiple blood-based biomarkers (NLR, PLR, cfDNA concentration, RAS status, RAS MAF, and CA19-9) was used to improve the prognostic stratification of metastatic PDAC patients. In this case, score 2 was defined as positive for all markers; score 1 positive for 3, 4, or 5 markers; and score 0 positive for 1 or 2

markers or negative for all of them. As shown in Figure 8C, patients with score 2 had a very short OS outcome compared with patients with score 1 ($p = 0.0026$), and especially compared with patients with score 0 ($p < 0.0001$). In regard to PFS, this combination of multiple blood-based biomarkers also efficiently stratified patients into dismal (score 2), poor (score 1), and good (score 0) prognosis (Figure 8D).

4. Discussion

Infiltration of immune cells in PDAC tumors is highly abundant, contributing to immune evasion and chemotherapy resistance [4]. In the present study, we described the utility of NLR and PLR along with others circulating tumor-specific markers to evaluate the prognosis in metastatic PDAC patients.

Previous reports have related high NLR and PLR values with poor prognosis in advanced pancreatic cancer [16,18–20]. However, most of the studies that have related high PLR values with poor OS involved locally advanced patients [20–22], who were not included in the present study. In this regard our analysis showed that metastatic PDAC patients with higher NLR values had significantly poorer OS and PFS rates, whereas both NLR and PLR were associated with poor-prognosis clinical features. Thus, higher NLR values were related with male gender and higher ECOG status. This is in agreement with other cancer studies, in which higher NLR values were reported in male cancer patients [23,24] and patients with high ECOG status [21]. In addition, those patients younger than 60 years had higher PLR values than older patients, likely because aging is known to be accompanied by a decrease in platelet count [25]. Further, patients with a primary tumor located in the head of the pancreas showed higher PLR values than those patients with a tumor in the body/tail of this organ.

Cancer cells can activate platelets leading to pro-cancerous effects. Activated platelets participate in the regulation of inflammation, releasing proinflammatory cytokines, and in modulating tumor microenvironment by recruiting leukocytes, including neutrophils. Moreover, activated platelets participate in tumor immune evasion by releasing transforming growth factor β (TGF-β), which is a cytokine with a potent immunosuppressive activity. Besides, TGF-β participates in the transition of tumor-associated neutrophils from an anti-tumorigenic (N1) towards a protumorigenic (N2) phenotype [4]. Furthermore, activated platelets have been implicated in the formation of NETs, with a positive feedback loop, because NETs in turn promote platelet activation [26,27]. Therefore, the platelet–neutrophil crosstalk plays an important role in the development and progression of cancer. In this regard, our analysis indicated that NLR and PLR positively correlatde and the combination of both factors increased their prognostic value. On the other hand, platelets and neutrophils have been related with the metastatic process [28–30], and our results confirmed that NLR was associated with liver metastasis in PDAC patients, in agreement with a previous report [31].

There is increasing evidence connecting KRAS mutations with tumor-promoting inflammation in several human cancers, including PDAC [13,32]. KRAS activation in cancer cells induces the expression and secretion of proinflammatory cytokines, stimulating the recruitment of neutrophils to the tumor [6]. On the other hand, gene dosage of mutant KRAS has an important role in PDAC biology [33], and we and others have recently reported the correlation of KRAS MAF in cfDNA with clinical stage and outcome in PDAC [8,15,34]. Importantly, in the present study, those patients with RAS-mutated cfDNA had higher NLR values and a positive correlation between NLR and RAS mutational load in cfDNA was found. Furthermore, the combination of NLR with RAS mutational status or load (MAF) in cfDNA greatly improved the prognostic classification of metastatic PDAC patients.

The prognostic significance of cfDNA levels and fragmentation has been previously described in metastatic cancer [35–37], including metastatic PDAC [15,38]. Specifically, we have recently reported that higher cfDNA concentration and smaller cfDNA fragment size are associated with poor outcomes in metastatic PDAC patients [15]. In the present study,

PLR was negatively correlated with cfDNA fragment size, and this may explain why higher PLR values are associated with more aggressive tumors. Moreover, although apoptosis and necrosis seem to provide most of cfDNA, some stimuli can activate neutrophils leading to DNA release and NETosis [39,40]. A previous study demonstrated a relation between NLR and altered values of cfDNA in endometrial cancer [41]. In this regard, our results showed a high positive correlation between NLR and cfDNA concentration but not cfDNA fragmentation. Moreover, a positive correlation between neutrophil elastase circulating levels and cfDNA concentration was found, suggesting that neutrophil activation significantly contributes to cfDNA content in plasma of metastatic PDAC patients. Besides, the positive correlation found between elastase and CA19-9 suggests that neutrophil activation and NETosis are related with disease progression in metastatic PDAC patients. In fact, higher elastase circulating levels were related with liver metastasis and poor OS and PFS. These findings are consistent with the reported interaction of neutrophils with circulating tumor cells facilitating their contact with hepatic endothelial cells, thus helping cancer cells dissemination and liver metastasis [42,43]. Also, inhibition of NETs has been shown to reduce liver metastasis in a preclinical model of metastatic colorectal cancer [44], while recent studies have suggested that NETs can also contribute to hepatic metastasis in PDAC [45].

Finally, we also showed that the combination of NLR and PLR with cfDNA-based liquid biopsy markers greatly improves prognostic power and provides accurate survival risk stratification.

5. Conclusions

In conclusion, our study supports that the use of NLR and PLR, along with other noninvasive biomarkers in a multi-parameter prognostic model, may constitute a valuable tool for the clinical management of metastatic PDAC patients. Future larger studies are warranted to validate the prognostic value in PDAC patients of this combination of systemic inflammatory and liquid biopsy biomarkers.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/6/1210/s1, Figure S1: Correlation between platelet-lymphocyte ratio (PLR) and circulating cell-free DNA (cfDNA) size, Figure S2: Correlation between neutrophil-lymphocyte ratio (NLR) and CA19-9.

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Institutional Review Board Statement: The study was conducted in compliance with the Declaration of Helsinki, and previous approval was obtained from the Ethical Research Committee of Córdoba (Comité de Ética de la Investigación de Córdoba, CEI Córdoba, PANCREAS-BIOPSIA-LIQ protocol, approved on April 26, 2017, Act no263, ref, 3490).

Informed Consent Statement: The written informed consent was obtained from all subjects prior to participating in the study.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author.

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References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]
2. Ferlay, J.; Colombet, M.; Soerjomataram, I.; Dyba, T.; Randi, G.; Bettio, M.; Gavin, A.; Visser, O.; Bray, F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J. Cancer* **2018**, *103*, 356–387. [CrossRef] [PubMed]
3. Rahib, L.; Smith, B.D.; Aizenberg, R.; Rosenzweig, A.B.; Fleshman, J.M.; Matrisian, L.M. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* **2014**, *74*, 2913–2921. [CrossRef]
4. Shi, J.; Xue, J. Inflammation and development of pancreatic ductal adenocarcinoma. *Chin. Clin. Oncol.* **2019**, *8*, 19. [CrossRef]
5. Oh, D.; Pyo, J.S.; Son, B.K. Prognostic roles of inflammatory markers in pancreatic cancer: Comparison between the neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio. *Gastroenterol. Res. Pract.* **2018**, *2018*. [CrossRef] [PubMed]
6. Dias Carvalho, P.; Guimaraes, C.F.; Cardoso, A.P.; Mendonca, S.; Costa, A.M.; Oliveira, M.J.; Velho, S. KRAS Oncogenic Signaling Extends beyond Cancer Cells to Orchestrate the Microenvironment. *Cancer Res.* **2018**, *78*, 7–14. [CrossRef] [PubMed]
7. Shaul, M.E.; Fridlender, Z.G. Cancer-related circulating and tumor-associated neutrophils – subtypes, sources and function. *FEBS J.* **2018**, *285*, 4316–4342. [CrossRef] [PubMed]
8. Wang, Y.; Fang, T.; Huang, L.; Wang, H.; Zhang, L.; Wang, Z.; Cui, Y. Neutrophils infiltrating pancreatic ductal adenocarcinoma indicate higher malignancy and worse prognosis. *Biochem. Biophys. Res. Commun.* **2018**, *501*, 313–319. [CrossRef]
9. Zhou, Y.; Cheng, S.; Fathy, A.H.; Qian, H.; Zhao, Y. Prognostic value of platelet-to-lymphocyte ratio in pancreatic cancer: A comprehensive meta-analysis of 17 cohort studies. *OncoTargets Ther.* **2018**, *11*, 1899–1908. [CrossRef]
10. Zhou, Y.; Wei, Q.; Fan, J.; Cheng, S.; Ding, W.; Hua, Z. Prognostic role of the neutrophil-to-lymphocyte ratio in pancreatic cancer: A meta-analysis containing 8252 patients. *Clin. Chim. Acta* **2018**, *479*, 181–189. [CrossRef]
11. Waters, A.M.; Der, C.J. KRAS: The critical driver and therapeutic target for pancreatic cancer. *Cold Spring Harb. Perspect. Med.* **2018**, *8*. [CrossRef] [PubMed]
12. Ji, H.; Houghton, A.M.; Mariani, T.J.; Perera, S.; Kim, C.B.; Padera, R.; Tonon, G.; McNamara, K.; Marconcini, L.A.; Hezel, A.; et al. K-ras activation generates an inflammatory response in lung tumors. *Oncogene* **2006**, *25*, 2105–2112. [CrossRef]
13. Kitajima, S.; Thummalapalli, R.; Barbie, D.A. Inflammation as a driver and vulnerability of KRAS mediated oncogenesis. *Semin. Cell Dev. Biol.* **2016**, *58*, 127–135. [CrossRef]
14. Liou, G.Y.; Döppler, H.; Necela, B.; Edenfield, B.; Zhang, L.; Dawson, D.W.; Storz, P. Mutant KRAS–induced expression of ICAM-1 in pancreatic acinar cells causes attraction of macrophages to expedite the formation of precancerous lesions. *Cancer Discov.* **2015**, *5*, 52–63. [CrossRef]
15. Toledano-Fonseca, M.; Cano, M.T.; Inga, E.; Rodríguez-Alonso, R.; Gómez-España, M.A.; Guil-Luna, S.; Mena-Osuna, R.; De la Haba-Rodríguez, J.R.; Rodríguez-Ariza, A.; Aranda, E. Circulating cell-free dna-based liquid biopsy markers for the non-invasive prognosis and monitoring of metastatic pancreatic cancer. *Cancers* **2020**, *12*. [CrossRef]
16. Luo, G.; Guo, M.; Liu, Z.; Xiao, Z.; Jin, K.; Long, J.; Liu, L.; Liu, C.; Xu, J.; Ni, Q.; et al. Blood Neutrophil–Lymphocyte Ratio Predicts Survival in Patients with Advanced Pancreatic Cancer Treated with Chemotherapy. *Ann. Surg. Oncol.* **2015**, *22*, 670–676. [CrossRef]
17. Heagerty, P.J.; Lumley, T.; Pepe, M.S. Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics* **2000**, *56*, 337–344. [CrossRef] [PubMed]
18. An, X.; Ding, P.R.; Li, Y.H.; Wang, F.H.; Shi, Y.X.; Wang, Z.Q.; He, Y.J.; Xu, R.H.; Jiang, W.Q. Elevated neutrophil to lymphocyte ratio predicts survival in advanced pancreatic cancer. *Biomarkers* **2010**, *15*, 516–522. [CrossRef]
19. Chen, Y.; Yan, H.; Wang, Y.; Shi, Y.; Dai, G. Significance of baseline and change in neutrophil-to-lymphocyte ratio in predicting prognosis: A retrospective analysis in advanced pancreatic ductal adenocarcinoma. *Sci. Rep.* **2017**, *7*, 753. [CrossRef] [PubMed]
20. Lee, B.M.; Chung, S.Y.; Chang, J.S.; Lee, K.J.; Seong, J. The neutrophil-lymphocyte ratio and platelet-lymphocyte ratio are prognostic factors in patients with locally advanced pancreatic cancer treated with chemoradiotherapy. *Gut Liver* **2018**, *12*, 342–352. [CrossRef]
21. Iwai, N.; Okuda, T.; Sakagami, J.; Harada, T.; Ohara, T.; Taniguchi, M.; Sakai, H.; Oka, K.; Hara, T.; Tsuji, T.; et al. Neutrophil to lymphocyte ratio predicts prognosis in unresectable pancreatic cancer. *Sci. Rep.* **2020**, *10*, 18758. [CrossRef] [PubMed]
22. Qi, Q.; Geng, Y.; Sun, M.; Wang, P.; Chen, Z. Clinical implications of systemic inflammatory response markers as independent prognostic factors for advanced pancreatic cancer. *Pancreatology* **2015**, *15*, 145–150. [CrossRef] [PubMed]
23. Howard, R.; Kanetsky, P.A.; Egan, K.M. Exploring the prognostic value of the neutrophil-to-lymphocyte ratio in cancer. *Sci. Rep.* **2019**, *9*, 19673. [CrossRef] [PubMed]
24. Piciucchi, M.; Stigliano, S.; Archibugi, L.; Zerboni, G.; Signoretti, M.; Barucca, V.; Valente, R.; Fave, G.D.; Capurso, G. The neutrophil/lymphocyte ratio at diagnosis is significantly associated with survival in metastatic pancreatic cancer patients. *Int. J. Mol. Sci.* **2017**, *18*. [CrossRef]
25. Jones, C.I. Platelet function and ageing. *Mamm. Genome* **2016**, *27*, 358–366. [CrossRef] [PubMed]
26. Palacios-Acedo, A.L.; Mège, D.; Crescence, L.; Dignat-George, F.; Dubois, C.; Panicot-Dubois, L. Platelets, Thrombo-Inflammation, and Cancer: Collaborating With the Enemy. *Front. Immunol.* **2019**, *10*, 1805. [CrossRef]
27. Olsson, A.K.; Cedervall, J. The pro-inflammatory role of platelets in cancer. *Platelets* **2018**, *29*, 569–573. [CrossRef] [PubMed]
28. Gay, L.J.; Felding-Habermann, B. Contribution of platelets to tumour metastasis. *Nat. Rev. Cancer* **2011**, *11*, 123–134. [CrossRef]

29. Cools-Lartigue, J.; Spicer, J.; McDonald, B.; Gowing, S.; Chow, S.; Giannias, B.; Bourdeau, F.; Kubes, P.; Ferri, L. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J. Clin. Investig.* **2013**, *8*, 3446–3458. [CrossRef]
30. Yang, J.J.; Hu, Z.G.; Shi, W.X.; Deng, T.; He, S.Q.; Yuan, S.G. Prognostic significance of neutrophil to lymphocyte ratio in pancreatic cancer: A meta-analysis. *World J. Gastroenterol.* **2015**, *21*, 2807–2815. [CrossRef] [PubMed]
31. Gao, Y.; Wang, W.J.; Zhi, Q.; Shen, M.; Jiang, M.; Bian, X.; Gong, F.R.; Zhou, C.; Lian, L.; Wu, M.Y.; et al. Neutrophil/lymphocyte ratio is a more sensitive systemic inflammatory response biomarker than platelet/lymphocyte ratio in the prognosis evaluation of unresectable pancreatic cancer. *Oncotarget* **2017**, *8*, 88835–88844. [CrossRef]
32. Hamarsheh, S.; Groß, O.; Brummer, T.; Zeiser, R. Immune modulatory effects of oncogenic KRAS in cancer. *Nat. Commun.* **2020**, *11*, 5439. [CrossRef] [PubMed]
33. Lennerz, J.K.; Stenzinger, A. Allelic Ratio of KRAS Mutations in Pancreatic Cancer. *Oncologist* **2015**, *20*, e8–e9. [CrossRef] [PubMed]
34. Bernard, V.; Kim, D.U.; San Lucas, F.A.; Castillo, J.; Allenson, K.; Mulu, F.C.; Stephens, B.M.; Huang, J.; Semaan, A.; Guerrero, P.A.; et al. Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* **2019**, *156*, 108–118e4. [CrossRef]
35. Tabernero, J.; Lenz, H.J.; Siena, S.; Sobrero, A.; Falcone, A.; Ychou, M.; Humblet, Y.; Bouche, O.; Mineur, L.; Barone, C.; et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: A retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol.* **2015**, *16*, 937–948. [CrossRef]
36. El Messaoudi, S.; Mouliere, F.; Du Manoir, S.; Bascoul-Mollevi, C.; Gillet, B.; Nouaille, M.; Fiess, C.; Crapez, E.; Bibeau, F.; Theillet, C.; et al. Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. *Clin. Cancer Res.* **2016**, *22*, 3067–3077. [CrossRef]
37. Valpione, S.; Gremel, G.; Mundra, P.; Middlehurst, P.; Galvani, E.; Girotti, M.R.; Lee, R.J.; Garner, G.; Dhomen, N.; Lorigan, P.C.; et al. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *Eur. J. Cancer* **2018**, *88*, 1–9. [CrossRef] [PubMed]
38. Lapin, M.; Oltedal, S.; Tjensvoll, K.; Buhl, T.; Smaaland, R.; Garresori, H.; Javle, M.; Glenjen, N.I.; Abelseth, B.K.; Gilje, B.; et al. Fragment size and level of cell-free DNA provide prognostic information in patients with advanced pancreatic cancer. *J. Transl. Med.* **2018**, *16*, 300. [CrossRef] [PubMed]
39. Kustanovich, A.; Schwartz, R.; Peretz, T.; Grinshpun, A. Life and death of circulating cell-free DNA. *Cancer Biol. Ther.* **2019**, *20*, 1057–1067. [CrossRef]
40. Goggs, R.; Jeffery, U.; LeVine, D.N.; Li, R.H.L. Neutrophil-Extracellular Traps, Cell-Free DNA, and Immunothrombosis in Companion Animals: A Review. *Vet. Pathol.* **2020**, *57*, 6–23. [CrossRef]
41. Cicchillitti, L.; Corrado, G.; de Angeli, M.; Mancini, E.; Baiocco, E.; Patrizi, L.; Zampa, A.; Merola, R.; Martayan, A.; Conti, L.; et al. Circulating cell-free DNA content as blood based biomarker in endometrial cancer. *Oncotarget* **2017**, *8*, 115230–115243. [CrossRef] [PubMed]
42. Spicer, J.D.; McDonald, B.; Cools-Lartigue, J.J.; Chow, S.C.; Giannias, B.; Kubes, P.; Ferri, L.E. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. *Cancer Res.* **2012**, *72*, 3919–3927. [CrossRef] [PubMed]
43. Heeke, S.; Mograbi, B.; Alix-Panabières, C.; Hofman, P. Never Travel Alone: The Crosstalk of Circulating Tumor Cells and the Blood Microenvironment. *Cells* **2019**, *8*. [CrossRef] [PubMed]
44. Miller-Ocuin, J.L.; Liang, X.; Boone, B.A.; Doerfler, W.R.; Singhi, A.D.; Tang, D.; Kang, R.; Lotze, M.T.; Zeh, H.J. DNA released from neutrophil extracellular traps (NETs) activates pancreatic stellate cells and enhances pancreatic tumor growth. *Oncoimmunology* **2019**, *8*, e1605822. [CrossRef] [PubMed]
45. Huber, M.; Brehm, C.U.; Gress, T.M.; Buchholz, M.; Alhamwe, B.A.; von Strandmann, E.P.; Slater, E.P.; Bartsch, J.W.; Bauer, C.; Lauth, M. The immune microenvironment in pancreatic cancer. *Int. J. Mol. Sci.* **2020**, *21*. [CrossRef] [PubMed]