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Non-invasive monitoring of metastatic colorectal cancer in Circulating Tumor DNA and Exosomal RNA

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Abstract

Metastatic colorectal cancer (mCRC) evolves dynamically over time in response to selective pressures, such as cytotoxic and targeted drugs. Although genotyping of tumor tissues dictates targeted therapies' eligibility, it fails to reflect tumor heterogeneity/dynamics and drug sensitivies, which are likely to change throughout the treatment. As such, it would be clinically relevant to evaluate the molecular profiles of mCRC patients during disease treatment. Liquid biopsy (e.g., cell-free tumor DNA – cfDNA; and extracellular vesicles - EVs) represents a promising non-invasive tool for a better surveillance of clonal evolution and real-time monitoring of treatment efficacy.

The general aim of this thesis was to assess whether cfDNA, EVs and EV-derived RNA (EV-RNA) are useful to track tumor dynamics and to monitor response to therapy of mCRC patients. To accomplish this, a prospective study with longitudinal quantification and molecular analyzes of cfDNA and EVs collected throughout treatment and follow-up of 13 mCRC patients was conducted.

RAS and BRAF mutation status detected by the Cobas Mutation test (RT-PCR) revealed a concordance of 46.2% between tissue (primary tumor or metastatic lesions) and cfDNA (at the baseline) samples, either confirming the wild-type or the mutant status. Moreover, in four out of ten patients with matched quantification of the levels and detection of RAS and BRAF mutations in cfDNA, a change in the KRAS mutation status was identified.
Concerning the number of EVs, despite we have already longitudinally isolated EVs from seven patients, only EVs collected from four patients were submitted to nanoparticle tracking analysis so far. All four patients showed a drastic variation in the number of EVs in one of the timepoints, and in three of the patients, the inflection point correspond to the onset of targeted therapy (cetuximab or bevacizumab). Concerning the amount of RNA (assessed by Nanodrop) per EV from those four patients, it varied along time, though not following the same peak dynamics as the total number of EVs. More so, there was no correlations on the variation dynamics between the quantity of EVs, the quantity of EV-RNA/EV, and the concentration of EV-RNA per milliliter of plasma in those four patients.
Since the total number of EVs was not yet determined for all patients, EV-RNA was normalized to the volume of plasma used for EV isolation. In all the seven patients analyzed, higher concentrations of EV-RNA than cfDNA were observed and, interestingly, these two forms of liquid biopsy varied overtime in opposite directions.

In conclusion, the work described in this thesis pinpoints to a correlation between the change in the number of EVs and the onset of the targeted therapy, which may indicate that the number of EVs is more accurate for treatment monitoring than the amount of cfDNA. Additionally, this work also set the ground towards a combinatorial method based on cfDNA

and EV-RNA to improve blood-based liquid biopsy for KRAS mutation detection in metastatic colorectal cancer patients.

Keywords: metastatic colorectal cancer, liquid biopsy, cell-free tumor circulating DNA, extracellular vesicles and extracellular vesicles-derived RNA, treatment monitoring

Resumo

O cancro colorretal metastático (mCRC) evolui dinamicamente ao longo do tempo em resposta a pressões seletivas instituídas pelo tratamento. Embora a genotipagem de tecidos tumorais dite a elegibilidade para terapias direcionadas, não reflete a heterogeneidade/dinâmica do tumor e as sensibilidades ao tratamento, que provavelmente mudarão ao longo do tratamento. Assim, torna-se clinicamente relevante avaliar os perfis moleculares de pacientes com mCRC durante o tratamento da doença. A biópsia líquida (por exemplo, DNA tumoral livre de células - cfDNA; e vesículas extracelulares - EVs) representa uma ferramenta não invasiva promissora para uma melhor vigilância da evolução clonal e monitorização em tempo real da eficácia do tratamento.

O objetivo geral desta tese foi avaliar se o cfDNA, as EVs e o RNA derivado de EVs (EV-RNA) são úteis para rastrear a dinâmica tumoral e monitorizar a resposta à terapia em pacientes com mCRC. Para isso, foi realizado um estudo prospetivo com quantificação longitudinal e análises moleculares em cfDNA e EVs recolhidos durante o tratamento e acompanhamento de 13 pacientes com mCRC.

O perfil mutacional dos genes RAS e BRAF foi determinado pelo teste de Mutação Cobas (RT-PCR), o qual revelou uma concordância de 46,2% entre tecido (tumor primário ou lesões metastáticas) e amostras de cfDNA (na linha de base), confirmando quer o tipo selvagem quer o estado mutante. Além disso, em quatro dos dez pacientes com quantificação correspondente dos níveis e deteção de mutações RAS e BRAF em cfDNA, foi identificada uma mudança no perfil de mutação no gene KRAS.

Quanto ao número de EVs, apesar de termos isolado longitudinalmente EVs de sete pacientes, apenas as EVs isoladas de quatro pacientes foram submetidas à análise de rastreamento de nanopartículas. Todos os quatro pacientes mostraram uma variação drástica no número de EVs num dos pontos temporais e, em três dos pacientes, o ponto de inflexão correspondeu ao início da terapia direcionada (cetuximab ou bevacizumab). Quanto à quantidade de RNA (avaliada por Nanodrop) por EV desses quatro pacientes, verificou-se uma variação ao longo do tempo, embora não seguindo as mesmas tendências que o número total de EVs. Além disso, não houve correlações na dinâmica de variação entre a quantidade de EVs, a quantidade de EV-RNA/EV e a concentração de EV-RNA por mililitro de plasma nesses quatro pacientes.

Como o número total de EVs ainda não foi determinado para todos os pacientes, o EV-RNA foi normalizado para o volume de plasma usado para isolamento de EV. Em todos os sete pacientes analisados, foram observadas concentrações mais altas de EV-RNA do que

cfDNA e, curiosamente, estas duas formas de biópsia líquida variaram ao longo do tempo em direções opostas.

Em conclusão, o trabalho descrito nesta tese aponta para uma correlação entre a mudança no número de EVs e o início da terapia direcionada, o que pode indicar que o número de EVs é mais preciso para a monitorização do tratamento do que a quantidade de cfDNA. Adicionalmente, este trabalho também estabeleceu as bases para um método combinatório baseado em cfDNA e EV-RNA para melhorar a biópsia líquida baseada em sangue para deteção da mutação KRAS em pacientes com cancro colorretal metastático.

Palavras-chave: cancro colorretal metastático, biópsia líquida, DNA circulante, vesículas extracelulares, RNA derivado de vesículas extracelulares, monitorização do tratamento.

Abbreviations

BRAF - Raf murine sarcoma viral oncogene homolog B

cfDNA – circulating-free DNA

CTC – circulating tumor cell

CRC – colorectal cancer

EGFR – epidermal growth factor receptor

EV – extracellular vesicles

EV-RNA – extracellular vesicle-derived RNA

HER2 - human epidermal growth factor receptor 2

KRAS - Kirsten rat sarcoma virus

mCRC – metastatic colorectal cancer

MET - Mesenchymal Epithelial Transition gene

1. Introduction

Colorectal cancer (CRC) is a highly prevalent disease and is the second leading cause of cancer-related deaths worldwide (Xi & Xu, 2021). Despite being a deadly disease, the survival rates of CRC patients have significantly improved over the years. This is mainly due to timely referral of patients to multidisciplinary teams, advancements in surgical techniques, the emergence of new therapeutic regimens, better imaging methods for diagnosis, and the implementation of prognostic and predictive molecular markers that allow for more individualized therapy management (Zhang et al., 2021). However, around 25% of patients are diagnosed with advanced-stage disease, while 50% of those diagnosed at earlier stages will eventually progress to advanced stages (Hernandez Dominguez et al., 2023).

CRC is now understood to be a complex and diverse disease, rather than a uniform clinical entity. Instead, CRC is a diverse and heterogeneous disease, compartmentalized into distinct molecular groups, each marked by a multitude of genomic and epigenomic alterations, underlining the complexity and variability of this disease (Noack & Langer, 2023). Although the diversity of molecular alterations in CRC has hindered the development of targeted therapies for advanced stages of the disease, some of these alterations are now considered useful biomarkers for predicting therapy response and prognosis. The RAS and BRAF genes are the primary markers for metastatic colorectal cancer. RAS mutations are negative predictors of response to therapies with anti-EGFR and are present in approximately 40% of metastatic patients. (Song et al., 2020). The BRAF V600E mutation of the BRAF gene is present in 8-12% of patients with mCRC, with a higher prevalence in the right colon, and is linked to a poorer prognosis (Mauri et al., 2021).

Tailoring the treatment plan based on the specific molecular changes within the tumor has provided considerable advancement in patient care. However, despite this progress, a significant number of patients do not respond to the treatment, and those who do respond often develop resistance and experience disease recurrence. (Ciardiello et al., 2022). Currently, disease progression is primarily detected through imaging and clinical diagnostic methods. However, these methods have limitations in terms of sensitivity and invasiveness. This has created a pressing need for alternative approaches, such as liquid biopsy, which offer greater sensitivity and minimally invasive detection. With liquid biopsy, not only can the treatment of advanced disease be monitored more effectively, but early diagnosis of disease progression can also be achieved (Mauri et al., 2022; Zhou et al., 2022).

1.1. Liquid biopsy and circulating tumor DNA detection for monitoring treatment responses and disease progression

The realm of liquid biopsies is advancing quickly, with increasing emphasis on analyzing tumor biomarkers present in body fluids. Several tumor-derived components circulate in the bloodstream, including circulating tumor cells (CTC), circulating tumor DNA (ctDNA), circulating RNA (cRNA), exosomes, and more (Lone et al., 2022). CtDNA has been the most extensively studied and offers promising insights. These DNA fragments originate from tumor cells and enter the circulation due to active secretion, apoptosis, or necrosis. By analyzing and quantifying these fragments, specific genetic alterations in the tumor can be identified, which can help in the early detection of therapeutic response or resistance (Dang & Park, 2022).

Liquid biopsies offer several advantages when compared to tissue biopsies. For example, they are less invasive, faster to obtain, and can more comprehensively depict the intrinsic and dynamic intratumoral heterogeneity. Tissue biopsies, in their turn, provide only a focal spatiotemporal snapshot of the tumor (Lone et al., 2022). Serial quantitative and qualitative ctDNA measurements allow for the in-depth tracking and analysis of clonal evolution throughout various systemic treatments. This approach is especially valuable when determining the best application of anti-EGFR agents for patients with molecularly selected mCRC. While some patients experience initial benefits from these agents, disease progression is an almost inevitable outcome (Kim et al., 2022). The factors contributing to secondary resistance are diverse and share some commonalities with primary resistance. Both laboratory-based and clinical retrospective studies highlight the emergence of several genomic changes, not just limited to the EGFR pathway. Notable alterations include mutations in RAS, BRAF, and the EGFR ectodomain, as well as amplifications in KRAS, HER2, and MET (Antoniotti et al., 2019). Liquid biopsies have been employed by numerous researchers to track the impact of anti-EGFR therapies. Through ctDNA analysis of samples taken during treatment, there is clear evidence of an increasing selection of genomic changes, especially RAS mutant clones, leading to secondary resistance to EGFR blockade. This often becomes apparent even before radiological signs of disease progression are evident (Knebel et al., 2019). For optimal prognostication and disease monitoring, consecutive sampling followed by KRAS mutation ctDNA analysis yielded the most reliable results (Bach et al., 2019). During follow-up, consecutive analysis demonstrated high accuracy in detecting recurrences in patients with pre-therapeutic detectable KRAS mutations. (Reece et al., 2019). The analysis of KRAS mutations in ctDNA is useful in identifying potential therapeutic targets and detecting acquired resistance in tumor mutations. The tumor's clonal evolution can become heterogeneous and dynamic under the pressure of targeted treatments, leading to multiple alterations in the mitogen-activated protein kinase

pathway effectors, such as RAS and MEK mutations, KRAS, BRAFV600E, and MET amplification. These changes were observed during the treatment with BRAF/EGFR, BRAF/MEK, and BRAF/EGFR/MEK inhibitors in patients with BRAFV600E-mutant mCRC (Antoniotti et al., 2019; Kim et al., 2022; Reece et al., 2019).

However, there are several steps that need to be taken to transition liquid biopsy from research to clinical practice. First, it is necessary to evaluate the agreement between this approach and tissue biopsy. A French prospective study with 425 patients shows a concordance of about 93% in the RAS status (Bachet et al., 2018). Still, there are several studies with concordances ranging between 60% and 95%, depending on the analysis method used. Several retrospective series have reported a more than 90% concordance between RAS status in matched tumor and ctDNA samples. Additionally, RAS mutations have been detected in ctDNA with high specificity (90% to 100%) but suboptimal sensitivity (89% to 96%). When compared to highly sensitive tissue-based techniques, the concordance rate (78% to 88%), specificity (83% to 91%), and sensitivity (70% to 85%) of plasma-based analyses were less encouraging to guide tailored treatment for RAS testing on ctDNA. (Kagawa et al., 2021; Lastraioli et al., 2023; Normanno et al., 2018).

At present, no prospective data supports the efficacy of halting ongoing therapy and starting a tailored treatment based on ctDNA signals of acquired resistance before symptoms or imaging shows disease progression. In patients who have developed resistance to anti-EGFR agents and have subsequently undergone at least one other therapy, the reintroduction of an anti-EGFR has shown clinical benefit (Patelli et al., 2023). Recent reports disclosed a biological rationale that supports the reintroduction of the EGFR blockade after an anti–EGFR–free interval. RAS-mutated clones emerge during disease progression but decline with anti-EGFR withdrawal, indicating the potential reversibility of resistance (Siravegna et al., 2015). The CRICKET trial phase II (Phase II Study of Cetuximab Rechallenge in Irinotecan-Pretreated mCRC, KRAS, NRAS, and BRAF Wild-Type Treated in First Line With Anti-EGFR Therapy) has shown that a cetuximab-based rechallenge strategy is effective in treating patients with mCRC (metastatic colorectal cancer) that had acquired resistance to first-line cetuximab-containing therapy, and who have RAS/BRAF wild-type tumors (Cremolini et al., 2019). This study emphasizes the importance of liquid biopsy in identifying appropriate candidates for this strategy. This is because detecting RAS mutations in ctDNA before rechallenging is associated with no clinical benefit. To strengthen these findings, the CHRONOS (Phase II Trial of Rechallenge with Panitumumab Driven by *RAS* Clonal-Mediated Dynamic of Resistance) study adopted ctDNA analysis as an inclusion criterion. This study showed that only those patients who experience a significant decrease in the fractional mutational abundance of RAS between the time of disease

progression after initial treatment with anti-EGFR therapy and the time of rechallenge are eligible for anti-EGFR rechallenge (Sartore-Bianchi et al., 2022).

1.2. Exosome detection in liquid biopsies

Exosomes are small vesicles ranging from 30 to 150 nm in diameter, originating from the endosomal system within cells and released into the extracellular environment (van Niel et al., 2018; Yáñez-Mó et al., 2015). These vesicles play a critical role in cell-to-cell communication through the transfer of various bioactive molecules, including proteins, lipids, and nucleic acids such as DNA (Thakur et al., 2014), RNA (Skog et al., 2008), and miRNA (S. Wang et al., 2021).

In the context of cancer, tumor cells release a high amount of exosomes into the bloodstream (D'Souza-Schorey & Schorey, 2018; Matsumoto et al., 2016; Rosell et al., 2009). These exosomes, often referred to as 'tumor-derived exosomes', carry molecular signatures specific to their cells of origin, and, importantly, can reflect the pathological status of the disease(Xu et al., 2018). Hence, their detection and analysis in liquid biopsies have shown immense promise as a non-invasive diagnostic and prognostic tool for advanced stages of cancer.

Exosome detection in liquid biopsies has several utilities in advanced cancer stages:

- **Disease Monitoring:** Regular monitoring of disease progression or regression is critical in managing advanced-stage cancers. Traditionally, such monitoring relies on imaging techniques and tumor markers, which can be invasive, expensive, and may not be able to detect micro-metastasis (J. Wang et al., 2020). Exosome profiling, on the other hand, can provide a real-time snapshot of the disease, and can potentially detect small metastatic events before they become visible in imaging studies(Tian et al., 2021).
- **Treatment Selection and Personalized Medicine:** Tumor-derived exosomes contain genetic and protein content that is representative of the tumor. Thus, their analysis can help to understand the molecular underpinnings of a patient's cancer, guiding treatment decisions, including the selection of targeted therapies(Shang et al., 2017).
- **Prediction of Treatment Response and Resistance:** Exosomes can give insight into whether a tumor is likely to respond to a particular treatment (Stevic et al., 2020). For instance, some tumors may release exosomes containing PD-L1, a protein that suppresses the immune response, thus indicating that immune checkpoint inhibitors may be effective (Chen et al., 2018). Moreover, changes in exosome content over

time may help detect emerging resistance to therapy, allowing for earlier intervention with alternative treatments.

- **Prognostic Indicators:** The number of circulating exosomes and their molecular contents can be associated with disease outcome. For instance, in some cancers, a high level of circulating exosomes is associated with a worse prognosis. Moreover, certain exosomal contents, such as specific miRNAs or proteins, may also correlate with survival outcomes (Nanou et al., 2020).

There were several studies analyzing exosomes in liquid biopsies of metastatic colorectal cancer.

- **Protein-Based Studies:** Exosomal protein markers have been identified in colorectal cancer patients. As such, mCRC patients displayed elevated blood concentrations of total EVs and CD133+ EVs than healthy controls, and these high levels before treatment are correlated with shorter overall survival of patients (Brocco et al., 2022).
- **miRNA-Based Studies:** Various studies have identified different miRNAs in exosomes from colorectal cancer patients that may serve as biomarkers for the disease (Tsukamoto et al., 2017). For instance, one study found that the level of miR-21, a microRNA known to play roles in tumor growth and invasion, was significantly higher in exosomes isolated from the serum of metastatic colorectal cancer patients compared to those from non-metastatic patients (He et al., 2021).
- **Genetic Alteration-Based Studies:** Exosomes can also carry DNA, including mutant DNA sequences that are present in cancer cells. A study has shown that KRAS mutations, commonly found in colorectal cancer, can be detected in exosomal DNA from patient plasma samples (Lucchetti et al., 2021).

Despite the promising potential of exosomes in liquid biopsy for advanced cancer stages, some challenges remain, such as the need for standardization in exosome isolation and analysis, and the requirement for large-scale clinical validation of the identified biomarkers. However, as our understanding and technological capabilities advance, the use of exosomes in liquid biopsies is likely to become an integral part of precision oncology.

2. Objectives

Liquid biopsy has emerged as a non-invasive approach to characterizing tumors, gauging patient prognosis, identifying minimal residual disease (MRD), tracking treatment responses, and following clonal evolution during various treatments. There's significant interest in using blood over tissue samples to detect RAS mutations, especially when choosing anti-EGFR treatments such as cetuximab and panitumumab for mCRC. Yet, despite using consistent methods, variations in detecting cfDNA molecular alterations have been observed across different studies. In this context, the idea for our work arises, which aims to identify and monitor molecular alterations in the RAS and BRAF genes in liquid biopsy in patients with mCRC throughout the course of therapy.

This study had three specific aims:

1. Quantitation of Extracellular Vesicles (EVs), EV-RNA, and Cell-free DNA (cfDNA) in patients with metastatic colorectal cancer (initial stage IV or at first progression) as a biomarker for monitoring response to therapy.

2. Identification of RAS and BRAF mutations RAS e BRAF in EV-RNA and cfDNA to evaluate concordance with the mutational status in tissue samples and as biomarkers of resistance.

3. Correlation between the levels of exosomes and cfDNA with Progression-Free Survival (PFS) and Overall Survival (OS).

3. Material and Methods

3.1. Patient samples

The patients enrolled onto this study were admitted at Instituto Português de Oncologia Francisco Gentil (Coimbra, Portugal). The study was approved by the hospital's ethics committee, and written informed consent was obtained from all patients before sample collection, in accordance with the Declaration of Helsinki on medical research involving human subjects.

Selection to participate in the study was performed according to the following inclusion and exclusion criteria:

Inclusion Criteria:

1. Confirmed diagnosis of mCRC (initial stage IV or at first progression)
2. The patient will be yielded to do chemotherapy
3. Documented tumor histopathology
4. Radiologically measurable metastases
5. ECOG PS 0-2
6. Informed consent signed by the patient

Exclusion Criteria:

1. Previous chemotherapy in the last 5 weeks
2. Another solid synchronous tumor
3. Overall Survival < 6 weeks

From each patient, formalin-fixed paraffin-embedded (FFPE) material originating from surgical specimens or biopsies performed in the context of follow-up and surgical treatment was retrospectively analyzed. Blood samples were obtained prospectively for analysis in the context of liquid biopsy, with collections carried out during the clinical follow-up, in the clinical follow-up timings, and when carrying out other blood analyses integrated within the follow-up. Peripheral blood samples (5ml) were collected between 2017 and 2019 (D+1 of each cycle) in room temperature spray-dried K2EDTA anticoagulant tubes (BD Vacutainer® PPT™ Plasma Preparation Tube) and processed into plasma following manufacturer's instructions. Briefly, within 30 minutes after collection, the PPT tubes containing whole blood were gently inverted 8 - 10 times and centrifuged at room temperature (18-25°C) for a minimum of 10 minutes at 1,100 RCF. Plasma (0.5-3ml) was decanted into a new tube and samples were stored at -80°C until further processing.

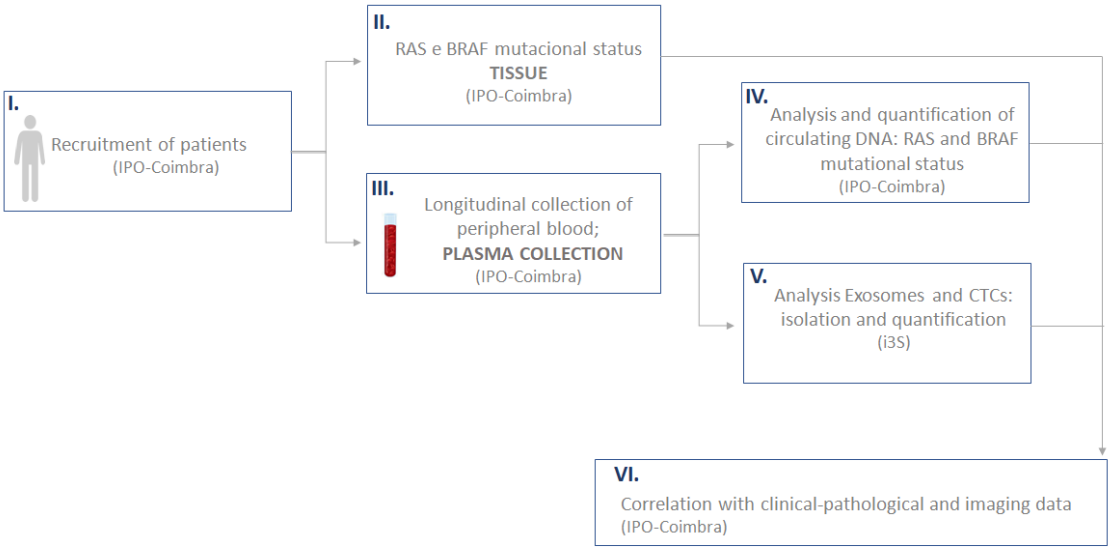

Figure 1. Schematic representation of the workflow of the study.

3.2. DNA extraction, MSI and KRAS, NRAS and BRAF mutation detection

DNA was extracted from FFPE tissue biopsy sample or macrodissected tumor content. Isolation of cfDNA from plasma was performed using the cobas® cfDNA Sample Preparation Kit. Nucleic acid quantification was performed by measuring the 260nm absorbance in the Nanodrop. MSI status was determined by amplification of a panel of validated microsatellite markers BAT25, BAT26, D2S123, and D5S346 through Polymerase Chain Reaction (PCR). The allelic instability analysis was performed by comparing the sizes of the fragments obtained for each marker in the normal and corresponding tumor samples. Molecular alterations in KRAS, NRAS, and BRAF genes were performed in DNA extracted from FFPE tissue and/or blood samples using the Cobas KRAS mutation test v2 and Cobas NRAS/BRAF mutation test v2 tests, which perform the detection by Real-time PCR (RT-PCR) of clinically relevant mutations in codons 12, 13 and 61 of the KRAS and NRAS genes, as well as in codon 15 of the BRAF gene.

3.3. Extracellular vesicles enrichment and characterization from plasma of mCRC patients

Thawed plasma samples (0.5 – 3.7ml) were diluted with 0.9% NaCl (pH 7.4) to a final volume of 15ml and filtered through a 0.22µm filter. Filtered supernatants were centrifuged in a SW32 rotor (Beckman Coulter, Fullerton, CA, USA) at 100,000x*g*, overnight at 4°C to pellet EVs. EV-enriched pellets were washed in 0.9% NaCl (pH 7.4), centrifuged at 100,000x*g* for 2 hours at 4°C, resuspended in a volume of 0.9% NaCl and stored at 4°C. Particle concentration was determined by Nanoparticle Tracking Analysis (NTA) using the NanoSight NS300 instrument (Malvern, Worcestershire, UK) with scientific CMOS camera. For this, EV-enriched samples were diluted (1:500) in 0.9% NaCl. Three technical measurements were recorded under a

controlled fluid flow with a pump speed set to 40 and a camera focus level adjusted between 10 and 16. The three videos were processed using the NTA 3.1 Build 3.1.54 software.

3.4. RNA extraction from EV-enriched mCRC plasma samples

Prior to RNA isolation, EV pellets were incubated with Proteinase K at 37°C for 10 min (final concentration 0.05mg/ml), following a heat-inactivation step at 75ºC for 10 min. Next, samples were treated with RNAse A at 37°C for 10 min (final concentration 0.02mg/ml). RNAse A was inhibited with Ribolock RNase inhibitor (final concentration 12U/ul). Total RNA was isolated from Proteinase K/RNAse A-treated samples with the Quick RNA MiniPrep kit (Zymo), by adapting some manufacturer's instructions. In particular, the ratio of RNA Lysis buffer to each sample was 1:1; 1.5 volumes of ethanol were used instead of 1; and RNA was eluted with 15ul of DNAse/RNAse free water (2x). RNA concentration was measured using the Nanodrop Microvolume Spectrophotometer and samples were kept at -80°C until further analysis.

4. Results

4.1. Patient characteristics

Thirteen patients were included in this study. The characteristics of the patients and the tumors are summarized in Table 1.

Table 1. Patient and tumors characteristics

Patients			**Number (n=13)**
Gender	Male		7
	Female		6
Median Age			58 (47-72) years
Primary Tumor Location	Colon	Right	3
		Transverse	2
		Left	4
	Rectum		4
Stage T	T1		0
	T2		1
	T3		9
	T4		3
Stage N	N0		5
	N1		2
	N2		4
Surgery primary tumor	Yes		8
	No		5
Metastasectomy	Yes		4
	No		9
Metastatic Lesion	1 site		5
	≥ 2 sites		8
Adjuvant Treatment	Yes		1
	No		12

4.2. KRAS, NRAS and BRAF mutational status

The mutational status of KRAS, NRAS and BRAF is summarized in Table 2. In seven of the 13 samples, the RAS and BRAF mutational assessments were performed in DNA isolated from tissue samples derived from the primary tumor. In the remaining 6 samples, the mutational assessment was performed in DNA isolated from the metastatic lesion.

KRAS or BRAF mutations were found in 69.2% (9/13) of the samples. KRAS mutations were found in 61.5% (8/13) of the samples. BRAF mutations were found in only one sample (7.7%). The remaining four samples were Ras and BRAF wild-type.

Table 2. RAS and BRAF mutation status in the tissue

Patient	Primary Tumor Location	Tissue Source	RAS Status (Tissue)	BRAF Status (Tissue)
1	Left Colon	Primary T. (colon)	WT	WT
2	Rectum	Pulmonary M.	KRAS Mut (Exon 2 - codon 12/13)	WT
3	Rigth Colon	Hepatic M.	KRAS Mut (Exon 2 - codon 12/13)	WT
4	Transverse Colon	Primary T. (colon)	WT	BRAF Mut - V600E
5	Rigth Colon	Peri-renal M.	WT	WT
6	Transverse Colon	Primary T. (colon)	KRAS Mut (Exon 2 - codon 12/13)	WT
7	Left Colon	Pulmonary M.	KRAS Mut (Exon 2 - codon 12/13)	WT
8	Rectum	Pulmonary M.	KRAS Mut (Exon 2 - codon 12/13)	WT
9	Rigth Colon	Hepatic M.	KRAS Mut (Exon 2 - codon 12/13)	WT
10	Sigmoid Colon	Primary T.	KRAS Mut (Exon 2 - codon 12/13)	WT
11	Colon	Primary T.	KRAS Mut	WT
12	Rectum	Primary T.	WT	WT
13	Colon	Primary T.	WT	WT

4.3. Comparison of RAS and BRAF mutation status between tissue and liquid biopsy

The comparison of RAS and BRAF mutation status between tissue and liquid biopsy (at the baseline) revealed a concordance in 46.2% (6/13) of the samples, either confirming the wild-type or the mutant status (Table 3). A discordance in the KRAS mutational status was found in 53.8% of the samples (7/13). In 42.9% (3/7) of discordant cases, the mutational status was performed in the primary tumor and in 57.1% (4/7) it was performed in the metastatic lesion. Only 1/7 discordant cases were KRAS wild-type in the primary tumor and KRAS mutant in the liquid biopsy.

When focusing on the origin of the tissue on which the mutational analysis was performed, it was observed that 57.1% (4/7) of the analyses done in the primary tumor were concordant and 42.9% (3/7) were discordant with the analysis done in the liquid biopsy. Fifty % (1/2) of the analyses done in hepatic metastasis and 100% (1/1) done in peri-renal metastasis were concordant with liquid biopsy. One hundred % (3/3) of the analyses performed in pulmonary metastasis were discordant.

Table 3. RAS and BRAF mutation status in tissue and liquid biopsy

Patient	Primary Tumor Location	Tissue	RAS Status (Tissue)	RAS Status (Liquid Biop.)	BRAF Status (Tissue)	BRAF Status (Liquid Biop.)	Metastatic Location
1	Left Colon	Primary T.	WT	WT	WT	WT	H + P
2	Rectum	Pulmonary M.	KRAS Mut	WT	WT	WT	P
3	Right Colon	Hepatic M.	KRAS Mut	KRAS Mut	WT	WT	H + P
4	Transversal Colon	Primary T.	WT	WT	BRAF Mut	BRAF Mut	LN + H
5	Right Colon	Peri-Renal M.	WT	WT	WT	WT	IP + P + H
6	Transversal Colon	Primary T.	KRAS Mut	KRAS Mut	WT	WT	H + Local Recurrence
7	Left Colon	Pulmonary M.	KRAS Mut	WT	WT	WT	P
8	Rectum	Pulmonary M.	KRAS Mut	WT	WT	WT	P
9	Right Colon	Hepatic M.	KRAS Mut	WT	WT	WT	H + P
10	Sigmoid Colon	Primary T.	KRAS Mut	WT	WT	WT	IP + LN + H
11	Colon	Primary T.	KRAS Mut	WT	WT	WT	H
12	Rectum	Primary T.	WT	WT	WT	WT	H
13	Colon	Primary T.	WT	KRAS Mut	WT	WT	LN + H

WT – wild-type; Mut – mutant; H – hepatic; P – pulmonary; IP – intraperitoneal; LN – lymph node

4.4. Quantification of cfDNA and mutational status of KRAS and BRAF along treatment

Quantification of cfDNA was performed in 12/13 patients, though only 10 had more than one longitudinal sample collected. A variation in the concentration of cfDNA was found in all 10 patients over time. In four patients, a change in the KRAS mutation status was identified (Figure 2).

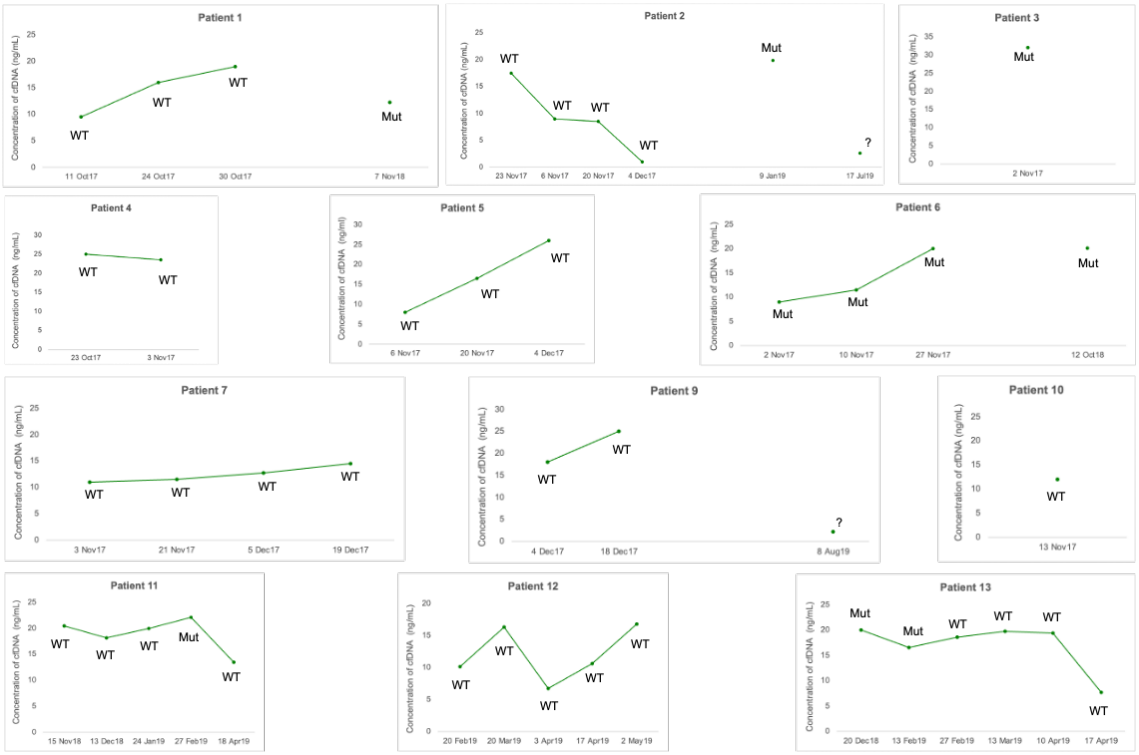

Figure 2. Longitudinal quantification of cfDNA. cfDNA was isolated from several timepoints along the treatment period and quantified using the Nanodrop. Missing points in the graph correspond to periods of sampling, though no sample was available for our analysis.

4.5. Longitudinal quantification of extracellular vesicles and their RNA cargo

Extracellular vesicles were isolated from longitudinal liquid biopsies in seven patients (Figure 3). All seven patients showed a drastic variation in one of the timepoints. In four of them, there was an increase in the number of exosomes, whereas in three there was a decrease. At least in three of the patients, the inflection points of the curve are associated with the onset of targeted therapy (cetuximab or bevacizumab).

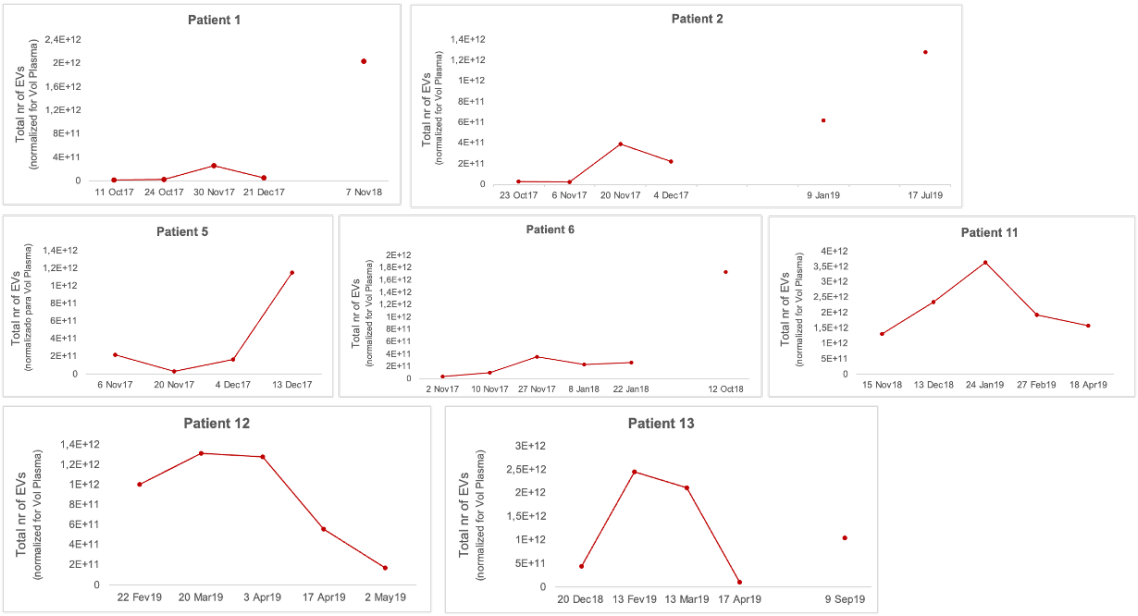

Figure 3. Quantification of extracellular vesicles in liquid biopsies. EVs were isolated from plasma samples through ultracentrifugation and quantified using NanoSight.

Isolation and quantification of RNA enclosed within EVs was done using samples collected longitudinally from 7 patients. The concentration of RNA within EVs was normalized for the total number of EVs. This analysis revealed that the amount of RNA per EV is also variable along time and do not follow the same peak dynamics of EVs (Figure 4).

More so, the concentration of RNA was also normalized to the volume of plasma from which EVs were isolated. The data reveals a dynamic variation in EVs-RNA along time, however for some patients it was not concordant neither with EV number nor EVs-RNA normalized to the number of EVs (Figure 5).

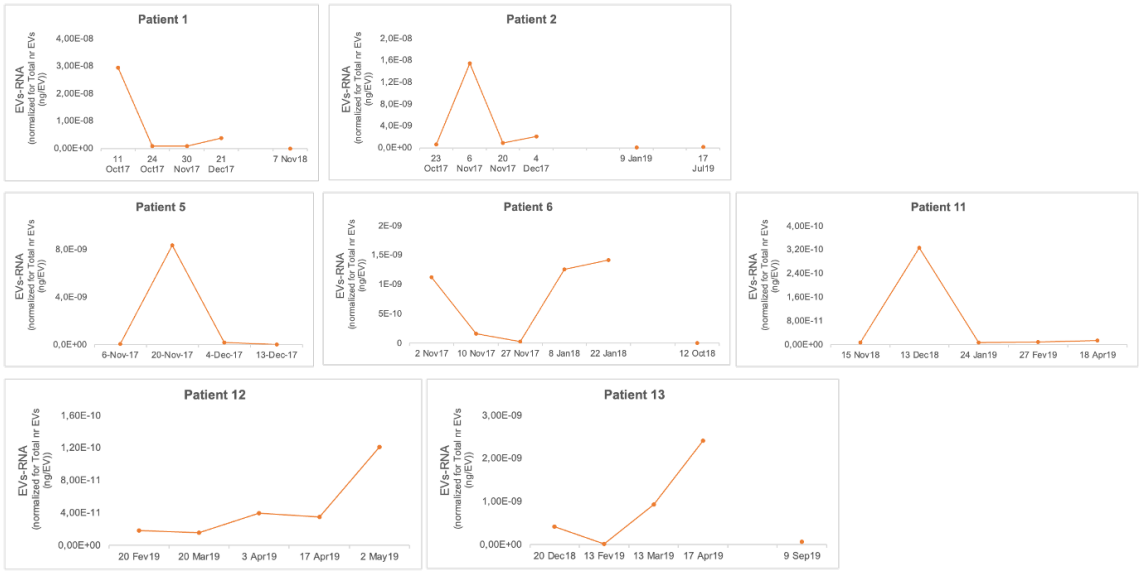

Figure 4. Quantification of EVs-RNA. For each longitudinal sample, RNA was isolated from EVs and normalized to the total number of EVs isolated from the respective sample.

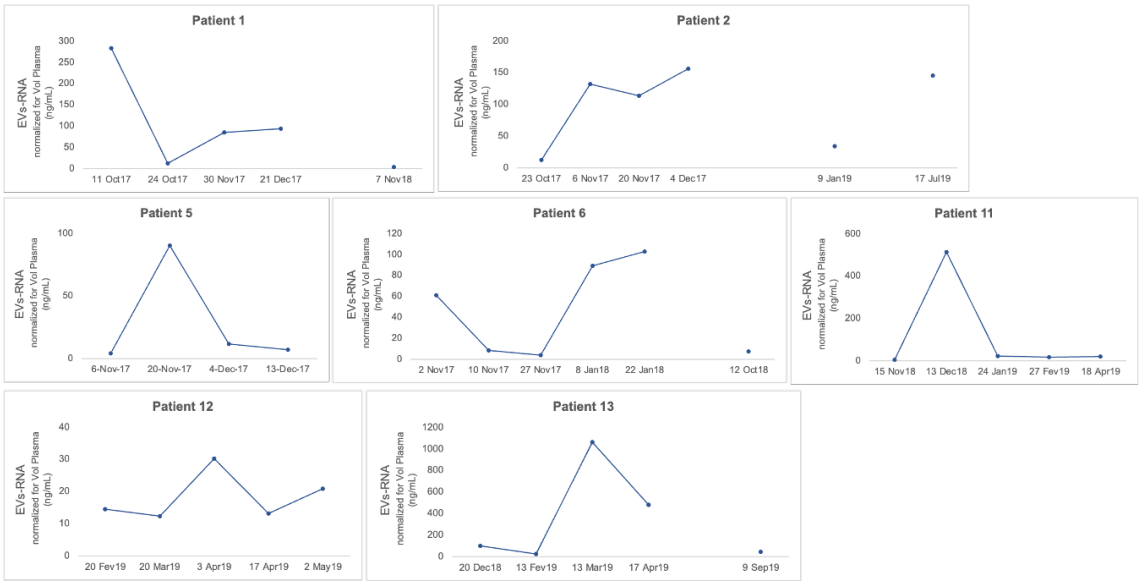

Figure 5. Quantification of EVs-RNA. RNA was isolated from EVs and normalized to volume of plasma from which EVs were isolated.

The concentration of RNA in the plasma was also compared with the concentration of cfDNA. In all the samples analyzed, EV-RNA was present at much higher concentrations than cfDNA. Curiously, EV-RNA and cfDNA concentrations followed opposite tendencies (Figure 6).

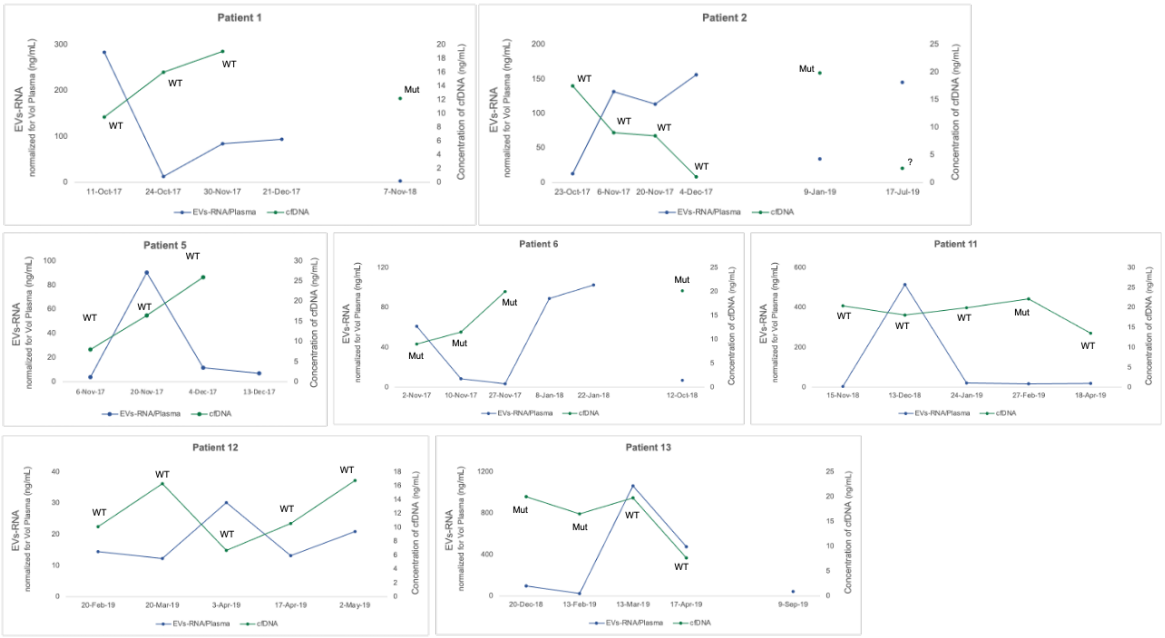

Figure 6. Comparison between the concentration of EV-RNA and cfDNA. EV-RNA and cfDNA concentrations were normalized to the volume of plasma from which they were isolated. In all the patients, EV-RNA and cfDNA vary in opposite directions.

5. Discussion

The implementation of liquid biopsy in the clinical setting brought with it the advantage of using a non-invasive, cost-effective strategy for the diagnosis of cancer and to monitor disease progression and response to treatment (Connal et al., 2023). In the setting of longitudinal monitoring of responses to treatment, liquid biopsies offer the possibility of overcoming the barrier of tissue availability from multiple timepoints (Caputo et al., 2023). However, there is still space to improve liquid biopsy methodology to take full advantage of it without compromising accuracy. For instance, whether analyzing DNA or RNA free in circulation or enclosed in extracellular vesicles, remains an open issue. Therefore, we set out to interrogate which methodology could more accurately portray the molecular dynamics of tumors.

Our data reveals a low concordance rate of 46.2% between the analysis of *KRAS* mutation status in the tissue and in the liquid biopsy. Moreover, when the mutational analysis is performed in the metastatic lesion, there is a higher discordance rate, especially when the tissue corresponds to a lung metastasis. These observations agree with previous data reporting that the concordance rates differ by the metastatic site (Kagawa et al., 2021). In particular, higher concordance rates were found in patients with liver metastasis, and higher discordance rates were found in patients with peritoneal or lung metastasis alone. Factors associated with concordance between peritoneal or lung metastasis and the liquid biopsy included the baseline longest diameter and lesion number, and sample collection interval (Kagawa et al., 2021). Accordingly, all but one of the discordant samples in our study had only pulmonary metastasis, in contrast to all the concordant cases in which there was metastasis in several organs, including the liver. However, the inclusion of more cases in the study is essential to draw more sustained conclusions.

Beyond the possible drawbacks of discrepancies between KRAS mutation status in tissue and plasma, studies have demonstrated that the resurgence of plasma KRAS mutations during anti-EGFR treatment could be identified before CT scans indicated disease progression (Misale et al., 2012). Accordingly, in our longitudinal study using cfDNA we could also observe a change in the KRAS mutation status in some of the patients. It remains to be determined whether we can recapitulate the same observations or even improve detection if EV-RNA is used.

However, resistance is not only attributable to the emergence of gene mutations as gene amplification and expression upregulation also account for the plethora of molecular mechanisms of resistance (Misale et al., 2014). The analysis of mRNA allows both the evaluation of gene expression levels and mutation detection. Moreover, in contrast to DNA, of which a low number of copies (generally two) exist per cell, RNA is produced in higher amounts (Siravegna et al., 2017). Nevertheless, the amount of cell-free tumor RNA present in circulation reflects not only the molecular contents of tumor cells but also the transcriptomic

alterations occurring in other cell types, such as immune cells, that mount a systemic anti-tumor response (Roskams-Hieter et al., 2022). As such, the sensitivity of detecting tumor-specific alterations might be impaired. Therefore, aiming to improve the sensitivity of liquid biopsies for longitudinal monitoring of responses to treatment, we set out to determine whether EVs and their RNA cargo might overcome this caveat of cfRNA analysis. EVs isolated from liquid biopsies are considered useful tools to monitor therapy response and predict prognosis (Yang et al., 2022). Although released from a variety of cell types, there is evidence that cancer cells release more EVs than non-cancer cells (Bebelman et al., 2021). Several in vitro and in vivo studies have shown that cancer cells increase EV secretion upon treatment with chemotherapy, such as paclitaxel, irinotecan and carboplatin, doxorubicin, among others (Ab Razak et al., 2019). Therefore, the characterization of mutational and expression profiles of genes associated with resistance, such as RAS and BRAF mutations, and KRAS, C-MET, and HER2 overexpression, in EV-RNA may provide a more sensitive approach to improve the detection of resistance biomarkers. Additionally, it may also improve the concordance rate between the detection of KRAS mutations between tissue and the liquid biopsy. Although our study is still in its infancy, we can already appreciate a correlation between a change in the number of EVs associated with the onset of the targeted therapy, which was not observed in the cfDNA amount. Concerning the analysis of EV-RNA, contrary to the expected, the concentration of EV-RNA did not correlate with the number of EVs isolated. Therefore, the number of EVs secreted and the amount of RNA they carry are likely two factors to take into consideration separately, as they may contribute with distinct information about the biological activity of cancer cells under treatment. Moreover, EV-RNA and cfDNA followed opposite directions. cfDNA is mostly derived from apoptotic and necrotic cells (Stejskal et al., 2023), whereas RNA and EVs are likely derived from viable cells. As such, the concentration of cfDNA and EV-RNA may provide complementary information about the state of viability of the cancer cells. However, only a more detailed study regarding the association between cfDNA and EV-RNA concentration with response to treatment may provide further insights.

6. Conclusion

The low concordance rate found between the analysis of the RAS and BRAF mutation status in the tissue and in the plasma reflects the need to investigate different methods that could more reliably allow biomarker detection using non-invasive testing. Our study provides evidence that the analysis of EV-RNA arises as a promising strategy to overcome this caveat. However, the analysis of more patients and further timepoints is essential to ascertain the potential of using EV-RNA as a tool for mutation and gene expression level detection in liquid biopsies.

7. Future perspectives

The future of this work encompasses:

1. The inclusion of more patients in the study.
2. Evaluation of RAS and BRAF mutational status in the EV-RNA and comparison with cfDNA
3. Evaluation of KRAS, C-MET and HER2 gene expression levels in EV-RNA.
4. Correlation with imaging data, PFS and OS.

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