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CUSTOMISATION OF THERAPEUTIC STRATEGY IN METASTATIC COLORECTAL CANCER BY USE OF LIQUID BIOPSIES (DETECTION OF RAS MUTATIONS WITH DIGITAL PCR)

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«λέγειν τὰ προγενόμενα, γινώσκειν τὰ παρεόντα, προλέγειν τὰ ἐσόμενα· μελετᾶν ταῦτα»

Ιπποκράτης (460–370 π.Χ.), *Ἐπιδημίαι 1.11*

PREFACE

"Declare the past, diagnose the present, foretell the future; practise these acts."

Hippocrates (460–370 B.C.), *Epidemics 1.11*

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ABBREVIATIONS

AJCC	American Joint Cancer Committee
BCT	Blood collection tube
BEAMing	Beads–emulsion–amplification–magnetics
BEBP	Biorepositories and Biospecimen Research Branch Evidence-Based Practices
biPAP	Bidirectional pyrophosphorolosis-activated polymerisation
CA 19-9	Carbohydrate antigen 19-9
CAPP-Seq	Cancer personalised profiling by deep sequencing
CAPOX	Capecitabine/oxaliplatin
CEA	Carcinoembryonic antigen
CECs	Circulating endothelial cells
CE-IVD	CE (Conformité Européene)-marked for *in vitro* diagnostic
CEUS	Contrast-enhanced ultrasound
cfDNA	Cell-free deoxyribonucleic acid
ChT	Chemotherapy
CI	Confidence interval
CLM	Colorectal liver metastasis
CRC	Colorectal cancer
CT	Computed tomography
CTCs	Circulating tumour cells
ctDNA	Circulating deoxyribonucleic acid
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
ECOG	Eastern cooperative oncology group
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
ERK	Extracellular regulated kinase
ESMO	European Society for Medical Oncology

EVs	Extracellular vesicles
FDA	Food and Drug Administration
5-FU	5-Fluorouracil
FFPE	Formalin-fixed, paraffin-embedded
FOLFOX	Leucovorin/infusional 5-fluorouracil/oxaliplatin
FOLFOXIRI	Leucovorin/infusional 5-fluorouracil/oxaliplatin/irinotecan
FP	Fluoropyrimidine
GE	Genomic equivalent
GEFs	Guanine nucleotide exchange factors
GSK3	Glycogen synthase kinase 3
HIPEC	Hyperthermic intraperitoneal
LAT	Local ablative treatment
LoD	Limit of detection
MAF	Mutant allele fraction
mCRC	Metastatic colorectal cancer
MEK/ERK	Mitogen- activated kinase/ERK kinase
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MUT or Mut	Mutant
NCI	National Cancer Institute
NED	No evidence of disease
NGS	Next-generation sequencing
NPA	Negative percent agreement
NTC	Negative control
OMD	Oligometastatic disease
OPA	Overall percent agreement
OS	Overall survival
P70S6K	p70 ribosomal protein S6 kinase
PC	Positive control
PD1	Progressive disease at first-line therapy

PD2	Progressive disease at second-line therapy
PCR	Polymerase chain reaction
PDK1	Phosphatidyl triphosphate dependent kinase 1
PET	Positron-emission tomography
PFS	Progression-free survival
PI3Ks	Phosphoinositide-3 kinases
PKC	Protein kinase C
PLA2	Phospholipase A2
PLD	Phospholipase D
PPA	Positive percent agreement
PS	Performance status
RALGDS	Guanine nucleotide exchange factors (GEFs) for RAL
RFA	Radiofrequency ablation
RSK	p90 ribosomal protein S6 kinase
R0	Microscopically margin-negative surgical resection
SBRT	Stereotactic ablative body radiotherapy
SD	Standard deviation
TTP	Time-to-progression
TEPs	Tumour-educated platelets
UICC	Union for International Cancer Control
UMI	Unique molecular identifiers
US	Ultrasound
VEGF	Vascular endothelial growth factor
WT	Wild type

PART I

1. COLORECTAL CANCER

1.1. General information

Colorectal cancer (CRC) is the malignant neoplasm originating from the colonic mucosa and generally growing towards the lumen and/or spreading to adjacent organs. It is a debilitating disease associated with high morbidity in the general population.

Based on the model proposed by Fearon and Vogelstein's known as the 'adenoma-to-carcinoma sequence', the development of CRC is a multi-step process arising from the normal epithelium with the formation of dysplastic aberrant crypts (1). These may then form a 'niche' for the development of an early adenoma, which may progress to an intermediate and late adenoma by the acquisition of additional mutations, finally becoming carcinoma. Other changes contribute to the development of metastatic potential and spread to distant organs (Figure 1).

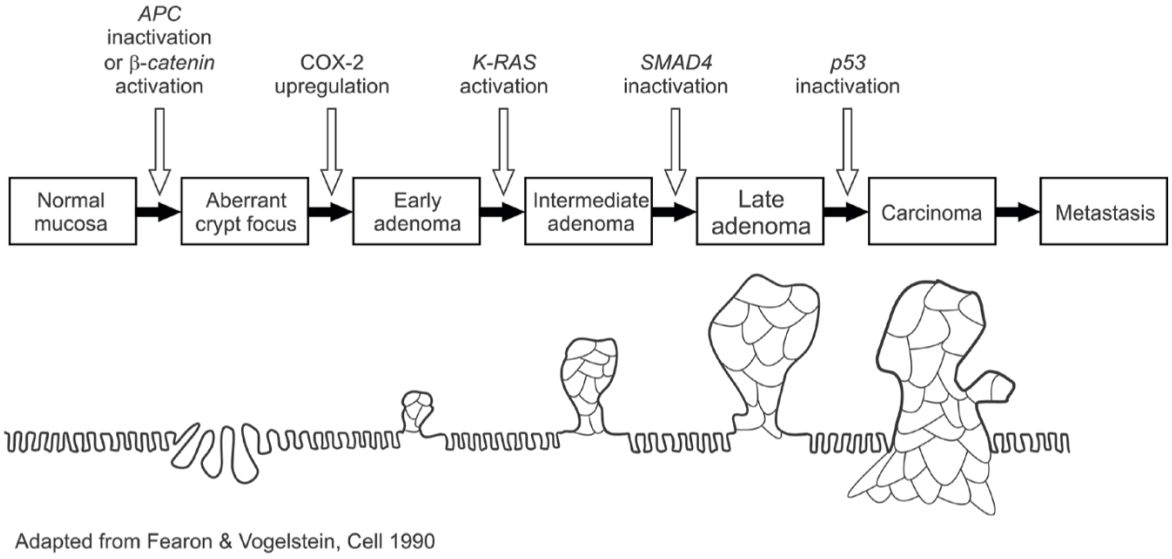

Figure 1. Fearon and Vogelstein's adenoma-to-carcinoma sequence. According to this model, specific genetic events involving *APC*, *COX-2, KRAS*, *SMAD4* and *p53* and other genes need to occur sequenctially for the development of CRC. Each of them correlates with a stage in the morphological evolution of from normal epithelium to metastatic cancer, through progression to dysplastic aberrant crypts, early adenoma, intermediate adenoma, late adenoma and carcinoma (2).

CRC is regarded as a single disease entity despite some differences in the management of tumours arising from each of the respective organ site, especially in early stages of the disease, due to differences in the regional anatomy and the adjacent organs to the colon or rectum (3). The evolution of genomics has shed light on molecular differences between tumours located in the colon or rectum, as well as of the right or left colon, which will be discussed later in this chapter.

1.2. Incidence and epidemiology

CRC is the third most common malignancy worldwide and the second most common cause of cancer death worldwide, with 1,849,518 estimated new cases and 880,792 estimated deaths for 2018 (4). For the same year in Europe, 499,667 estimated new cases and 242,483 estimated deaths from colorectal cancer were reported (Table 1) (4).

The high mortality rate can be, to a certain extent, attributed to the high frequency of metastatic disease, partly due to failure to prevent metastatic spread when the cancer is diagnosed at an earlier stage. Approximately 25% of patients present with metastases at initial diagnosis, and almost 50% of patients with CRC will develop metastases (5), leading to over 60% of patients with CRC requiring treatment of metastases in the course of their disease.

Over the last two decades, there has been a substantial improvement in the treatment outcome of the disease, due to the advent of targeted therapies and use of local ablative techniques for the resection of metastases. However, there is still unmet need for accurate biomarkers that will allow for early initial diagnosis prior to development of metastases, optimal treatment selection, monitoring of response to treatment and detection of relapse.

Table 1. Incidence (cases) and mortality (deaths) for colorectal cancer and all cancers in 2018 (including nonmelanoma skin cancer). Adapted from GLOBOCAN 2018 (4).

	Incidence (Cases)		**Mortality (Deaths)**	
	Males	Females	Males	Females
Cancer site				
Colon	575,789	520,812	290,509	260,760
Rectum	430,230	274,146	184,097	126,297
All sites	**9,456,418**	**8,622,539**	**5,385,640**	**4,169,387**

1.3. Clinical Manifestations

Symptoms are associated with relatively large tumours and/or advanced disease stages and are generally not CRC-specific. Depending on the location and stage of the primary tumour, patients can present with change in bowel habits, general or localised abdominal pain, weight loss, iron deficiency and anaemia, among others. The existence of symptoms has been associated with worse prognosis, whilst their number (but not their duration) is inversely related to survival (6).

1.4. Diagnosis

The diagnosis of CRC is based on clinical suspicion but requires a stepwise process to be confirmed.

Endoscopy is the main diagnostic procedure for the diagnosis of CRC and is either total colonoscopy or sigmoidoscopy, more than 35% of CRC primaries are located in the sigmoid or rectum. Endoscopy allows for the exact localisation and biopsy of the primary lesion, as well as for the detection and excision of any synchronous lesions. If not prior to surgical removal of the primary tumour, a complete examination of the colorectum is necessary within 3–6 months.

Regarding metastatic CRC (mCRC), the European Society for Medical Oncology (ESMO) Consensus Guidelines state that the presence of metastatic disease should always be radiologically confirmed, setting as a prerequisite the histological examination of the primary tumour or metastases before initiation of any systemic treatment (3).

Therefore, the first diagnostic step is an abdominal/pelvic and thoracic computed tomography (CT) scan. In cases of doubt or if further information is required, this is followed by a second method, such as ultrasound (US) (including contrast-enhanced US [CEUS]), magnetic resonance imaging (MRI) or positron emission tomography (PET)-CT scan, depending on the localisation of the metastases. US may be helpful to characterise liver lesions, MRI to detect liver, peritoneal or pelvic metastases, and PET-CT to detect extrahepatic disease. This stepwise imaging approach is recommended in relation to therapeutic possibilities, rather than the use of all imaging modalities in all patients.

1.4.1. Molecular pathology and biomarkers

Tissue selection for biomarker testing

In the current era of precision oncology, molecular profiling plays a key role in mCRC, where various biomarkers need to be tested to decide on the appropriate systemic therapy. Molecular profiling can be conducted on a biopsy or surgically resected specimen of the primary tumour or a metastatic lesion of *de novo* metastatic tumours. In case of relapsed tumours that were initially non metastatic, archival primary tumour tissue may also be used for molecular testing.

Due to the large number of clinically relevant biomarkers to be tested, the ESMO Consensus Guidelines emphasise on the need to maximise the number of collected tissue samples (3). Ideally, 10 diagnostic tumour/biopsy/endoscopy samples should be collected. It is also very important to ensure appropriate handling and storage of these samples, to allow for further analysis with future tests on frozen tissue as accurately as possible, if required (3).

Currently, the biomarkers that are routinely tested in mCRC are *KRAS*, *NRAS*, *BRAF* and microsatellite instability (MSI).

RAS genes

One of the most important biomarkers tested in mCRC is the mutation status of the *KRAS* and *NRAS* genes, collectively known as *RAS*.

RAS constitute the most frequently mutated gene family in cancer. *RAS* mutations are found in approximately 30% of cancers, predominantly in pancreatic, lung and colon cancers (7). The RAS proteins (KRAS, NRAS and HRAS) bind Guanosine Diphosphate (GDP) and Guanosine Triphosphate (GTP) with high affinity and have intrinsic GTPase activity, hydrolysing GTP to GDP (8). They therefore function as molecular switches that can activate several effector pathways by controlling the expression of downstream genes, as illustrated in Fig. 2.

More specifically, RAS signalling regulates important physiological cellular processes, such as cell proliferation and survival, and is implicated in cancer development by either increasing or prolonging signal activation. Proteins with oncogenic mutations are resistant to downregulation of GAP-mediated hydrolysis of bound GTP, and thus signal persistently (9).

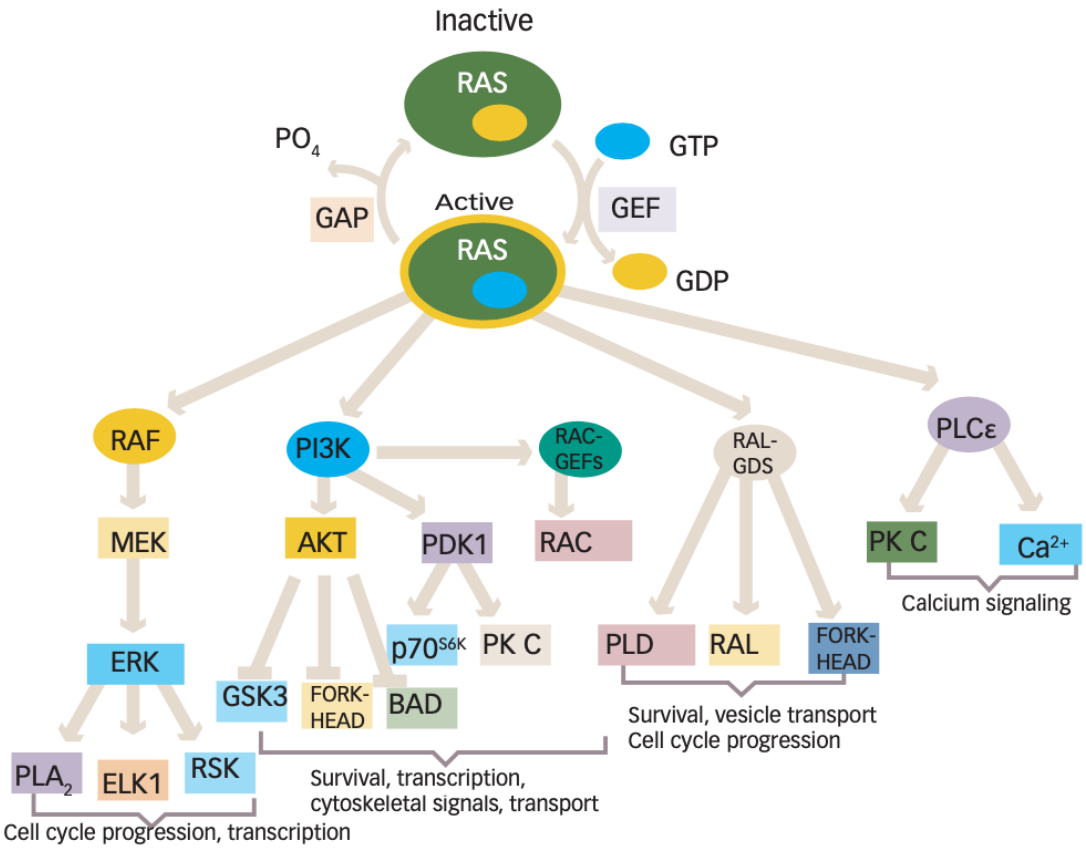

Figure 2. *RAS* activation and signalling downstream of RAS. ERK, extracellular regulated kinase; GSK3, glycogen synthase kinase 3; MEK, mitogen-activated kinase/ERK kinase; P70S6K, p70 ribosomal protein S6 kinase; PDK1, phosphatidyl triphosphate dependent kinase1; PI3Ks, phosphoinositide-3 kinases; PKC, protein kinase C; PLA2, phospholipase A2; PLD, phospholipase D; RALGDS-guanine nucleotide exchange factors (GEFs) for RAL; RSK, p90 ribosomal protein S6 kinase (9).

In CRC, between 40–45% of tumours harbour activating mutations in *KRAS* and 1–3% in *NRAS*. This suggests that the RAS family plays an important part in the development of CRC. The fact that they are found in both early and late CRC stages supports that they constitute early events in aberrant crypt foci formation that can become hyperplastic polyps and eventually CRC (see Fig. 1), although other genes are capable of initiating malignancy in the colon or rectum (7).

The presence of *RAS* mutations in the tumours of patients with metastatic CRC is a negative predictive biomarker for therapeutic choices involving anti-epidermal growth factor receptor (EGFR) monoclonal antibody therapies for metastatic disease. 'Expanded *RAS* analysis' is a term referring testing of *KRAS* exons 2, 3 and 4 (codons 12, 13, 59, 61, 117 and 146) and *NRAS* exons 2, 3 and 4 (codons 12, 13, 59, 61 and 117). Testing for mutations in

the above gene locations is mandatory prior to treatment and should be conducted on all patients eligible/being considered for treatment with anti-EGFR antibodies (cetuximab and panitumumab) (3).

It should be noted that for *RAS* mutation testing, tissue from a primary tumour or liver metastasis is recommended. This is because studies show discordance rates for *KRAS* exon 2 mutation testing between primary tumours and metastases of approximately 5% for liver metastases and 25% for lymph node metastases. These data can be extrapolated to expanded *RAS* analysis. Tissue from other metastatic sites, such as lymph node or lung, should be used only when primary tumour or liver metastases samples are not available.

BRAF

BRAF mutations (generally V600E) are found in 8–12% of patients and are a significant negative prognostic marker. *BRAF* mutations are a negative predictor for EGFR antibody therapy in later lines although their role in earlier lines has not been fully ascertained. *BRAF* mutation status should be assessed alongside the assessment and/or potential determination of treatment intensity and for selection for clinical trials.

Microsatellite instability (MSI)

Microsatellite instability (MSI) is found in 4–8% of tumours and confers an inferior prognosis, which may be driven by the frequent presence of *BRAF* mutations. MSI testing can assist in the setting of genetic counselling. Most studies in mCRC show that MSI status is not relevant as a single predictive marker for response to chemotherapy (ChT) but is strongly predictive for the clinical benefit of immune checkpoint inhibitors.

1.5. Staging and risk assessment

The pathological stage remains one of the best-established determinants of prognosis in CRC, and its report should follow the 7th edition of the American Joint Cancer Committee (AJCC)/ Union for International Cancer Control (UICC) TNM classification (Fig. 3). T stage represents the extension of the tumour into the bowel wall and adjacent organs. N stage represents the

site and number of removed regional lymph nodes and their potential infiltration by cancer cells. Of note, pathological examination of a minimum of 12 nodes is required for adequate pN-staging. M stage accounts for the metastatic involvement of distant organs (10).

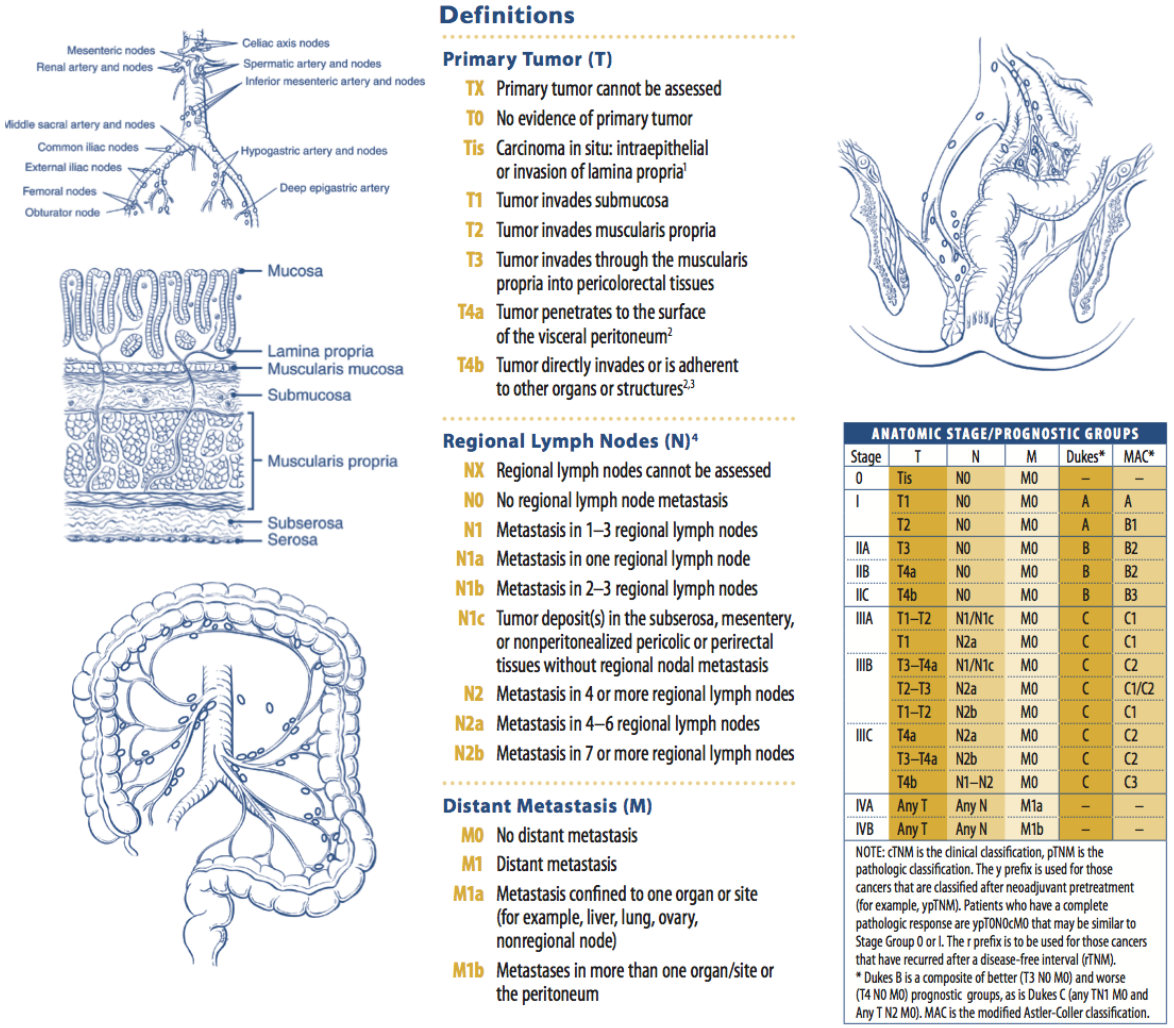

Figure 3. American Joint Committee on Cancer (AJCC) Colon and Rectum Cancer Staging (7th edition). T stage represents the extension of the tumour into the bowel wall and adjacent organs. N stage represents the site and number of removed regional lymph nodes and their potential infiltration by cancer cells, with a requirement for pathological examination of a minimum of 12 nodes for adequate pN-staging. M stage accounts for the metastatic involvement of distant organs (11).

1.6. Treatment

The majority of patients undergo surgery of the primary tumour. However, to optimally define the therapeutic strategy for patients with mCRC, an expert multidisciplinary team should be involved, including a colorectal surgeon, a specialist hepatobiliary and/or lung surgeon, a pathologist, a diagnostic radiologist and radiation oncologist and medical oncologists as standards.

The following sections cover the management of patients with oligometastatic disease, as well as with non resectable metastatic disease.

1.6.1. Oligometastatic disease

Oligometastatic disease (OMD) is characterised by metastases at up to three different sites, with five or more lesions, predominantly visceral and occasionally lymphonodal. Typically, involved sites are the primary tumour and other involved sites, such as the liver, lung peritoneum, nodes and ovary.

Treatment strategy should be based on the possibility of achieving complete ablation of all tumour masses using surgical R0 resection and/or interventional local ablative treatment (LAT). For OMD confined to a single organ (commonly liver), or a few organs, surgery is the standard treatment and is the only option to be proven potentially curative. For more extensive OMD, involving more sites or lesions, systemic treatment is the standard of care and should be considered as the initial part of every treatment strategy.

The best local treatment should be selected from a toolbox of procedures, according to disease localisation, treatment goal, treatment-related morbidity and patient-related factors, such as comorbidities and age. Liver-directed therapy is the best-established approach, but options include stereotactic ablative body radiotherapy (SBRT) and radiofrequency ablation (RFA), peritonectomy with/without hyperthermic intraperitoneal ChT (HIPEC) and nodal dissection. Patients with lung metastases have better outcomes, and a “watch and wait” strategy (or sequential approach) may be appropriate.

Surgical resection of liver metastases

The treatment strategy for colorectal liver metastases (CLM) should be directed towards complete resection, whenever possible. Decisions should be based on oncological and technical criteria. CLM are technically resectable as long as complete macroscopic resection is feasible while maintaining at least a 30% future liver remnant or a remnant liver-to-body-weight ratio higher than 0.5. Oncological criteria include the number of lesions and the presence (or suspicion) of extrahepatic disease and all criteria used in the FONG score.

Imaging in the identification of resectable/unresectable disease

Imaging should comprise firstly an abdominal/pelvic and thoracic CT scan and, in the case of doubt, a second method–such as US (CEUS), MRI or PET/CT scan depending on the localisation of the metastases. A stepwise imaging approach is the recommended policy, in relation to the therapeutic possibilities, rather than the use of all imaging modalities in all patients. For metachronous metastases, histopathological or cytological confirmation of metastases should be obtained if the clinical or radiological presentation is either atypical or very late (i.e. later than 3 years) after the initial diagnosis of this primary tumour. Resectable metastases do not need histological or cytological confirmation before resection.

Liver metastases that are technically resectable up front

In the context of OMD, the treatment aim is curative. Thus, it is very important to define the resectability of liver metastasis, based on both technical criteria for surgery and prognostic considerations. In patients with clearly resectable disease and favourable prognostic criteria, perioperative treatment may not be necessary and upfront R0 resection is justified. In technically resectable disease where the prognosis is unclear or probably unfavourable, perioperative combination ChT (3 months prior to and post-surgery) with leucovorin/infusional 5-FU/oxaliplatin (FOLFOX) or capecitabine/oxaliplatin (CAPOX) should be administered. Targeted agents should not be used in this setting. Where the criteria for prognosis and resectability are not sharply defined, including patients with synchronous onset of metastases, perioperative therapy should be considered (as part of a continuum of treatment option). Adjuvant ChT is not recommended for patients with

favourable oncological and technical (surgical) criteria who have not received perioperative ChT but may be beneficial for patients with unfavourable criteria. In patients who have not received any previous ChT, adjuvant treatment with FOLFOX or CAPOX is recommended (unless patients were previously recently exposed to oxaliplatin-based adjuvant ChT). Decision making should include the patient's characteristics and preferences.

Unresectable CLM with 'conversion' as a strategic goal

Any patient with limited liver and/or lung metastases should be considered a candidate for potential secondary resection. Conversion therapy offers the best means of converting unresectable disease to resectable. Response to systemic treatment is a strong prognostic indicator with conversion therapy. Resectability should be assessed after 2 and then 4 months of optimal treatment. Up to 75% of patients will relapse (mainly in the liver) and ablative techniques, such as RFA or SBRT, may be used as an adjunct to surgery to achieve no evidence of disease (NED). There is currently no standard for the treatment of synchronous CLM, but the recommendation is for more aggressive treatment and preoperative ChT.

Conversion treatment

Resection rates are correlated with response to systemic therapy and regimens leading to high response rated and/or fast onset of response and/or large tumour reduction (Early Tumour Shrinkage, Depth of Response) are recommended for potentially resectable disease with conversion as the goal. There is uncertainty surrounding the best combination. In *RAS* wild-type disease, a cytotoxic doublet + an anti-EGFR antibody seems to have the best benefit-to-risk ration, although the combination of leucovorin/infusional 5-FU/oxaliplatin/irinotecam (FOLFOXIRI) + bevacizumab may also be considered and, to a lesser extent, a cytotoxic doublet + bevacizumab. In *RAS* mutant disease, a cytotoxic doublet + bevacizumab or FOLFOXIRI + bevacizumab. Patients must be re-evaluated regularly to prevent the overtreatment of resectable patients as the maximal response is expected to be achieved after 12–16 weeks of therapy in most patients. Total therapy duration should not exceed 6 months.

Metastases at unfavourable/uncommon sites and role of ablative treatment with or without surgery

Patients with a limited number of lesions and involved sites should be regarded as having OMD and should be treated according to the standard treatment algorithm. In this situation, ablation of visible sites is unlikely to lead to cure but may allow discontinuation of systemic therapy with the possibility of a relapse-/disease-free interval. RFA, microwave ablation, cryoablation, SBRT and, to a lesser extent, embolisation techniques are all feasible. Selection of ablative treatments from the toolbox differ according to the size and localisation of metastases, the rates of control achieved, the invasiveness of the technique, prognostic considerations, patient factors and preferences and life expectancy, as well as to the local expertise of the treating team. This strategic treatment approach should be evaluated and pursued further in suitable patients.

1.6.2. Treatment of metastatic disease

The definition of a treatment aim and strategy are important considerations in multimodal treatment approaches and the choice of a systemic treatment strategy. Relevant factors include: tumour- and disease-related characteristics, patient-related factors and treatment-related factors.

Determination of a therapeutic strategy

The therapeutic strategy should be selected following a diagnostic work-up including clinical examination, blood counts, determination of liver and renal function, tumour marker measurements (primarily carcinoembryonic antigen [CEA] levels), abdominal and thoracic CT/MRI scan and assessment of the patient's general health. General health and performance status (PS) are strong prognostic and predictive factors for ChT and "fit" or "unfit" are used to determine the intensity of treatment choice. Three clinically relevant categories are evolving for the treatment of 'fit' patients whose metastatic disease is not resectable at presentation.

- Group 1A: Intensive treatment with the goal of cytoreduction and conversion to resectable disease or the use of local ablative treatments (LAT).
- Group 1B: Intensive treatment for rapid reduction of tumour burden because of impending clinical threat or organ dysfunction, or severe symptoms (although patients will not reach resection or benefit from LAT).
- Group 2: Intensive treatment is unnecessary, and the goal is disease control.

Knowledge of *RAS* and *BRAF* mutational status is required to further refine treatment strategies.

The typical first-line treatment options are a cytotoxic doublet (such as FOLFOX, CAPOX or leucovorin/infusional 5-FU/irinotecan [FOLFIRI]) or, in selected patients, a cytotoxic triplet (FOLFOXIRI). Fluopyrimidine (FP) monotherapy is an option in selected patients with asymptomatic, primarily unresectable metastases that are likely to be eligible for multiple lines of treatment and who are not candidates for combination ChT. Biologics (targeted agents) are indicated in the first-line treatment of most patients (unless contraindicated).

- If bevacizumab is used, it should be combined with
    - Cytotoxic doublets: FOLFOX/CAPOX/FOLFIRI
    - Cytotoxic triplet FOLFOXIRI in selected fit and motivated patients where cytoreduction (tumour shrinkage) is the goal and potentially also in fit patients with tumour *BRAF* mutations.
    - FP monotherapy in patients unable to tolerate aggressive treatment.
- If EGFR antibodies are used, they should be combined with
    - Cytotoxic doublets: FOLFOX/FOLFIRI
    - Capecitabine-based and bolus 5-FU-based doublets should not be combined with EGFR antibodies.

Recent retrospective analyses of large first- and second-line trials have shown that for patients with *RAS* wild-type status, combinations with anti-EGFR antibodies have a higher activity in patients with left-sided primary tumours. Conclusions from this finding are to reinforce the use of EGFR antibody therapy in patients with mCRC and left-sided *RAS* wild-type tumours, to promote the idea that patients with right-sided *RAS* wild-type tumours might be better treated with ChT alone or ChT plus bevacizumab and to emphasise that, in the absence of data on specific treatment sequences, there is no reason that EFGR-antibody therapy should be avoided in cases of disease progression or treatment intolerance independent of primary tumour location. Patients should receive all three available cytotoxic agents and all suitable targeted agents during the course of their treatment, when possible, although the optimal sequence remains to be elucidated. Around 70-80% of "fit" patients should receive second-line therapy and 50-60% should receive third-line therapy.

Discontinuation of treatment and the concept of maintenance therapy

After initial induction therapy, an active maintenance treatment is seen as a possible option to shorten duration of induction combination therapy. Patients receiving FOLFOX or CAPOX plus bevacizumab-based therapy as induction therapy, should be considered for maintenance therapy after 6 cycles of CAPOX or 8 cycles of FOLFOX. The optimal maintenance treatment is a combination of an FP plus bevacizumab. Bevacizumab as monotherapy is not recommended. Patients receiving FOLFIRI can continue on induction therapy–at a minimum–for as long as tumour shrinkage continues, and the treatment is tolerable. For patients receiving initial therapy with FOLFOXIRI plus or minus bevacizumab, FP plus bevacizumab may be considered as maintenance therapy. For patients receiving initial therapy with a single-agent FP (plus bevacizumab). Induction therapy should be maintained until progression. Individualisation and discussion with the patient are essential. Initial induction therapy or a second-line therapy must be reintroduced at radiological or first signs of symptomatic progression. If a second-line therapy is chosen, re-introduction of the initial induction treatment should be a part of the entire treatment strategy as long as no relevant residual toxicity is present.

Second line

Second-line therapy is recommended for patients with a good PS and adequate organ function. The type of therapy depends on the first-line choice and, in patients in whom the initial ChT backbone has failed, the ChT backbone should be changed from that used first line. Bevacizumab-naïve patients should be considered for treatment with an antiangiogenic (bevacizumab or aflibercept) second line. Aflibercept should be restricted to combination with FOLFIRI for patients progressing on an oxaliplatin-containing regimen. Patients who received bevacizumab in first line should be considered for treatment with bevacizumab post-continuation strategy, aflibercept or ramucirumab (in combination with FOLFIRI) when treated in first lien with oxaliplatin. EGFR antibodies in combination with FOLFIRI/irinotecan for patients with *RAS* wild-type (*BRAF* wild-type) disease. The relative benefit of EFGR antibodies is similar in later lines compared with second line. Patients who are fast progressors on first-line bevacizumab-containing regimens should be considered for treatment with aflibercept or ramucirumab (only in combination with FOLFIRI), and–in the case of patients with *RAS* wild-type disease and no pre-treatment with anti-EGFR therapy–EGFR antibody therapy, preferably in combination with ChT.

Third line

In *RAS* wild-type and *BRAF* wild-type patients not previously treated with EGFR antibodies, cetuximab or panitumumab therapy should be considered. Cetuximab and panitumumab are equally active as single agents. Cetuximab plus irinotecan is more active than cetuximab alone in irinotecan-refractory patients. There is no evidence to administer the alternative EGFR antibody if a patient is refractory to one of the EGFR antibodies. Regorafenib is recommended in patients pre-treated with FP, oxaliplatin, irinotecan, bevacizumab and, in *RAS* wild-type patients, with EFGR antibodies. Regorafenib is superior to placebo in terms of overall survival (OS)–although there are toxicity concerns. Trifluridine/tipiracil is recommended for patients pre-treated with FP, oxaliplatin, irinotecan, bevacizumab and, in *RAS* wild-type patients, with EGFR antibodies. Trifluridine/tipiracil is superior to placebo in terms of OS.

1.6.3. The role of biomarkers in mCRC

Biomarkers can be diagnostic, predictive, or prognostic. Ideally, a biomarker should only serve one of these purposes, but there are good and clinically relevant examples of prognostic biomarkers that predict a response to a specific therapy (3).

The presence of any *RAS* mutation represents a negative predictive biomarker of anti-EGFR treatment outcome for patients with metastatic colorectal cancer.

Targeted therapies have changed the face of oncology and significantly improved prognosis of various malignancies including metastatic colorectal cancer. The addition of monoclonal antibodies against vascular epithelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) in chemotherapy schemes are established therapeutic options in the first and second treatment lines in these patients. EGFR is a downstream effector of the *RAS* genes and anti-EFGR are only effective in tumours with wild-type *RAS* genes. *KRAS* exon 2 codons 12 and 13 were the first *RAS* mutations to be identified as negative predictive markers to anti-EGFR and comprise % of mutations. Further studies revealed that another % of tumours harbour mutations in other codons of *KRAS* as well as *NRAS*. Currently, extended *RAS* testing (including *KRAS* codons 12, 13, 59, 61, 117 and 146, and *NRAS* codons 12, 13, 59, 61 and 117) in tumour tissue is a prerequisite for anti-EGFR administration. Thus, the median progression-free survival of patients *RAS* wildtype tumours is 15 months for those who receive anti-EGFR plus a chemotherapy doublet as first-line treatment and 6 months for those who receive the combination as second-line treatment.

However, there is still a subset of patients who do not respond to anti-EGFR although their tumours are tested negative for *RAS* mutations (Fig. 4). Tumour heterogeneity may account for this lack of response, at least partially, as it is known that tumours are made of clusters of cells that evolve (mutate) and differ at the molecular level. A group of cells which are identical with each other constitute a clone and hence tumours are polyclonal, exhibiting a high degree of heterogeneity in space. As a result, tissue biopsy is subject to selection bias and *RAS* negative testing may be a false negative. Although this is limited by multiple sampling recommended by ESMO, it cannot be fully eliminated, especially in the clinical reality where there is only limited tissue for molecular testing. Another issue in clinical

practice is that new biopsies are often not feasible, making archival formalin-fixed, paraffin-embedded tissue from diagnosis the only material available for testing. Improved prognosis of patients with mCRC nowadays means that this sample may be a few years' old and, over this period of time, tumour evolution may have led to the acquisition or loss of mutations and an overall different molecular profile, which shapes the heterogeneity of tumours in time. Molecular testing results may be outdated and in certain cases mislead rather than guide therapeutic options. However, both dimensions of tumour heterogeneity that undermine the accuracy of tissue biopsy are likely to be overcome with liquid biopsy approaches.

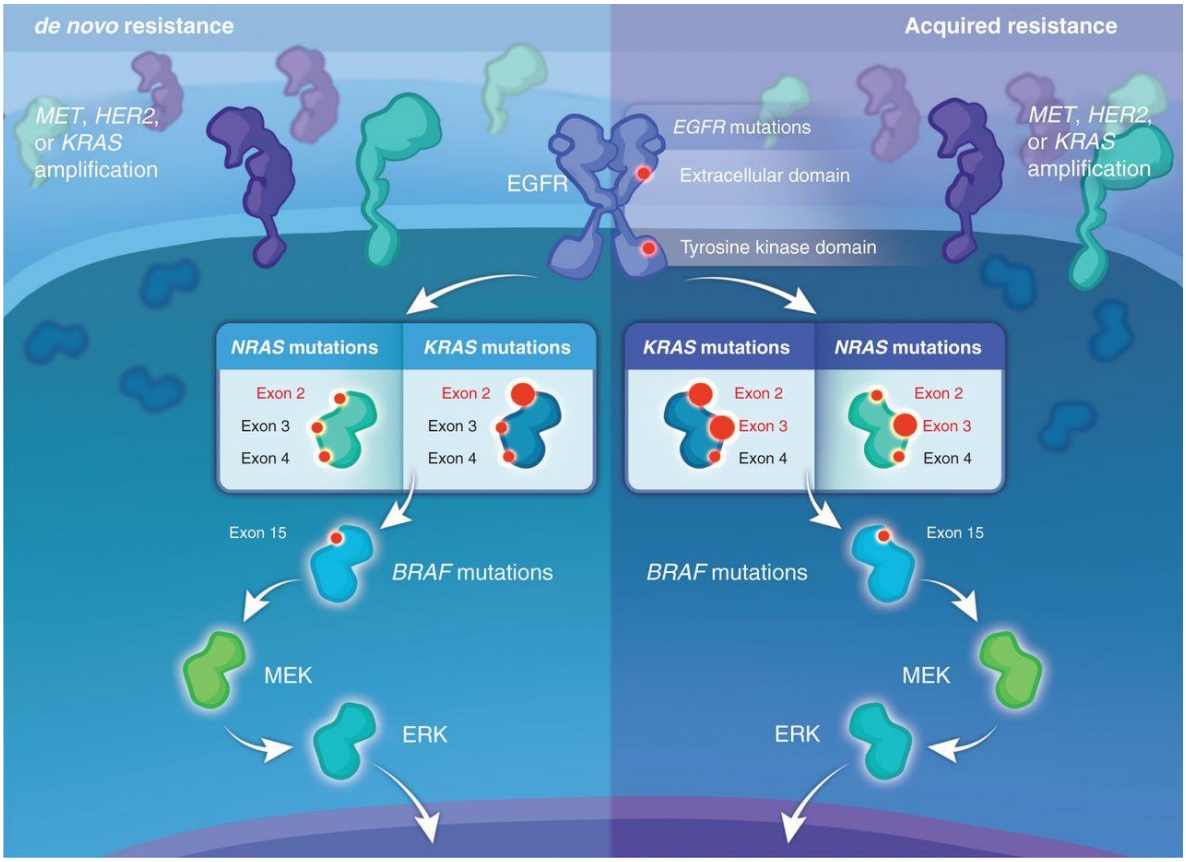

Figure 4. Molecular mechanisms of primary and secondary resistance to anti-EGFR therapies in mCRC. The genetic mechanisms responsible for *de novo* and acquired resistance largely overlap. With the exception of EGFR mutations, which were described only in the acquired setting, all of the genetic alterations defined as a mechanism of de novo resistance are also responsible for acquired resistance. Differences can be found in the frequency of individual genetic alterations, such as *KRAS* and *NRAS* exon 3 mutations, which occur more frequently in the acquired rather than in the *de novo* setting. Text in red highlights the most frequent mutations (12).

2. LIQUID BIOPSIES

2.1. General Information

Liquid biopsies comprise an ensemble of novel, minimally invasive techniques that allow for the analysis of tumour-derived biologic material circulating in the blood or other liquid samples from patients with cancer (the 'tumour circulome' (13)).

They were developed to overcome some of the inherent limitations of tissue biopsy, which however remains the 'gold standard' for cancer diagnosis and molecular testing. Firstly, acquisition of histological samples can often be challenging due to difficulty in accessing some tumours, increased cost and technical complexity. Further to that, the available tissue sample is often archival, dating back many years, and is formalin-fixed and embedded in paraffin. This may affect the abundance and quality of DNA, introducing further error due to DNA degradation and negatively impact on analysis (14). Moreover, repeated tissue biopsy for evaluating genetic changes during the course of the disease is not practical due to its invasive nature. Thus, it only provides a 'snapshot' of the disease in space and time.

The advent of liquid biopsies promises to transform molecular testing in oncology. It is a minimally invasive technique as it is based on a single blood aspiration which is a simple and easy procedure, without the events of infection, haemorrhage, wounds, and complications that accompany invasive biopsies (15). It can therefore be repeated as often as required to allow for serial monitoring of the patient, contrary to tissue biopsy that only depicts the molecular profile of the tumour in the specimen examined (16, 17). Furthermore, it provides real-time access to the genetic information and can capture tumour heterogeneity in a comprehensive manner that reflects all sites of the disease.

Circulating tumour-derived elements include circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), membrane-bound vesicles released by tumour cells called exosomes, tumour-educated platelets (TEPs), each of them providing one or more levels of information (14).

All the above can be isolated for analysis as liquid biopsy biomarkers, the most important of which are illustrated in Fig. 5.

The most commonly used liquid biopsy biomarkers are CTCs and ctDNA and are more extensively discussed in the following sections.

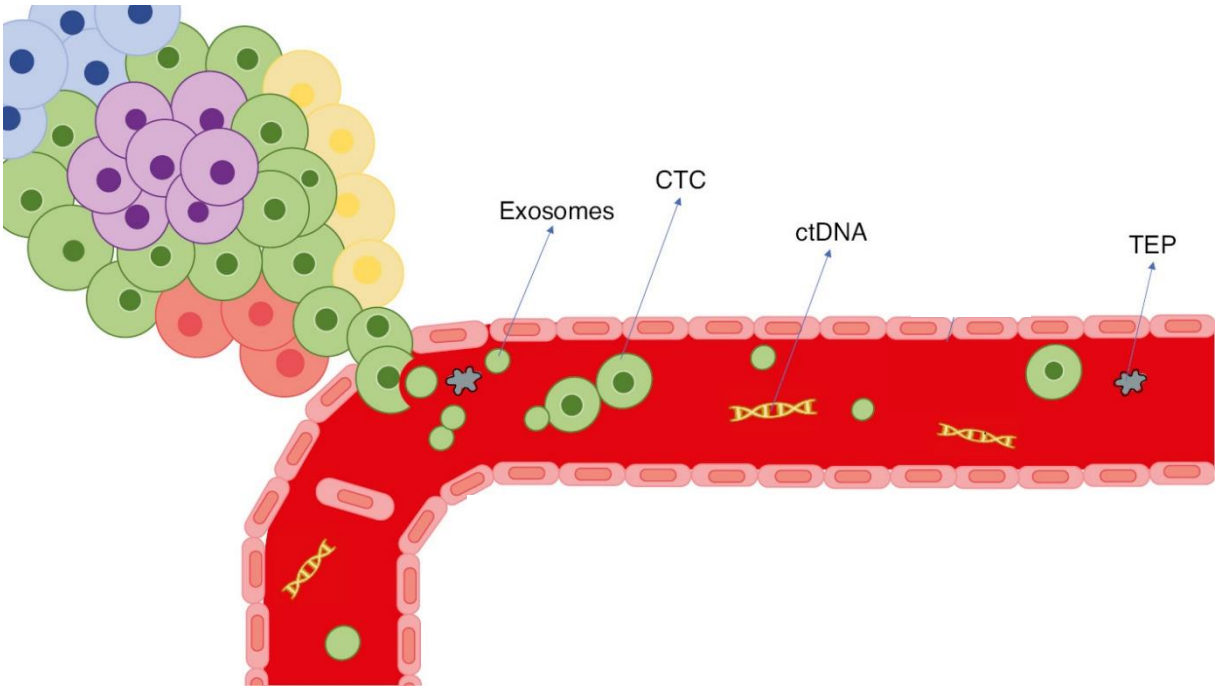

Figure 5. Schematic representation of the origin and different types of liquid biopsy biomarkers. Tumours can grow into a blood vessel, resulting in the release of CTCs, ctDNA, exosomes and TEPs into the peripheral circulation, which can subsequently be isolated for analysis. CTC, circulating tumour cell; ctDNA, circulating tumour DNA; TEP, tumour-educated platelet. Adapted from (14).

2.2. Circulating tumour cells

Circulating tumour cells (CTCs) constitute a rare subset of cells that are isolated from the circulation that are known to have detached from the primary tumour (18). They are thought to be the main contributors to distant metastasis, as they extravasate into the blood from the primary tumour. Also, the detection of CTC in the circulation has been associated with poor prognosis in a wide array of cancers.

However, study of CTCs is complicated by their scarcity in the bloodstream in a high background of hematopoietic cells, as even in the blood of patients with metastatic

cancers, their detection rate is 1–10 CTC per mL of whole blood (19). Their isolation and characterisation are based on immunohistochemistry and other physical or biological properties, and as CTCs are highly heterogeneous, this further complicates their detection, isolation, enumeration, expansion and molecular characterisation (20).

To overcome the scarcity of CTC, enrichment methods based on leucocyte depletion or selection of epithelial cells have proven useful (18, 19). Moreover, recent advances in CTC technologies led to the emergence of various platforms to increase yield and tumour characterization efficacy (21). However, the CTC enumeration system CellSearch still remains the first and only clinically validated FDA-cleared system for CTC identification, enumeration and analysis.

2.3. Cell-free DNA

Cell-free DNA refers to extracellular DNA molecules (double-stranded DNA and mitochondrial DNA) (22) originating from any cell type in bodily fluids of diseased and healthy individuals. It was first detected in the blood of diseased and healthy individuals in 1948 (23), although without attracting much attention at the time.

2.3.1. Origins of cfDNA

Shedding of nucleic acids into the circulation is an active process mainly associated with cell death and more specifically with the lysis of apoptotic or necrotic primary tumour cells, metastatic cells, CTCs, as well as normal blood cells and stromal cells (Fig. 6) (18, 24).

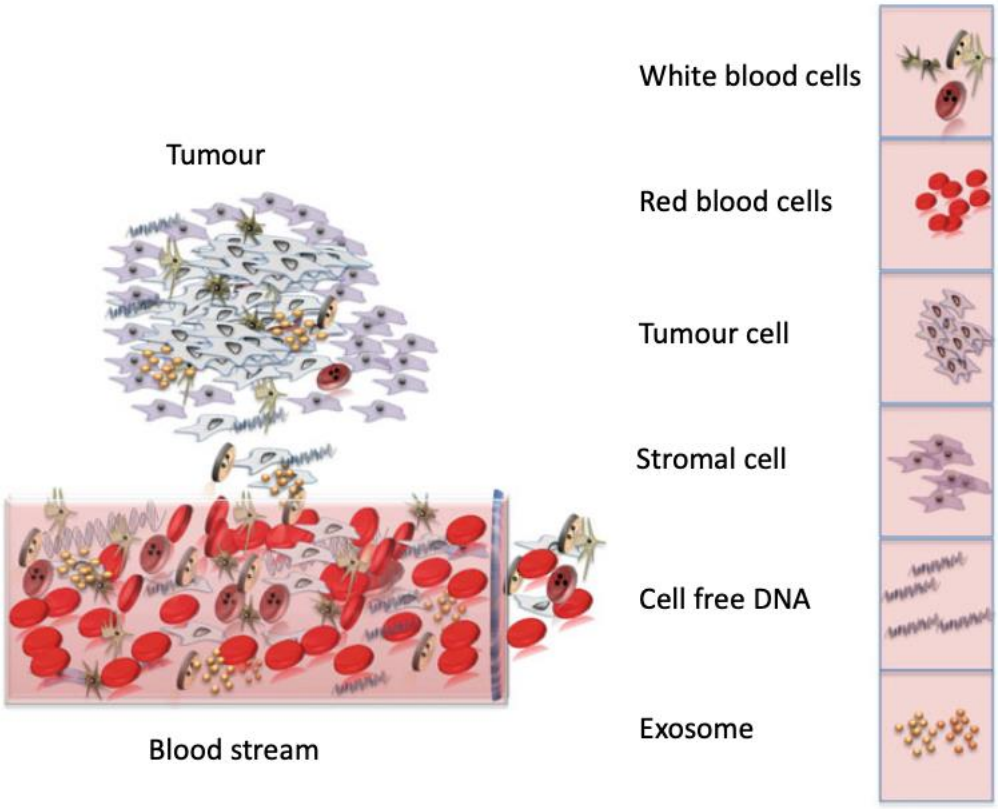

Figure 6. Schematic of circulating elements in the bloodstream. The bloodstream contains different types of cells, such as white and red blood cells, tumour and stromal cells, as well as other circulating products. Cell-free DNA and exosomes are non-cellular circulating products that are actively released from the lysed apoptotic and necrotic cells into the bloodstream (24).

The origin of cfDNA from cell death is supported by many of its properties. Firstly, it is double-stranded and characterized by high fragmentation (25). Additionally, cfDNA fragments are usually approximately 167 base pairs long, which corresponds to the length of the DNA wrapped around a nucleosome plus its linker, with fainter signals in multiples of this length (25). This suggests that caspase-dependent cleavage during apoptosis, which occurs at the internucleosomal linker region, is an important contributor to the generation of cfDNA (22).

However, these fragment sizes represent DNA derived from non-cancerous cells, which corresponds to the majority of cfDNA (25). In contrast, fragments derived from tumour cells are generally shorter and, although the underlying mechanism remains unclear, the difference in size is useful to differentiate cfDNA origins (25).

Many diseases including cancer are linked to detectable cfDNA in the blood (26). Although the amount of cfDNA in patients with cancer is generally higher than in healthy individuals,

there is considerable variation among individuals. Diverse parameters account for this, including biological differences in patients and their tumours, as well as technical differences in the methods used to detect cfDNA (22). Although it is accepted that cfDNA is generally more abundant in more advanced stages of cancer, the degree of cfDNA shedding can vary significantly in different cancer types (27).

2.3.2. Kinetics of cfDNA

With respect to the release rate of cfDNA into the circulation, it can vary depending on tumour location, tumour burden, cancer stage, tumour size and vascularity, cellular turnover, and response to therapy, while its levels are known to range from 0.01% to 90% (28).

Another property of cfDNA is its short half-life. Although the mechanisms of clearance are not fully understood, it is well established that its half-life ranges from 16 min to 2.5 hours (28). This has very important implications in its clinical applications, meaning that it reflects the current status of cell turnover in a given timepoint when the sampling is performed (29).

2.3.3. Circulating tumour DNA (ctDNA)

Cancer cells are known to release genetic material in the blood circulation that can be isolated from patients' plasma and allow for identification of the neoplastic molecular signature (15, 30). Ranging from 0.005–85%, ctDNA typically constitutes <1% in limited amounts of cell-free DNA (cfDNA) in the blood and its detection can often be very challenging (30, 31).

In addition to the varying amounts of cfDNA, the fraction of DNA molecules that derive from tumour cells also varies. This is known as circulating tumour DNA (ctDNA) and originates mainly from apoptotic or necrotic tumour cells, living tumour cells and circulating tumour cells. It has an array of biological characteristics that distinguish it from its normal cell derived counterpart. Most studies agree that ctDNA is more fragmented and is thus shorter than normal cell-derived cfDNA, which can be exploited analytically to refine its detection and characterisation (25).

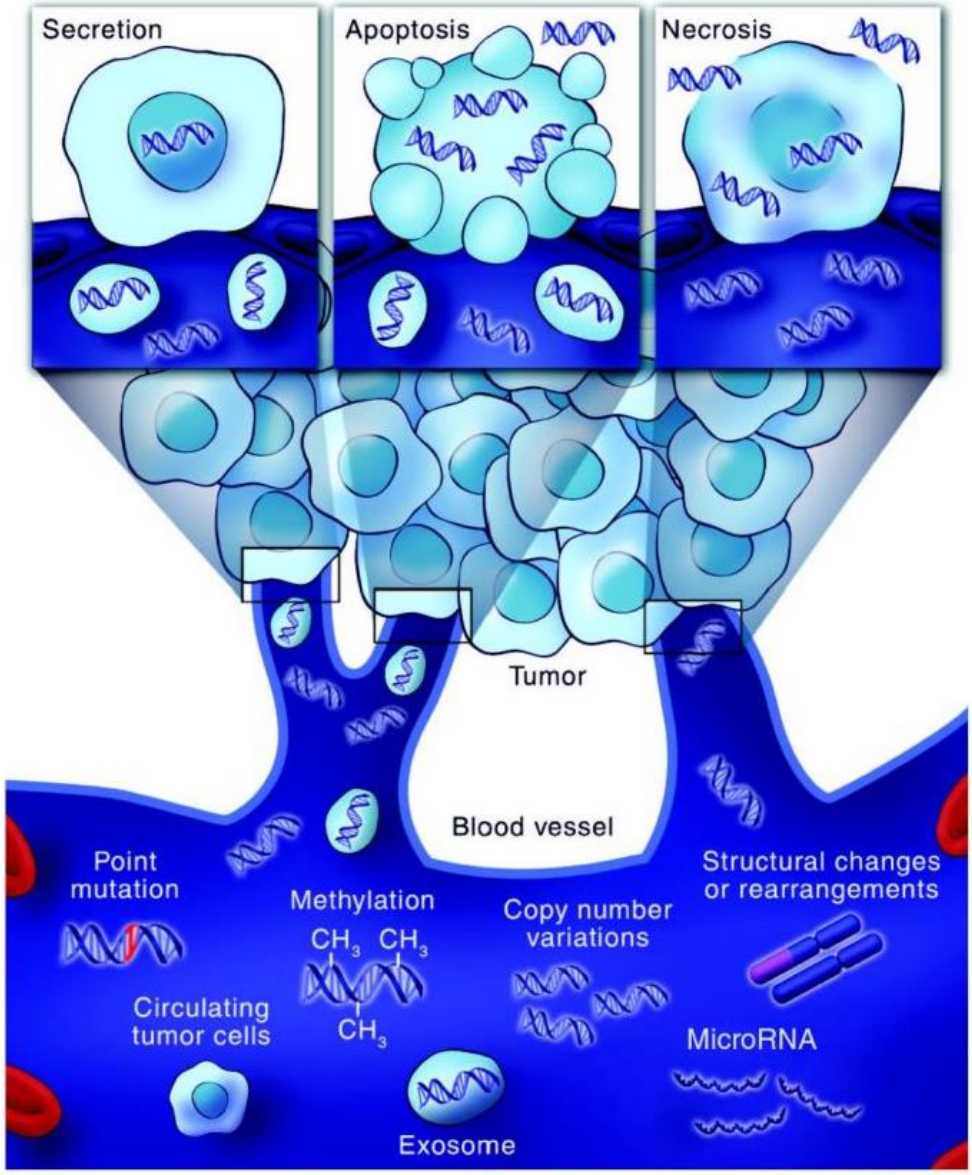

Figure 7. Genetic alterations detectable in circulating cell-free tumour DNA. Tumour cells release small fragments of cell-free DNA into circulation by multiple mechanisms. Cancer-associated genetic alterations such as point mutations, copy number variations, chromosomal rearrangements, and methylation patterns can be detected in circulating cell-free DNA (15).

Tumour-associated genetic aberrations can be detected in cfDNA extracted from the plasma of patients with cancer using one of several different techniques. To distinguish ctDNA from background normal cell cfDNA, high-sensitivity techniques have been developed that are able to detect 1 mutant allele in 10,000 wild-type ones.

Among them, digital polymerase chain reaction (PCR) and modified next-generation sequencing (NGS) with unique molecular identifiers (UMI) have gained ground in Oncology. The main methods of ctDNA detection are summarised in Fig. 8.

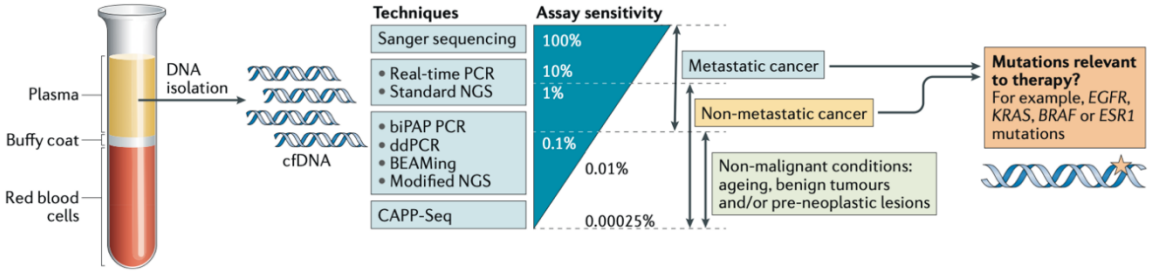

Figure 8. Methods of ctDNA detection and assay sensitivity considerations. Several different techniques can be used to detect cell-free DNA (cfDNA) isolated from the plasma of patients with cancer. The scheme depicts the assay sensitivities of some of the main technologies used for the detection of circulating tumour DNA (ctDNA) in correlation with the typical range of ctDNA concentrations at different stages of tumour development and progression, as well as in individuals without cancer. BEAMing, beads–emulsion–amplification–magnetics; biPAP, bidirectional pyrophosphorolysis-activated polymerisation; CAPP-Seq, cancer personalised profiling by deep sequencing; ddPCR, droplet digital PCR; NGS, next-generation sequencing (18).

2.4. Preanalytical considerations

It is well established that the quality of cfDNA analysis can be significantly affected by steps in the preanalytical workflow, while divergence in handling practices across laboratories hampers reproducibility of research data in the field (32). For this reason, the National Cancer Institute (NCI) Biorepositories and Biospecimen Research Branch Evidence-Based Practices (BEBP) issued recommendations for the harmonization of cfDNA collection and processing practices, summarized in Table 2 at the end of this section (32).

In terms of the optimal source for cfDNA isolation, plasma is better than serum, as the latter contains higher amounts of genomic DNA from blood cells, mainly leukocytes, lysed with blood clotting during separation of serum from whole blood (33). Given that ctDNA only represents a small fraction of cfDNA, this can negatively affect ctDNA detection, especially that with low-frequency genomic alterations (34).

Regarding blood collection tubes, di-potassium or tri-potassium ethylenediaminetetraacetic acid (EDTA) tubes are preferable to those containing heparin or citrate as anticoagulant as

they prevent cell lysis (34). Currently, there are at least nine types of commercially available collection tubes, specifically designed for the preservation of cell-free nucleic acids that shipment and storage of whole blood samples in room temperature, for plasma isolation after 3–7 days. However, to preserve cfDNA concentration and integrity, NCI guidelines recommend room temperature and shorter storage times prior to plasma processing, i.e., 2–4 h and up to 3 days for EDTA and preservative tubes, respectively.

A two-step centrifugation protocol is recommended. The first one at 800–1,600 g separates plasma from cellular components, while a second high-speed step at 14,000–16,000 g removes any remaining cellular material (35). Optimal storage temperatures are −80 °C or colder (32). Plasma cfDNA is sensitive to temperature variations, with more than three freeze-thaw cycles increasing cfDNA fragmentation (36). It is therefore recommended to aliquot plasma in small vials before freezing and ideally avoid repeat freeze-thaw cycles (32).

Additional considerations to take into account regarding long-term storage of samples. Prolonged storage time decreases cfDNA content, it should not exceed three months for quantification or fragmentation studies (37). However, longer times were not found to affect detection of specific sequences or aberrations in cfDNA that can be detected in samples stored for several years, although this may compromise detection of sequences present in smaller quantities.

Table 2. Recommendations for optimal specimen handling for cfDNA analysis. Adapted from (32).

Parameter	Recommendation
Collection tube	EDTA tubes when immediate processing is possible cfDNA stabilizing tubes when processing delays are unavoidable
Pre-centrifugation processing delay	≤ 2 h at room temperature or on ice (EDTA tube) ≤ 3 days at room temperature (stabilizing tube)
Tube agitation	Minimize (after initial inversions)
Centrifugation	Two centrifugation steps • 800–1,600 g for 20 min • 14,000–16,000 g for 10–20 min
Plasma storage	≤ –80°C, with ≤1 freeze-thaw event
cfDNA storage	≤ – 20°C, with ≤1 freeze-thaw event
Quantification	Real-time or digital PCR

2.5. Applications of ctDNA

The detection of ctDNA has emerged as a promising alternative to tissue molecular profiling, and is recognized as the liquid biopsy biomarker with the most clinical applications (38).

2.5.1. Screening and early diagnosis

In early cancer stages, ctDNA holds promise as a powerful screening tool before cancer is clinically detectable (39). This would enable early diagnosis and timely therapeutic interventions with potential to decrease cancer morbidity and mortality (39).

2.5.2. Treatment selection and prognosis

Following diagnosis of cancer, ctDNA analysis enables molecular profiling of the tumour in a minimally invasive way for personalized treatment selection. As previously mentioned, owing to the short half-life of ctDNA, it can serve as a reliable "proxy" of the molecular make-up of all tumour sites in real time, as long as they shed ctDNA in the bloodstream (40, 41).

Resistant subclones within any known or occult disease site can be captured as long as they shed ctDNA into the bloodstream even in very small quantities. Thus, serial ctDNA sampling can inform therapeutic decisions, allowing to adjust treatment based on tumour evolution. To date, ctDNA testing for mutations to the epidermal growth factor receptor (EGFR) gene have been approved by the EMA and FDA to guide treatment in advanced non-small cell lung cancer (42).

2.5.3. Tracking residual disease and risk of relapse

In the absence of metastatic disease, it remains unclear which patients will benefit from adjuvant therapy following successful surgical resection of a tumour. Analysis of ctDNA is also being extensively investigated in this scenario. Owing to its short half-life, absence of ctDNA in plasma samples taken after surgery may point out when the patient is free of

disease and will not derive any benefit from adjuvant therapy. Thus, ctDNA sampling after surgery can be useful to decide when it is safe to avoid adjuvant therapy, thus avoiding over-treatment.

2.5.4. Advanced setting

Mutation acquisition is a hallmark of cancer which accounts for its molecular evolution in space and time. During the course of the disease, new subclones can emerge as a result of selective pressure from targeted therapies. This is highly relevant in advanced cancer settings, where ctDNA is a useful tool to reassess the molecular profile of the tumour and decide upon the right therapeutic strategy, as well as to monitor response to the administered treatment and assess prognosis. A summary of the various clinical applications is depicted in Fig. 9 and their role in colorectal cancer management is covered in the next section.

Ongoing clinical studies utilize the detection of ctDNA in blood circulation as a liquid biopsy method to guide the administration of targeted agents according to the mutations identified in relapsing CRC patients (43). Furthermore, ctDNA can be detected in bodily fluids other than blood, such as urine, cerebrospinal fluid, and pleural effusion (44). The detection of ctDNA in urine samples from CRC patients using NGS has been associated with tumour load, while the comparison between tumour tissue and urine mutant *KRAS* was highly concordant (45).

More robust results are still needed for the integration of ctDNA as a clinical biomarker in optimal therapy selection, emergence of resistance, and molecular classification. However, already published data advocate that it will fulfil its promise in the near future (46). On the other hand, the application of ctDNA for CRC diagnosis remains elusive, as it cannot bypass the sensitivity and specificity issues of traditional biomarkers (42).

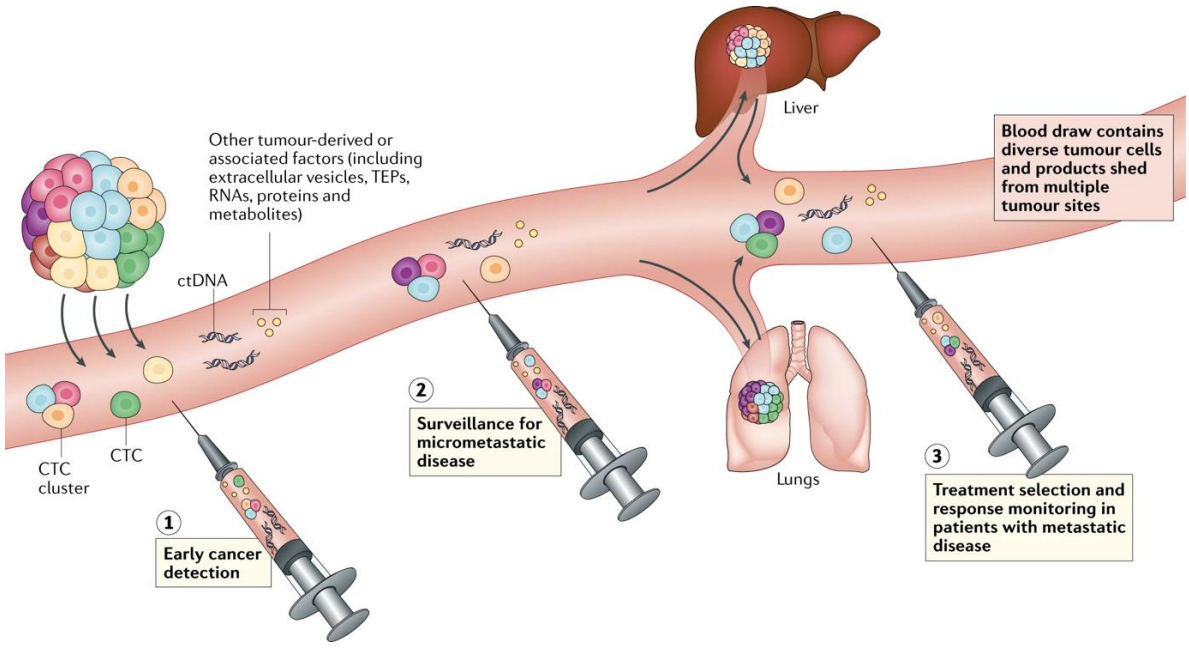

Figure 9. Various clinical applications of liquid biopsy using CTCs, circulating nucleic acids or other tumour-derived materials in the bloodstream. A single blood sample can contain a range of cell types and cell products emanating from multiple tumour sites around the body. Analysis of these tumour-derived factors with liquid biopsy has several applications in cancer management. (1) Early detection; liquid biopsy approaches could also be used to further investigate abnormalities detected on imaging examinations such as mammography or lung CT. (2) Surveillance for micrometastatic disease following curative-intent treatment of a primary tumour, to evaluate the risk of disease recurrence and enable timely management of recurrent disease, if needed. (3) Guiding the selection of the most appropriate treatment and/or monitoring treatment responses in patients with overt metastatic disease through dynamic characterisation of changes in tumour burden and disease biology. CTC, circulating tumour cell; ctDNA, circulating tumour DNA; TEPs, tumour-educated platelets (34).

2.5.5. Role of ctDNA in metastatic colorectal cancer

Although ctDNA may not be homogeneously shed by all sites of cancer throughout the body, it molecularly represents all of them provided that they possess vascular or lymphatic drainage. The fact that ctDNA rise is shown to precede radiological progression in many studies supports its ability to represent even occult niches of cancer cells. This permits the detection of subclones with distinct molecular characteristics that may alter the overall response to treatment. As a result of evolutionary pressure exerted by a specific treatment, such subclones may dominate the tumour and confer resistance to the respective treatment. In another setting, they may represent the tumour's 'Achilles' heel' inferring potential response to a drug that may have substantial impact on the overall prognosis of a patient.

Such subclonal discrepancies are currently being overlooked in mCRC, as are in all types of cancer where liquid biopsies are not yet part of everyday practice. The use of ctDNA to guide treatment in mCRC is being more and more explored in randomised clinical trials. It is of particular value in studies exploring the concept of "anti-EGFR rechallenge" (47). This term refers to re-treatment with anti-EGFR monoclonal antibodies in subsequent lines of treatment of mCRC patients who had responded to these drugs at first line. In this setting, repeat liquid biopsies before and/or during anti-EGFR rechallenge is used to guide therapy (Fig. 10).

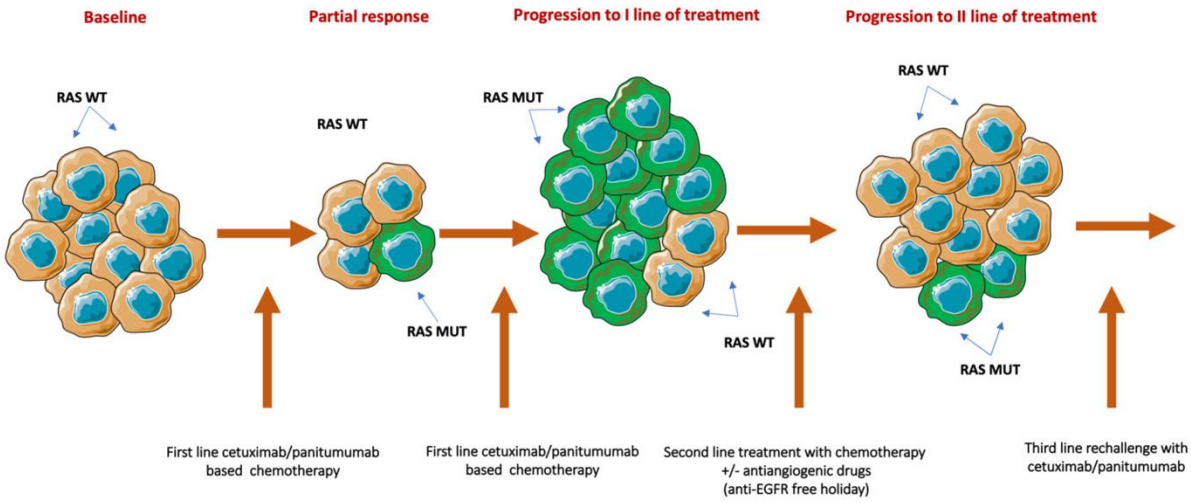

Figure 10. Biological rationale for rechallenge therapy. Treatment with anti-EGFR inhibitors rapidly eliminates *RAS* WT-sensitive clones and favours the expiation of resistant cancer cells. After disease progression, and due to the administration of a second line of chemotherapy without anti-EGFR monoclonal antibodies, *RAS* mutant clones progressively decay, inducing the proliferation of *RAS* WT cells. WT: Wild type; MUT: Mutant; /: Or. (47)

The concept of rechallenge had been explored in a study by Santini *et al (48).* Its results were published in 2012 and reported the clinical outcome of cetuximab plus irinotecan as a third-line treatment in 39 patients with *KRAS* exon 2 wild type mCRC (48). The study by Santini *et al.* was based on the notion that *KRAS* mutation emerge are stable and do not emerge secondarily in mCRC and thus did not attempt to retest *KRAS* status at rechallenge. In 2019, proof-of-concept study CRICKET was the first to perform liquid biopsies on mCRC patients being rechallenged with cetuximab plus irinotecan at third line (49). Even though *RAS* wild type status could not a prerequisite for enrolment in the study, post hoc analysis

of the RAS status with liquid biopsy showed that all patients who responded to rechallenge cetuximab plus irinotecan were *RAS* wild type at baseline, which paved the way for ctDNA *RAS* testing as a clinically relevant tool for treatment strategy design. At present, there are randomised clinical trials being conducted that focus on ctDNA-guided anti-EGFR rechallenge in mCRC. Results of these studies are likely to establish ctDNA as a biomarker for therapeutic design in this setting.

The value of ctDNA to assess response to first-line treatment has been studied in early-stage disease settings and to a lesser extent in mCRC. A study of 24 mCRC patients who underwent weekly ctDNA measurements during first-line treatment with FOLFIRI demonstrated a shorter PFS in those patients whose samples exhibited multiple increases in ctDNA, thus stressing out "the importance of monitoring ctDNA levels as early as one week after treatment onset to enable early detection of treatment failure" (50). In another study which assessed the role of ctDNA detection as a predictive biomarker for resectability of liver metastases in mCRC, detectable *KRAS* mutated ctDNA after 4 weeks of systemic therapy was associated with a lower R0/R1 resection rate than no detection of ctDNA (36% vs 85%, $p = 0.01$) (51).

Results from serial liquid biopsies in 41 patients who received first-line treatment for mCRC suggested that changes in ctDNA levels were able to distinguish progressing patients approximately four weeks earlier than changes in CEA and CA 19-9 levels (52).

Despite the advances in the field, high sensitivity ctDNA profiling in mCRC is currently limited in the context of clinical trials. The current study aims to evaluate the feasibility and accuracy of repeat liquid biopsies in a real-world setting of a University Hospital in Greece. The use of a high-sensitivity digital PCR for the detection of *RAS* mutations in the plasma of patients with mCRC permits real-time molecular profiling across treatment lines with an aim to monitor their response to first line therapy as well as to identify molecular subgroups of patients who may benefit from "rechallenge" therapy with anti-EGFR.

RAS status was used as it is one of the most important and well-established predictive biomarkers in mCRC. Around 50% of mCRC patients harbour *RAS* mutations which lead to insensitivity to the therapeutic blockade of the EGFR with monoclonal antibodies, in

contrast with patients with *RAS* wild-type tumours, who respond to these agents. However, the *RAS* status is dynamic and can change over the course of the disease due to clonal evolution of cancer. For example, patients with initially *RAS* wild-type tumours who receive anti-EGFR may develop *RAS* mutations. This may happen during treatment, leading to resistance to the administered treatment, or after completion of treatment, leading to disease progression. On the other hand, patients initially diagnosed with *RAS* mutated tumours may become wild type, which renders them more likely to benefit from anti-EGFR.

Currently, we are not able to capture these changes, as *RAS* testing is performed on tissue samples. For patients with mCRC, the only timepoint of tissue acquisition is CRC diagnosis, which may be long before they developed metastases. Upon relapse, *RAS* testing is thus performed on archival tissue, as sites of relapse may be inaccessible or technically difficult for re-biopsy. Therefore, our decision for treatment is guided by *RAS* status of the initial biopsy without knowing whether this is still accurate, which may have a tremendous impact on treatment response, disease progression and survival.

In this setting, the use of liquid biopsies is very promising for the timely detection of *RAS* status changes in a minimally invasive way. Repeat testing of ctDNA with a high-sensitivity technique such as BEAMing Digital PCR can allow to effectively assess the current *RAS* status of the patient, compare it with the initial one and possibly guide the customisation of therapeutic strategies in mCRC.

PART II

1. AIMS AND OBJECTIVES OF THE DOCTORAL DISSERTATION

The goal of this doctoral dissertation is to study the utility of repeat liquid biopsies, alongside standard tissue testing, for the customisation of therapeutic strategy in mCRC.

More specifically, we used the BEAMing Digital PCR platform to detect *RAS* mutations in ctDNA in plasma of mCRC patients at multiple timepoints.

The doctoral dissertation has the following aims:

A. To define the prevalence of *RAS* mutations in the plasma at baseline, first and second disease progressions with BEAMing Digital PCR.

B. To study the concordance of *RAS* status with plasma BEAMing Digital PCR and tissue methods used for clinical decision making.

C. To assess plasma *RAS* mutant allele fraction (MAF) as a predictive biomarker in the first line of treatment.

D. To define change in *RAS* status across treatment lines.

The objectives were to define:

a. The frequency of the *RAS* mutations in the plasma at baseline, first and second disease progressions with BEAMing Digital PCR.

b. Overall percent agreement (OPA) between plasma and tissue *RAS* status.

c. Percentages of patients whose *RAS* MAF increases or decreases from baseline to mid first-line treatment and association with radiological response as per RECIST 1.1 and with hazard ratio.

d. Percentages of patients whose *RAS* status changed from wild-type to mutated and from mutated to wild-type during the course of the disease.

2. MATERIALS AND METHODS

2.1. Setting

The study was led and co-ordinated by the University Hospital of Ioannina with the collaboration of the University Hospital of Larissa and the EUROMEDICA General Clinic in Thessaloniki, Greece. Blood samples were collected from patients with mCRC followed up in the oncology outpatient department of all three hospitals, who fulfilled the inclusion criteria, as described in *Section 2.2*.

The study held the approval of the Ethics Committee of the University Hospital of Ioannina (11/18-04-2018) and of the respective committees of the collaborating centres as per their internal regulations.

Blood sample analysis was performed at the Liquid Biopsy Unit of the Laboratory of Clinical Chemistry of the University Hospital of Ioannina. Samples from the University Hospital of Ioannina were immediately processed, while external samples from the two collaborating centres were shipped in appropriate conditions to the University Hospital of Ioannina for further processing.

2.2. Patient inclusion criteria

Patients with a diagnosis of metastatic colorectal cancer were enrolled in the study after acquisition of written informed consent. Inclusion criteria were as follows:

- Histologically confirmed adenocarcinoma of the colon and/or the rectum.
- Clinically/radiologically confirmed metastatic disease.
- Signed informed consent from the patient stating that they are willing and able to fully adhere to the observation protocol's requirements, including regular follow-up visits and blood sampling as per protocol.
- Available formalin-fixed, paraffin-embedded (FFPE) tumour tissue sample from the primary tumour or a metastasis.

- Patients with sufficient hepatic, renal and haematological reserves who the treating physician evaluates as fit and eligible for bio-chemotherapy regimens and plasma monitoring.
- ECOG Performance Status 0-2.
- Age ≥18 years.

2.3. Blood sample analysis

All patients enrolled in the study were sampled at diagnosis of metastatic disease, prior to initiation of first line treatment with chemotherapy and/or a targeted agent.

Repeat sampling timepoints include disease progression on first line therapy (first progression of disease, PD1) and disease progression on second line therapy (second progression of disease, PD2). For a subset of patients who were *RAS* mutated plasma samples at diagnosis, an additional sample was collected at approximately 3 months following initiation of first line therapy.

First, samples were processed for plasma separation and ctDNA isolation. Subsequently, ctDNA was tested for the presence of *RAS* mutations by use of BEAMing Digital PCR, which determined the *RAS* status as mutated or non-mutated (wild type). In the case of *RAS* mutation detection, quantification of *RAS* mutant ctDNA was performed and was expressed as mutant allele frequency (MAF), which is fraction of the *RAS* mutant ctDNA in the total *RAS* wild type ctDNA.

In the following sections, a breakdown of the stages required for blood sample analysis is provided according to the University of Ioannina Liquid Biopsy Unit's pre-analytical protocol.

2.3.1. Specimen Collection

Following acquisition of informed consent, two samples of 10 mL each were collected in BD Vacutainer K_2 EDTA blood collection tubes with Lavender Hemogard closure or Streck Cell-Free DNA Blood Collection Tubes (BCT) per patient at each timepoint.

2.3.2. Plasma Preparation

Next, the collected blood samples were processed for plasma preparation with a two-step centrifugation process. As far as the time from blood collection to process is concerned, this was 4 hours for samples in BD Vacutainer tubes and 72 hours for samples in Streck Blood Collection Tubes (BCT).

The first centrifugation was performed at 1,600 g for 10 minutes on a bench-top centrifuge with a swing-bucket rotor and zero deceleration. This was to separate plasma from the cellular components of the blood, such as red and blood cells, and platelets (Table 3A).

Table 3A. First centrifugation step for plasma separation.

Parameters	BD Vacutainer K_2 EDTA / Streck Cell-Free DNA BCT
Rotor type	Fixed angle
Speed (rcf)	1600
Time (min)	10
Deceleration	Maximum

The supernatant from each of the two blood collection tubes was transferred manually with an electrical pipette controller and use of disposable serological pipettes to a clean tube, leaving at least 500 uL of the supernatant to avoid disrupting the cellular layer. Plasma fraction (supernatant) was visually checked to reject if highly haemolytic (reddish). Plasma was transferred from both tubes to one 50 mL centrifuge tube without disturbing the cellular layer. Subsequently, we conducted a second centrifugation at 3,000 g for samples placed in BD Vacutainer tubes and at 6,000 g for samples placed in Streck BCT for 10 min to the pooled supernatant, using the same centrifuge with fixed-angle rotor. This step ensured removal of any remaining cellular component from plasma (Table 3B).

Table 3B. Second centrifugation step for removal of any cellular component.

Parameter	BD Vacutainer K_2 EDTA	Streck Cell-Free DNA BCT
Rotor type	Swing bucket	Swing bucket
Speed (rcf)	3000	6000
Time (min)	10	10
Deceleration	Zero	Zero

We then transferred the supernatant of the second centrifugation to a fresh 15-mL centrifuge tube with an electrical pipette controller and a new disposable serological pipette. At this step, caution was taken not to disrupt the pellet and leave at least 300 uL of plasma at the bottom of the tube and was then disposed to ensure any cellular component was not stored for analysis.

With the electrical pipette controller, we then mixed the sample by gently pipetting up and down ten times. Once homogenised, the next step was to aliquot the 3.5 mL of the sample in cryogenic vials. Plasma cryogenic vials were immediately placed in an upright position into freezer at –80^{o}C for storage for up to 24 months. One aliquot was then thawed for isolation of circulating nucleic acids and further analysis.

An additional step was storage of the buffy coat from the sample following the first centrifugation. This is a concentrated suspension appearing as a thin layer between erythrocytes and plasma and contains most of the leukocytes and platelets. We transferred it with a manual pipette by use of filter tips to fresh cryogenic vials for storage in –80^{o}C for future analysis.

2.3.3. Purification of Circulating DNA from Plasma

The next step was the purification of the circulating DNA. This was performed using the QIAAmp® Circulating Nucleic Acid Kit (Qiagen) according to manufacturer's instructions. The DNA that was eluted was then stored at –20^{o}C up to a maximum of 8 days.

2.3.4. Analysis of ctDNA with BEAMing Digital PCR

Within 8 days from DNA purification, the eluted DNA was analysed for *RAS* mutations using OncoBEAM^{TM} RAS CRC (Sysmex-Inostics), which is a clinically validated assay for *RAS* mutation analysis in ctDNA in Stage IV colorectal cancer patients. The OncoBEAM^{TM} assay is based on the BEAMing Digital PCR technology, which involves beads-based amplification in water-in-oil emulsions and allele-specific hybridisation followed by flow cytometry, and allows the detection of 34 actionable mutations in codons 12, 13, 59, 61, 117 and 146 of the *KRAS* and *NRAS* oncogenes against a background of wild-type genomic DNA. The main steps of BEAMing Digital PCR are as follows:

1. DNA isolation from plasma.
2. Pre-amplification in a multiplex approach to enrich target DNA.
3. Emulsion-based digital PCR in combination with specific amplification for each gene exon on magnetic beads.
4. Mutation detection using allele-specific hybridiation probes that are reverse complementary to the known wild-type and mutant sequences.
5. Differentiation of beads, carrying copies of the mutant or the wild-type alleles is done via flow cytometry analysis.

Fig. 11 provides a simplified overview of the above steps, which are described in more details below.

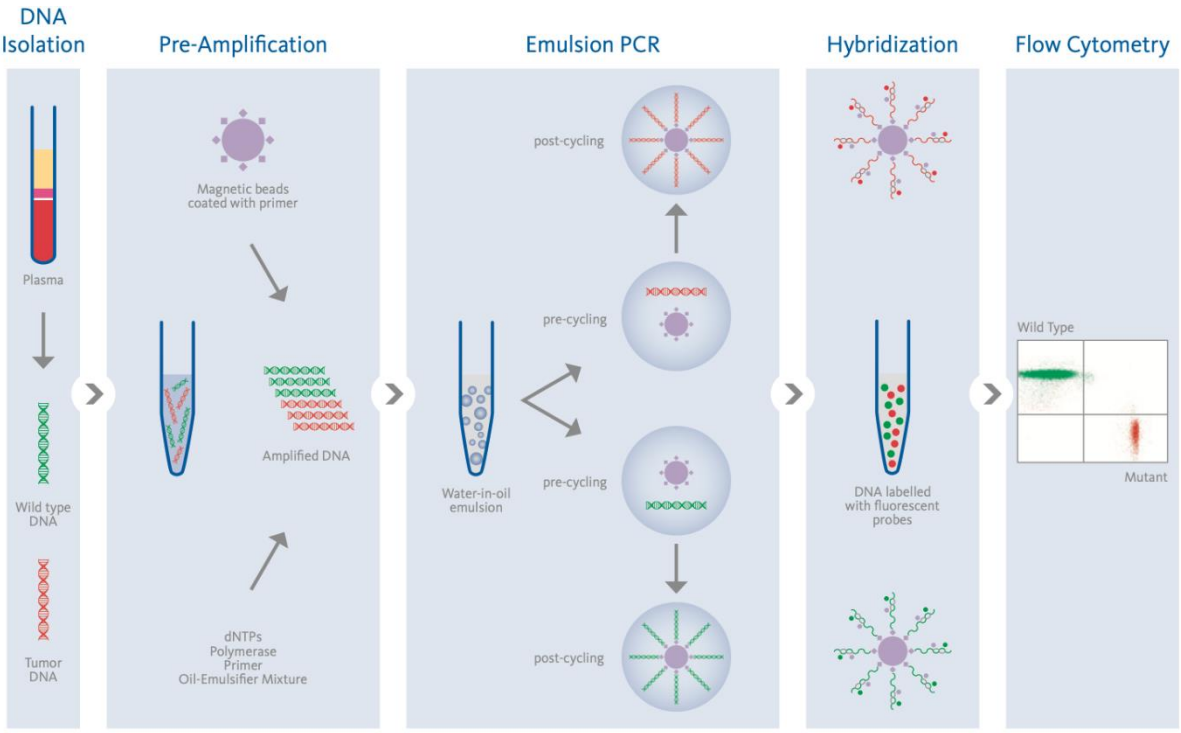

Figure 11. Schematic overview of BEAMing Digital PCR (simplified). BEAMing Digital PCR technology combines emulsion PCR with magnetic beads and flow cytometry for the highly sensitive detection of mutant tumour DNA molecules. The process can be divided into five steps. First, DNA is isolated from plasma which contains both wild-type and tumour DNA fragments. A pre-amplification step follows, where target DNA that contains the sequence of interest is amplified. A primer-coated magnetic beads, dNTPs, polymerase, primers and an oil emulsifier are added in the mixture, allowing for the creation of a water-in-oil emulsion with multiple compartments, each one containing a primer-coated magnetic bead and a DNA fragment (pre-cycling) which multiplies following the emulsion PCR cycles (post-cycling). Next, amplified DNA on the magnetic beads is labelled with fluorescent probes in a hybridisation step. Lastly, flow cytometry is used to separate wild type from tumour DNA. PCR, polymerase chain reaction; dNTPs, deoxynucleoside triphosphates (53).

Step 1: Multiplex PCR Pre-Amplification

DNA eluates are subjected to Multiplex PCR in 6 replicates using the Multiplex Primer Mix, the Multiplex Master Mix and Multiplex PCR DNA Polymerase which are provided with the OncoBEAM^{TM} RAS CRC Kit for use on a ThermoFisher's Veriti Dx thermal cycler instrument. Multiplex PCR is performed to co-amplify the regions of interest under high-fidelity PCR conditions.

Primers target a region within *KRAS* Exon 2, a region within *KRAS* Exon 3, two regions within *KRAS* Exon 4, one region within *NRAS* Exon 2, one region within *NRAS* Exon 3, and a region within *NRAS* Exon 4. Non-allele specific primers bind within these regions and are thereby ensuring unbiased amplification of wildtype as well as mutant molecules.

Step 2: Emulsion PCR Amplification

After multiplex PCR, a pooling and dilution step is performed, where the 6 replicate PCR reactions from each eluate are manually pooled in a new PCR plate and diluted with specific fixed dilution factors defined in the IFU. Dilution is done manually using 1x TE Buffer, low EDTA concentration (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Diluted Multiplex PCR product is used as input to the Emulsion PCR, which requires the EmulsiFIRE, Emulsion Beads, the Emulsion DNA Polymerase and the specific Emulsion PCR Mastermixes which are provided with the OncoBEAM™ RAS CRC Kit, for use on ThermoFisher's Veriti Dx thermal cycler instrument.

Preparation of the emulsion requires use of an electronic pipette properly programmed resulting in formation of water in oil emulsion with an optimized number of water compartments. Emulsion PCRs are exon specific with exon specific primer pairs. These emulsion PCR primers bind outside the targeted sites, thereby ensuring amplification of each of the 7 exons of the multiplex PCR from wild-type and mutant molecules. Amplicons, which harbour 2 codons are prepared in 2 parallel reactions.

A water-in-oil emulsion is created with each aqueous microdroplet containing an individual fragment of DNA. This allows millions of compartmentalized PCRs to be performed in parallel in one single test tube resulting in beads coated with thousands of copies of DNA that are identical to the original template DNA molecule, either mutant or wildtype.

Step 3: Breaking and Hybridisation

After emulsion PCR, emulsions are broken releasing magnetic beads coated with amplified PCR products which are then recovered using a magnet plate. Bead-bound PCR products are denatured, leaving only 1 DNA strand bound to the beads. Beads with single stranded

DNA are purified with a magnet plate. Breaking and DNA denaturation is achieved using Breaking Buffers 1 and 2, which are provided with the OncoBEAM™ RAS CRC Kit, and requires use of an electronic pipette properly programmed according to specifications in this IFU. The allele- specific hybridisation step is performed using codon-specific probe mixes and Hybridisation Buffer, which are provided with the OncoBEAM™ RAS CRC Kit, and electronic pipette properly programmed according to specifications in this IFU. Probe hybridisation is achieved after completing hybridisation program using ThermoFisher's Veriti Dx thermal cycler instrument.

Each of the 12 emulsion PCR products is hybridised with a mixture of 3 different fluorescent-labelled oligonucleotide probe types (1 distinct probe mixture for each codon to be detected). To distinguish mutant from wild-type coated beads, allele-specific fluorescent probes complementary to the known wild-type and mutant sequences are simultaneously added to the beads for hybridisation. One of the oligonucleotide probes, the "universal probe" binds to the target amplicon outside of the target region and is used to distinguish between beads that contain PCR products and beads without PCR products. The two other oligonucleotide probes are wild-type and mutant specific probes that target the respective allele and label the beads according to their mutational status. Each of the three probe types within each probe mix is labelled with a specific fluorescent reporter molecule.

For each of the 12 codons, all probes detecting target mutations are provided in the same mixture, together with the respective wild-type probe and the universal probe.

After hybridisation, magnetic beads are washed and re-suspended with PBS using an electronic pipette properly programmed according to specifications in this IFU, resulting in an acceptable concentration for flow cytometry readout.

Step 4: Flow Cytometry

The beads are detected using a flow cytometry instrument, a detection platform that measures fluorescent signals. Forward and side scatter are used to differentiate between single beads and clusters of multiple beads and gating is used to select the single bead

population. Single beads are then analysed for the fluorescent dye indicating the presence of PCR products (universal signal) and gating is used to select single beads containing PCR amplified products. Single beads containing PCR amplified products are then subjected to the analysis of the two fluorescent dyes indicating the presence of beads with wild-type, mutant, or both (mixed) PCR products. Fixed instrument settings are defined in a template. The fluorescent intensities of the 3 dyes are recorded for each bead and stored in a .FCS file for each measurement.

Analysis of the flow cytometry data fcs-file is performed using FCS Express V5.0. For analysis, raw data are loaded into a template with fixed analysis settings.

Generated PDF summary sheets contain analysis template specific information about measurement validity (sample or control “Valid” or “Invalid”) and for samples also information about mutation status (“Mutation Detected” or “No Mutation Detected”). The absolute number of mutant beads has to exceed a minimum level, in order to ensure that the measurement is within an acceptable range. Measurement validity criteria include internal control for evaluation of adequate sample DNA concentration. External control measurements confirm the validity of test results. Results are reported for each valid sample (i.e., for a sample with 12 valid measurements) cumulatively as “Mutation Detected” in case one or more of the 12 measurements resulted in a positive result or as “No Mutation Detected” for samples for which all 12 measurements were found to be negative.

2.3.5. Quality Control

Two types of external BEAMing Digital PCR controls are provided with the kit - positive control(s) = PC and no template control = NTC.

The positive controls are specific for each exon of the *KRAS* and *NRAS* genes, thus 7 different positive controls (low level synthetic mutant DNA representing each of the target regions in a wild-type background) are included in each plate run. Tris EDTA Buffer without target DNA is used as a no template control and included in each plate run. All controls are run in parallel with all workflow steps from DNA amplification to result interpretation. Each codon test is valid if the PC and NTC are valid.

An internal control measurement is included into each sample and external control measurement as a full process control.

For this purpose, the confirmation of the presence of the target amplicon within the human genome at an acceptable level is used, i.e., a region within each of the 7 target amplicons is detected from the DNA of the sample or control. This region is outside of the region interrogated by the mutation specific probe or the wild-type specific probe.

2.2.6. Assay Limitations

The assay cannot differentiate between somatic and germline mutations without the analysis of matched normal cell. False negative and false positive results may occur for the following reasons:

i. Incorrect handling of blood samples or plasma samples (e.g., prolonged storage)

ii. Rare polymorphisms within the region of interest

iii. Heterogeneity of specimen

iv. High mutant samples might cause false positive results in neighbouring samples. Use caution during workflow procedure and reanalyse test results for confirmation in case of doubt.

The assay is not recommended for use for patients undergoing therapy, as low tumour burden may exist which may yield inaccurate results. A “No Mutation Detected” result does not preclude presence of a *RAS* mutation in patients harbouring exclusively non-liver metastases. This is especially true for patients with lung as the only site of metastases. BEAMing Digital PCR technology is a highly sensitive detection procedure that uses polymerase chain reaction (PCR), therefore use caution to avoid contamination of samples and reaction mixes by external sources of DNA and/or PCR product in the test environment and the DNA in the positive control.

Other mutations within the gene of interest or other genes are not analysed with this assay.

2.3.7. Specific Performance Characteristics

Limit of Detection (LoD)

DNA extracted from plasma of healthy donors was adjusted with wild-type genomic DNA to generate background DNA of 3 different levels: low DNA amount (5,000 GE/sample), medium DNA amount (23,000 GE/sample) and high DNA amount (116,000 GE/sample). Copies of synthetic double strand DNA for *KRAS* and *NRAS* target analyte were spiked into each DNA background in serial dilutions. Eight (8) replicates of each panel member were prepared and run using 1 lot of the OncoBEAM^{TM} RAS CRC Kit. The LoD was calculated for each tested target mutation from a probit regression model as the analyte concentration at which, with a predefined probability of 95%, measurement results yield a "Mutation Detected" result.

Claimed LoD in a medium background DNA concentration was confirmed by detecting 20 replicates with a probability of 95% (including confidence interval) of the sample measurements as "Mutation Detected" for 7 representative target analytes (KRAS 2 cd12 g35t, KRAS 3 cd61 a183c, KRAS 4A cd117 a351c, KRAS 4B cd146 g436a and NRAS 2 cd12 g35a, NRAS 3 cd61 c181a, NRAS 4 cd117 g351t). For all 34 target mutations 3-fold LoD was verified by detecting at least 5 replicates of 3-fold LoD samples for each target analyte in a medium DNA background level with a probability of 100% of the sample measurements as "Mutation Detected".

Clinical Cut-off

The clinical cut-off was transferred from tissue to plasma by testing 99 clinical samples of known tissue *RAS* status with plasma BEAMing Digital PCR.

A clinical cut-off (mutant bead count) was set for each codon in a way to maximize the number of true results. Samples above the cut-off with a defined specific mutation signal are determined as "Mutation Detected".

Samples below the cut-off or above the cut-off but with an unspecific signal are determined as "No Mutation Detected".

Diagnostic Accuracy

A study was conducted to demonstrate the ability of the OncoBEAM™ RAS CRC assay to correctly discriminate *RAS* mutation positive and *RAS* mutation negative clinical plasma samples as compared to results obtained with reference methods for tissue sample testing. The *RAS* status of specimens from mCRC patients was determined using the following reference methods on FFPE tumour tissue sections: allele-specific PCR and sequencing (Sanger sequencing, pyrosequencing, and next generation sequencing). Reference method testing was conducted with CE-IVD approved kits and in-house validated methods.

Agreement analysis between the OncoBEAM™ RAS CRC assay and the reference methods for detection of *RAS* mutations in a total of 236 samples from mCRC patients. The diagnostic accuracy of OncoBEAM™ RAS CRC assay with reference methods was evaluated by estimating the overall percent agreement (OPA) for *RAS* mutation detection. The results demonstrate an overall percent agreement (OPA) of 93.3%, positive percent agreement (PPA) of 92.6%, and negative percent agreement (NPA) of 94.0% (54).

A multicentre study compared concordance between tissue- based standard of care (SOC) results and results obtained from plasma analysis using the OncoBEAM™ RAS CRC assay. Overall concordance results were confirmed in this study [Overall Percent Agreement (OPA) 92.0%, Positive Percent Agreement (PPA) 89.8%, Negative Percent Agreement (NPA) 94.4%] (54). The highest concordance of plasma and tissue *RAS* results was observed in patients having liver metastases only (OPA 94.5%), whereas the lowest concordance rate was associated with the presence of lung metastases only (OPA 68.8%) (54).

2.4. Clinical data analysis

Apart from the generated data from patient blood sample analysis, we performed prospective collection of patient data including demographic and clinical information. Data were retrieved from hospital patient records, stored in a clinical database, and regularly updated as needed.

At baseline, the following data were collected:

- Age
- Gender
- Date of birth
- Date of diagnosis
- Smoking status
- Comorbidities
- Family history of neoplasia
- Right colon or left colon/rectum localisation of primary tumour
- Histological grade
- Histological type
- ECOG Performance Status
- Tumour stage (I–IV) at diagnosis of CRC
- Localisation of metastases
- Upfront surgery (if available)
- Adjuvant treatment scheme (if available)
- CEA levels (if available)
- CA 19-9 levels (if available)

For each treatment line, the following data were collected:

- Treatment scheme
- Treatment start and end dates
- Best radiological response
- CEA levels (if available)
- CA 19-9 levels (if available)
- Metastasectomy (if applicable)
- Date of disease progression
- Site of disease progression

Additionally, molecular results from tissue testing that were used in clinical decision making were retrieved from patient records, including *RAS*, *BRAF* and microsatellite instability (MSI) statuses. Liquid biopsy results of the ctDNA *RAS* status and MAF were also recorded.

2.5. Statistical considerations

Descriptive statistics including counts with the corresponding percentages (for categorical variables) and medians with ranges (for continuous) were used to summarize patient and tumour characteristics as well as mutational status at each timepoint of interest (baseline, middle of first-line treatment, first and second progression). Associations between categorical variables were assessed via the chi-square or Fisher's exact test, whichever more appropriate. The McNemar's test was used to assess differences in RAS mutational status between different time points, while the Wilcoxon signed-rank test was performed to estimate changes *RAS* MAF between the timepoints of interest.

Time to progression (TTP) was calculated as the time from first-line treatment to the first documented progression. Patients without disease progression were censored at the time of last follow-up. Progression-free survival (PFS) was defined as the time from the initiation of first-line treatment to the first documented progression, death (from any cause) or last contact, whichever occurred first. Overall survival (OS) was estimated as the time from first-line treatment to the date of death from any cause or last contact. Time to event endpoints were estimated using the Kaplan-Meier product limit method and the complementary log-log transformation was used to calculate the 95% confidence intervals (CI) for the median values. Cox univariate regression was used to estimate the effect of several parameters of interest on TTP, PFS and OS. All tests were two-sided at a 5% level of significance.

3. RESULTS

3.1. Patients and disease characteristics

A total of 68 patients diagnosed with metastatic colorectal cancer between January 2018 and October 2019 were enrolled in the study. The median age at the time of diagnosis was 64.5 years (range 31–87). More than half of the patients were males (72.1%), had history of smoking (56.4%) and family history of neoplasia (56%). Most patients had grade 2 tumours (81%) and the most common primary tumour localisation was the left colon (49.1%).

Fifty-seven out of 64 patients (89.1%) with available information about the stage at diagnosis were *de novo* metastatic. The remaining eight patients were initially diagnosed with stage III CRC before progressing to develop metastatic disease when they entered the study. Seven of them had their primary tumour resected and subsequently received adjuvant treatment, which was oxaliplatin-based for five out of six patients (85.7%), and fluopyrimidine-only for one patient (14.3%).

Another 31 patients underwent resection of the primary tumour at diagnosis of mCRC. Four of them also had their metastasis removed, which was limited to the liver for three patients and to the peritoneum for one patient, prior to initiation of first line systemic therapy in the metastatic setting.

This leads to a total of 38 patients out of the 60 with available information who had undergone primary tumour resection with or without metastasectomy before first line treatment initiation. Table 4A summarises patient characteristics and Table 4B summarises disease and treatment characteristics for patients with available data for each parameter.

Table 4A. Patient characteristics.

Parameter	Median (min, max)
Age at diagnosis (*N*=64)	64.5 (31.0, 87.0)
	***N* (%)**
Gender (*N*=68)	
Female	19 (27.9)
Male	49 (72.1)
Smoking history (*N*=55)	
No	24 (43.6)
Yes	31 (56.4)
Comorbidities (*N*=60)	
Cardiometabolic	36 (60.0)
Neoplastic	5 (8.3)
Other	5 (8.3)
None	14 (23.3)
Family history of neoplasia (*N*=50)	
No	22 (44.0)
Yes	28 (56.0)

Table 4B. Disease and treatment characteristics.

Parameter	*N* (%)
Primary tumour localisation (*N*=57)	
Right	19 (33.3)
Left/rectum	38 (66.7)
Histology (*N*=58)	
Adenocarcinoma	56 (96.6)
Mixed (adenomucinous)	2 (3.4)
Grade (*N*=42)	
1	1 (2.4)
2	34 (81.0)
3	7 (16.7)
Stage at diagnosis (*N*=65)	
III	8 (12.3)
IV	57 (87.7)
Metastatic localisation (*N*=63)	
Liver only	22 (34.9)
Liver, bone	1 (1.6)
Peritoneal	10 (15.9)
Visceral (lung, brain, etc. ± liver)	30 (47.6)
Upfront surgery (*N*=60)	
No	22 (36.7)
Primary location resection	34 (56.7)
Primary location resection and metastasectomy	4 (6.7)
Adjuvant treatment (*N*=7)	
Fluopyrimidine only	1 (14.3)
Oxaliplatin-based	5 (71.4)
None	1 (14.3)
First-line targeted treatment (*N*=63)	
Antiangiogenic	29 (46.0)
Anti-EGFR	18 (28.6)
None	16 (25.4)

3.2. Treatment characteristics

All patients received first-line chemotherapy between January 2018 and February 2020. Out of 63 patients with available information regarding the first-line treatment regimen, 50 patients (79.4%) received an oxaliplatin-based doublet, six (9.5%) were treated with an irinotecan-based doublet and four (6.3%) with a triplet, while two patients (3.2%) received fluoropyrimidine monotherapy and one patient was treated with a different regime. Antiangiogenic treatment was administered in most patients (46%), while 28.6% were treated with anti-EGFR first-line therapy.

Twenty-one patients (out of 54 with available information; 38.9%) received maintenance therapy after first-line treatment including anti-EGFR treatment (two patients; 9.5%), antiangiogenic (one patient; 4.8%), cetuximab (two patients; 9.5%), fluoropyrimidine alone (three patients; 14.3%) and fluoropyrimidine with antiangiogenic component (13 patients; 61.9%). It is of note that two patients were on active maintenance therapy with fluoropyrimidine and antiangiogenic component at the time of the analysis.

Thirty-six patients progressed on first line treatment. Two patients died shortly after first-line disease progression. Second-line treatment data were available for all remaining patients but one, with 18 (50%) receiving an irinotecan-based doublet, six (16.7%) receiving an oxaliplatin-based doublet, three (8.3%) receiving a single agent chemotherapy, three (8.3%) receiving regorafenib, one receiving pembrolizumab and two having localised treatments.

At the time of the analysis, 15 patients had progressed on second-line treatment.

3.3. Follow-up information

After a median follow-up of 13.3 months (95% CI 9.4–15.3), 15 deaths (22.1%) were reported. The median overall survival (OS) was 21.7 months (95% CI 20.8–NR). Thirty-six patients (52.9%) experienced a disease progression after first line treatment. The median time to progression (TTP) was 10.1 months (95% CI 8.8-11.8).

A total of 39 progression-free survival (PFS) events had been reported until the data cut-off for the analysis (July 2020) and the median PFS was 9.9 months (95% CI 8.5–11.5). Of note, TTP differs from PFS solely in that the event of interest is only disease progression, while PFS includes deaths from other causes.

Fig. 12 presents the Kaplan-Meier curves for OS, TTP and PFS.

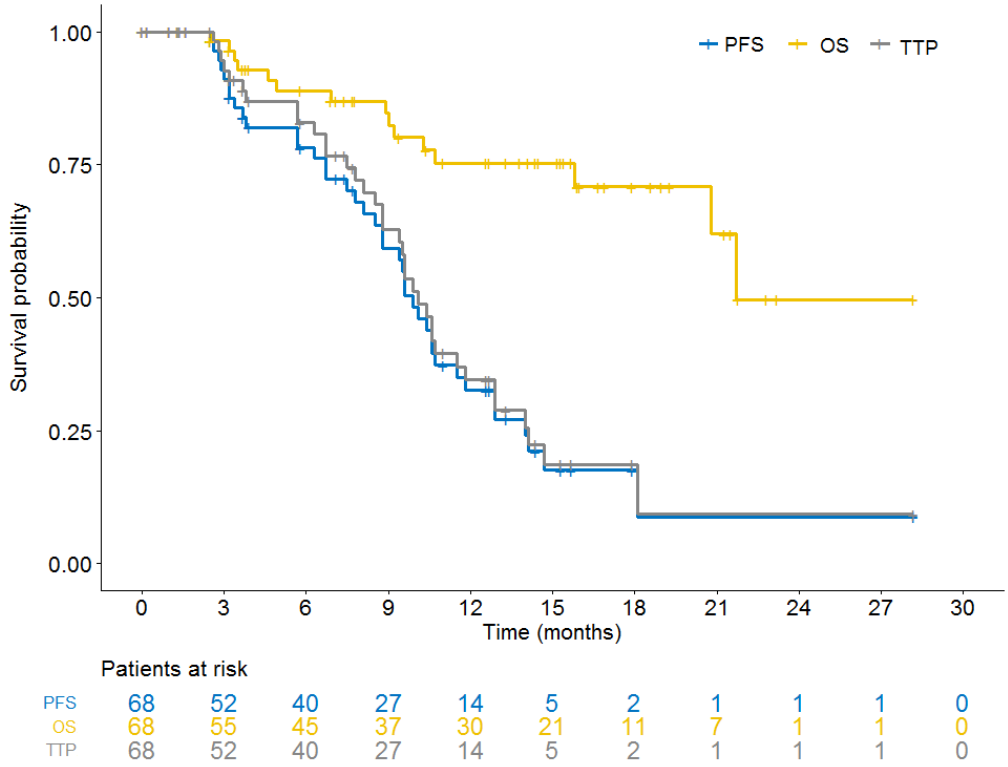

Figure 12. Kaplan-Meier curves for overall survival (OS), time to progression (TTP) and progression-free survival (PFS).

Among the 66 patients with available tissue results, *RAS* mutations were identified in 34 patients (51.5%). In the entire cohort, *KRAS* mutations were detected in 31 patients (47%), *NRAS* in 3 patients (4.5%), while no mutation was found in the remaining 32 patients (48.5%) (Fig. 13). As far as the patients with a *RAS* mutation are concerned, mutations were detected in *KRAS* in the vast majority (92.2%) compared to *NRAS* which was found mutated in three patients (8.8%).

Regarding the distribution of mutations within the *KRAS* gene, codon 12 mutations were detected in 17 out of the 31 patients (54.8%). Among the three patients with *NRAS* mutations, two mutations were detected in codon 13 (66.7%) and one (33.3%) in codon 61 (Fig. 14).

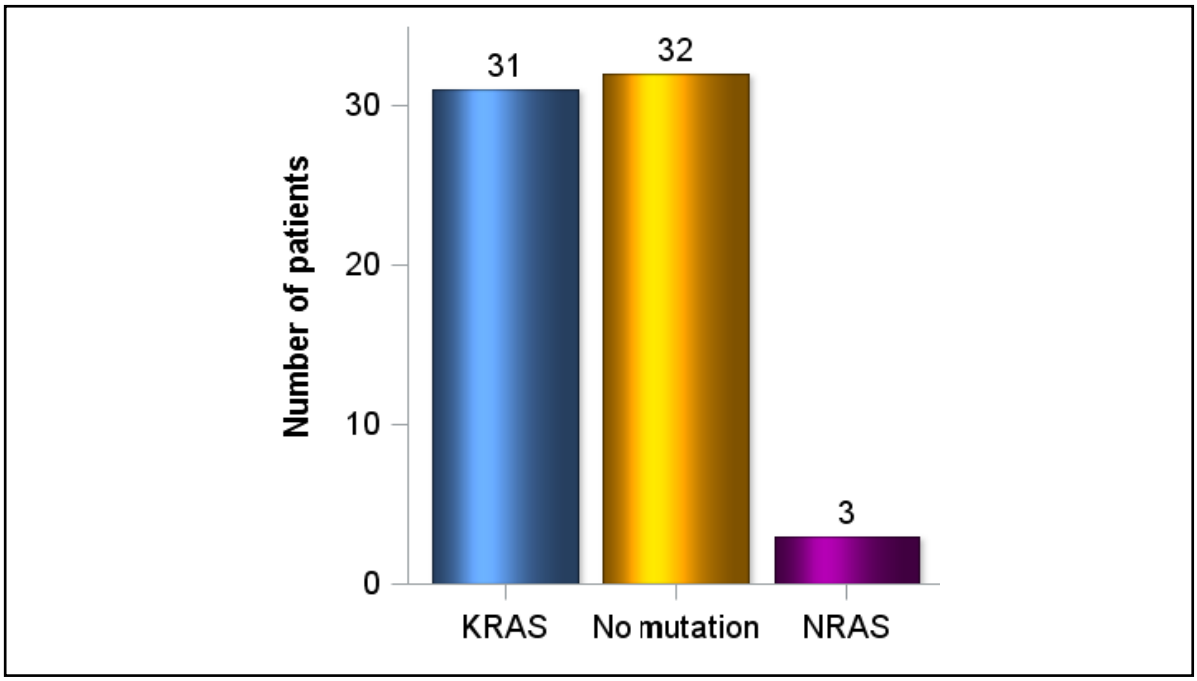

Figure 13. Bar plot of *RAS* mutations detected in tissue at baseline (*N*=66).

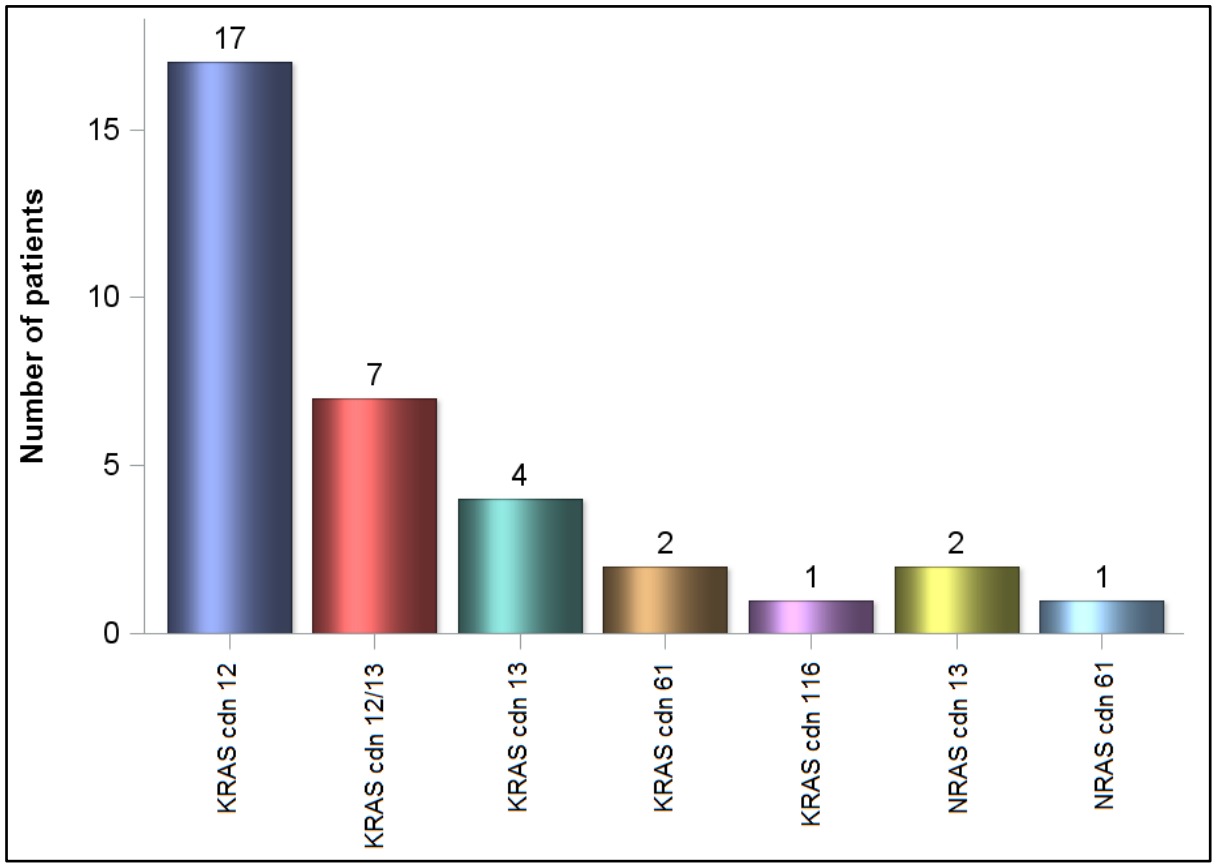

Figure 14. Bar plot of the distribution of *RAS* mutations detected in tissue at baseline (*N*=34).

3.3.2. Tumour tissue BRAF and MSI at baseline

In 42 patients of the total sample (61.8%), the *BRAF* and microsatellite instability (MSI) statuses were available from tissue testing. *BRAF* mutations were identified in three patients (7.1%), while five patients (11.9%) were found to have microsatellite stable MSI tumours.

3.4. Plasma *RAS* status by BEAMing Digital PCR at baseline

Using BEAMing Digital PCR, we analysed 67 patients' baseline plasma samples to define *RAS* status (Fig. 15). *RAS* mutations were detected in the ctDNA of 32 patients (47.8%). These included *KRAS* mutations in 27 patients (84.3%), *NRAS* mutations in 2 patients (6.3%), and 3 patients having both a *KRAS* and an *NRAS* mutation (9.4%). Overall, 93.8% of patients with a *RAS* mutation in ctDNA, carried a mutation in *KRAS*, while 15.6% in *NRAS*. No *RAS* mutations were detected in 35 out of the 67 patients with a baseline plasma sample (52.2%).

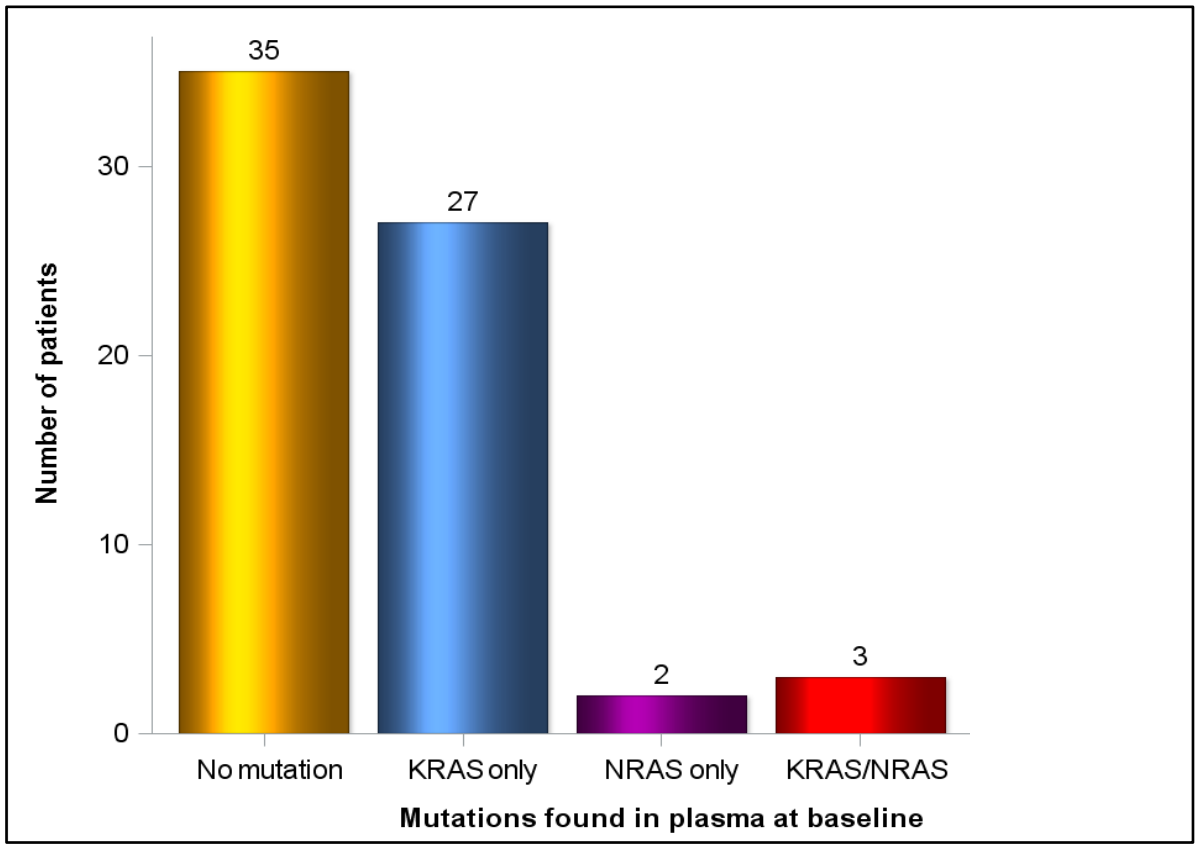

Figure 15. Bar plot of the distribution of mutations detected in plasma at baseline (*N*=67).

3.5. Comparison of *RAS* status in tumour tissue and plasma at baseline

After defining the *RAS* mutation frequencies in tissue and plasma, the next step was to compare our method of *RAS* testing in ctDNA against the "gold standard" approach, which is tissue testing, in the 65 patients with available tissue and plasma samples at baseline (Tables 4A and 4B). To this end, calculation of the positive, negative, and overall percent agreement was performed.

- Positive percent agreement (PPA) corresponds to the fraction of patients with *RAS* mutations detected in ctDNA in their baseline plasma samples, out of the total number of patients with *RAS* mutations in tissue testing (23 out of 34 patients, i.e., 67.6%).
- Negative percent agreement (NPA) corresponds to the fraction of patients with no *RAS* mutation detected in ctDNA in their plasma samples, out of the total of patient with no *RAS* mutation detected in their tissue sample (24 out of 31 patients, i.e., 77.4%).
- The overall percent agreement (OPA) of the two methods is defined as the fraction of patients whose tissue and ctDNA sample pairs agree either for the detection or the non-detection of *RAS* mutations (23 and 24 patients, respectively, total of 47), out of the total number of patients. Thus, in our cohort, the OPA was 72.3%, from the 47 out of 65 patients (Table 5).

Table 5. Comparison of *RAS* mutation detection in ctDNA against tissue results at baseline.

	***RAS* mutation detection in ctDNA**					
	Mutation detected		No mutation detected		Total	
	N	%	*N*	%	*N*	%
***RAS* mutation detection in tissue**						
Mutation detected	23	67.6	11	32.4	34	100
No mutation detected	7	22.6	24	77.4	31	100
Total	30	46.2	35	54.8	65	100

Additionally, we performed comparison analysis of the concordance of tissue and plasma regarding the presence and absence of *RAS* mutations.

- Concordance was 36.9% regarding the absence of mutations, as 24 out of 65 patients were found to carry no mutations in tissue and plasma at baseline (Table 6).
- As far as the concordance for the presence of *RAS* mutations is concerned, 23 patients out of 65 (35.4%) were found to carry a *RAS* mutation in both tissue and plasma at baseline.

Furthermore, we calculated the PPA for *KRAS* and *NRAS* separately.

- Twenty patients were found to carry *KRAS* mutations in ctDNA out of 31 patients with *KRAS* mutations in tissue, leading to a PPA of 64.5% for *KRAS*.
- Three patients carried *NRAS* mutations in both tissue and plasma, leading to PPA of 100% for *NRAS*.

Interestingly, one of the patients with *NRAS* codon 13 mutation found in tissue, BEAMing Digital PCR detected an additional mutation in *KRAS* codon 12 in the plasma at baseline.

Table 6. Comparison of *RAS* mutational status in tissue and ctDNA at baseline. Mut: mutation.

	***RAS* mutations in ctDNA**									
	KRAS only		*KRAS* and *NRAS*		*NRAS* only		No mut detected		Total	
	N	**%**	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**
***RAS* mutations in tissue**										
KRAS only	20	30.8	0	0	0	0	11	16.9	31	47.7
NRAS only	0	0	1	1.5	2	3.1	0	0	3	4.6
No mut detected	6	9.2	1	1.5	0	0	24	36.9	31	47.7
Total	26	40	2	3	2	3.1	35	53.8	65	100

The remaining patients had discordant *RAS* results in tissue and ctDNA. More specifically, 11 patients (16.9% of total) were found to carry *KRAS* mutations in tissue but not in plasma. It should be mentioned that three of these patients carried two distinct *KRAS* mutations in tissue located in codon 12 and codon 13. However, only the mutation in *KRAS* codon 12 was detected in ctDNA. In contrast, in another patient with a *KRAS* codon 13 mutation detected in tissue, ctDNA testing revealed an additional *KRAS* codon 117 mutation, which was not detected in tissue.

Of the 18 tumour-ctDNA discordant cases, 11 had *RAS* mutated status in tumour and wild-type *RAS* in ctDNA and the remaining seven cases had wild-type *RAS* status in tumour and *RAS* mutations identified in ctDNA. A mosaic plot comparing *RAS* mutational status identified by testing in tissue and ctDNA is presented in Fig. 16.

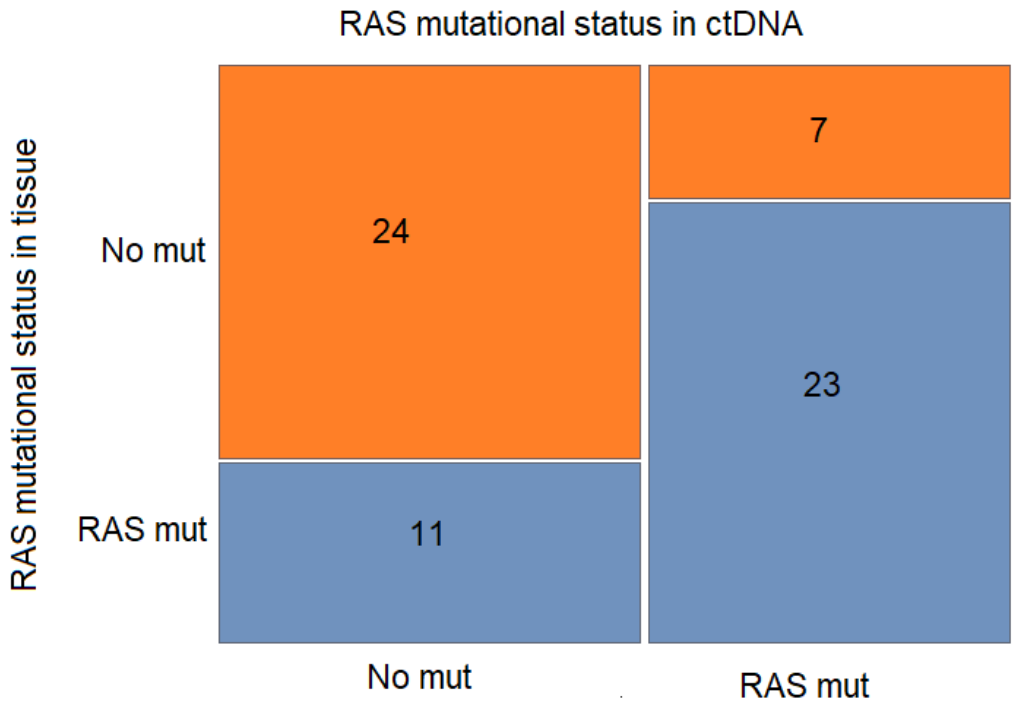

Figure 16. Mosaic plot comparing *RAS* mutation status by tissue and ctDNA testing at baseline. Among 65 patients with paired tissue and ctDNA samples, *RAS* mutations were detected in tissue and ctDNA in 23 patients, while no *RAS* mutations were detected in either sample type in 24 patients. In the remaining petiants, *RAS* mutations were detected in tissue but not in ctDNA in 11 patients, and in ctDNA but not in tissue of 7 patients.

3.6. Analysis of factors affecting tissue-ctDNA concordance

To better understand the discrepancies between tissue and plasma results, we analysed several clinical and pathological factors which have been described to affect ctDNA detection (Table 7). These included:

i. the sensitivity of the method used for tissue testing,

ii. previous resection of the primary tumour,

iii. the presence of liver metastases,

iv. the cancer stage when CRC was initially diagnosed.

3.6.1. Sensitivity of the method used for tissue testing

As a high-sensitivity method was used for *RAS* detection in ctDNA, tissue-ctDNA discordance could be partly explained by the lower sensitivity of methods used for tissue testing in some patients. Thus, we analysed concordance rates in the group of patients whose tissue was tested with low- versus high-sensitivity techniques. Out of the 45 concordant cases, the majority (62.2%) were tested with high-sensitivity techniques, compared to 37.8% of cases, in which tissue testing was performed with a low-sensitivity technique. Out of 18 discordant cases, the majority (61.1%) had their tissue tested with low-sensitivity techniques, compared to 38.9% of patients whose tissue was tested with high-sensitivity techniques. However, this association was not found to be statistically significant (p=0.092).

3.6.2. Previous resection of the primary tumour

Higher ctDNA levels in the circulation can be more easily detected with liquid biopsy, thus improving the overall concordance of tissue and plasma *RAS* status. Previous resection of the primary tumour, with or without resection of metastatic sites, could be used as an indicator of the actual tumour burden at the time of mCRC diagnosis and at baseline plasma sampling. This is because in patients in whom the primary tumour had not been resected and was still *in situ*, the tumour burden was more likely to be higher compared to those

who had undergone resection of their primary tumour with or without resection of metastatic sites. Thus, patients with both the primary and the metastatic site *in situ* may shed ctDNA into the circulation, as they have not only larger volume of disease, but also more sites of disease.

However, concordance of tissue-ctDNA *RAS* was found in 17 out of 23 patients (39.5%) with resected primary tumour, and in 26 out of 43 patients (60.5%) who had the primary *in situ*, but this difference was not found to be statistically significant (p=0.559) (Table 7).

3.6.3. Presence of liver metastases

Liver is a site of metastasis that has been associated with high release of ctDNA in the circulation. Therefore, tissue-ctDNA concordance was examined with regard to the presence or absence of liver metastases. A statistically significant association of liver metastases with tissue-ctDNA concordance was found (p=0.01), as liver involvement was present in 38 out of the 45 concordant cases (84.4%), and in 9 out of the 17 discordant cases (52.9%).

3.6.4. Cancer stage at diagnosis of CRC

RAS testing is routinely performed in the everyday clinical practice in patients with mCRC. For those patients who are diagnosed with stage III CRC and later develop metastases, metastatic disease is most commonly confirmed with imaging, whilst a new tissue biopsy of the metastasis is rarely performed only in cases of diagnostic doubt. Thus, in these patients, *RAS* testing is more likely to be performed on archival tissue from the initial diagnostic biopsy or the surgical resection specimen. Consequently, the tissue *RAS* status reflects the cancer mutational landscape at the time of the initial diagnosis, which is not essentially in agreement with the *RAS* status of the tumour when it becomes metastatic. Also, the elapsed time may affect the quality of tumour DNA isolated from tumour that is formalin-fixed, paraffin-embedded and the *RAS* status result. In contrast, for *de novo* metastatic patients, whose CRC is stage IV since diagnosis, the tissue sample used for *RAS* testing is synchronous with the baseline liquid biopsy performed in the setting of our study.

Therefore, we used the cancer stage at diagnosis as an indicator of the chronological relationship (synchronous or asynchronous) of tissue and plasma for each baseline sample pair. Concordance between tissue and ctDNA *RAS* status was higher in patients with *de novo* metastatic disease (39 out of 45 patients; 86.7%) as compared with those with stage III disease at diagnosis (6 out of 45 patients; 13.3%). However, this difference was not found to be statistically significant (p=0.662), probably due to the small patient numbers.

Table 7. Association of *RAS* status concordance between tissue and ctDNA testing with relevant parameters.

	Tissue-ctDNA concordance		
	Yes	**No**	***p*-value**
Tissue testing method sensitivity			0.092
Low	17 (37.8)	11 (61.1)	
High	28 (62.2)	7 (38.9)	
Resected primary			0.559
No	17 (39.5)	5 (31.3)	
Yes	26 (60.5)	11 (68.8)	
Stage at diagnosis			0.662
III	6 (13.3)	1 (5.9)	
IV	39 (86.7)	16 (94.1)	
Liver involvement			0.010
No	7 (15.6)	8 (47.1)	
Yes	38 (84.4)	9 (52.9)	

3.7. Case-by-case analysis of tissue *RAS* wild-type/plasma mutated patients

Based on the previous findings showing a near statistically significant association of the sensitivity of the tissue testing method with tissue-ctDNA concordance, we conducted a case-by-case analysis of the seven patients with no *RAS* mutations in tissue but positive ctDNA results.

- All seven patients were metastatic at diagnosis, having synchronous tumour and plasma samples.
- Five out of seven patients had the primary tumour resected; two had it *in situ*.
- One patient had liver only disease, one had lymph node involvement and the third one had liver, lung, and lymph node metastases.
- All seven patients received an oxaliplatin-based doublet and four out of seven received an anti-EGFR agent, one received an antiangiogenic and the remaining two did not receive any targeted agent with first-line chemotherapy.
- Three patients had *KRAS* codon 12 mutations, two had *KRAS* codon 13 mutations, one had *KRAS* codon 59 mutation and another patient had mutations in both *KRAS* codon 12 and *NRAS* codon 61.
- Mutant allele fraction (MAF) levels for all seven patients ranged from 0.01% to 19.91% with a median of 0.12%. There was no association between high MAF levels (10.4%-19.9%) and the site of metastases.

Interestingly, the two patients with the highest MAFs of 16.09% and 19.91% received the anti-EGFR monoclonal antibody cetuximab along with first-line chemotherapy, based on their tissue *RAS* wild-type status.

The first patient with baseline MAF of 19.91% had an unresected rectal primary with liver, lung and lymph node metastases, and developed disease progression after less than 3 months of first-line treatment. CEA levels dropped from 2,074 to 506 ng/mL, and CA19-9 from 5,179 to 2,090 U/mL, as did the *RAS* MAF, which was undetectable at disease progression.

The second patient with baseline MAF of 16.09%, responded to first-line therapy with oxaliplatin-based doublet and cetuximab with stable disease and no progression until date of last contact at 16 months following diagnosis of metastatic disease. A liquid biopsy sample was available for this patient at the middle of first-line therapy. MAF dropped from 16.09% at baseline to 0.01% after three months of therapy.

This patient also had available detectable tumour markers at diagnosis that were monitored throughout the course of the disease. In agreement with the declining trend of MAF, CEA dropped from 316 to 3 ng/mL and CA19-9 dropped from 97 to 7 U/mL, suggesting a biochemical response to first line therapy.

Of note, analysis of this patient's tumour tissue was performed with NGS, a high-sensitivity method. This suggests that the *RAS* mutant ctDNA may have originated from a subclone which was not captured in the tissue biopsy sample that was tested, constituting an example of tumour heterogeneity. The fact that the patient responded to anti-EFGR despite the presence of the *RAS* mutation in ctDNA shows that treatment selection based solely on liquid biopsy may have pitfalls, as the presence of *RAS* mutant subclone is not always predictive of response to first-line anti-EGFR, especially in a patient with low burden of disease at diagnosis (limited to lymph nodes).

It is also worth mentioning the case of another patient, with liver and lung metastases and a resected right colon primary, who received the anti-EGFR panitumumab based on tissue *RAS* wild-type status. However, on ctDNA testing, a *KRAS* codon 13 MAF of 0.06% was detected. This patient experienced disease progression after 15 weeks of therapy. At disease progression, *KRAS* codon 13 MAF increased to 0.33% and additional mutations in the *KRAS* codons 12 and 61, and *NRAS* codons 12 and 61 were also detected with MAF levels of 0.01%, 0.02%, 0.20% and 0.01%, respectively. Of note, this patient had an MSI tumour and received second-line immunotherapy with pembrolizumab.

3.8. Survival analysis

Given that ctDNA testing provides real-time information about the tumour mutational profile, also capturing tumour heterogeneity among disease sites, it has been suggested that it may guide therapeutic decisions even more reliably than tissue testing. However, as all mCRC patients are treated based on tissue molecular profile, one may expect worse treatment outcomes when there is tissue-ctDNA discordance for *RAS* status.

Therefore, we analysed tissue-ctDNA concordance for *RAS* status for associations with respect to OS, TTP or PFS. No prognostic significance was identified for tissue-ctDNA concordance as shown in Table 8, most probably due to the low number of the events of interest.

3.9. *RAS* MAF in the middle of first-line therapy

BEAMing Digital PCR testing was performed in 14 patients in the middle of first-line therapy, to assess monitoring of *RAS* Mutant Allele Fraction (MAF) as predictor of response to treatment.

At baseline, all 14 patients had at least one *RAS* mutation, with two patients having two different mutations, leading to a total of 16 mutations detected at baseline in our cohort. In the middle of first line therapy, *RAS* MAF was detectable for the same mutations as at baseline in 10 out of 14 patients (71.4%), while for the remaining four patients (28.6%), *RAS* MAF levels were undetectable. This corresponds to a marginally statistically significant difference in the *RAS* mutational status between baseline and the middle of first-line treatment (p=0.046). Three of the four patients (75%) with undetectable *RAS* status mid first-line treatment had received an oxaliplatin-based doublet, while one patient had received triplet chemotherapy.

RAS MAF levels both at baseline and middle of first-line therapy for the 16 detected *RAS* mutations followed a normal distribution. Mean *RAS* MAF decreased from 12.27% [standard deviation (SD) 13.97%] at baseline to 5.63% (SD 10.24%) in the middle of first-line treatment. However, no statistically significant difference (p=0.147) was identified, possibly due to the small sample size.

RAS MAF levels were also analysed with regard to OS, TTP or PFS, but they were not found to be a significant prognosticator, for either the entire cohort (p=0.585, p=0.617 and p=0.518, respectively) or among patients treated with non-anti-EGFR agents (p=0.595, p=0.879 and p=0.753, respectively).

Fig. 17 illustrates the change in *RAS* MAF from baseline to the middle of first-line therapy along with the respective mutations for all 14 patients.

Table 8. Cox univariate regression for RAS status concordance between tissue and ctDNA testing at baseline among comparable patients with respect to overall survival (OS), time to progression (TTP) and progression-free survival (PFS) (*N*=65).

	Event/Total	Hazard Ratio (95% CI)	*p*-value
	OS		
Tissue-ctDNA concordance			
No	1/18	0.24 (0.03–1.84)	0.171
Yes	14/47	Reference	--
	TTP		
Tissue-ctDNA concordance			
No	5/18	0.50 (0.19–1.31)	0.158
Yes	31/47	Reference	--
	PFS		
Tissue-ctDNA concordance			
No	5/18	0.45 (0.17–1.17)	0.101
Yes	34/47	Reference	--

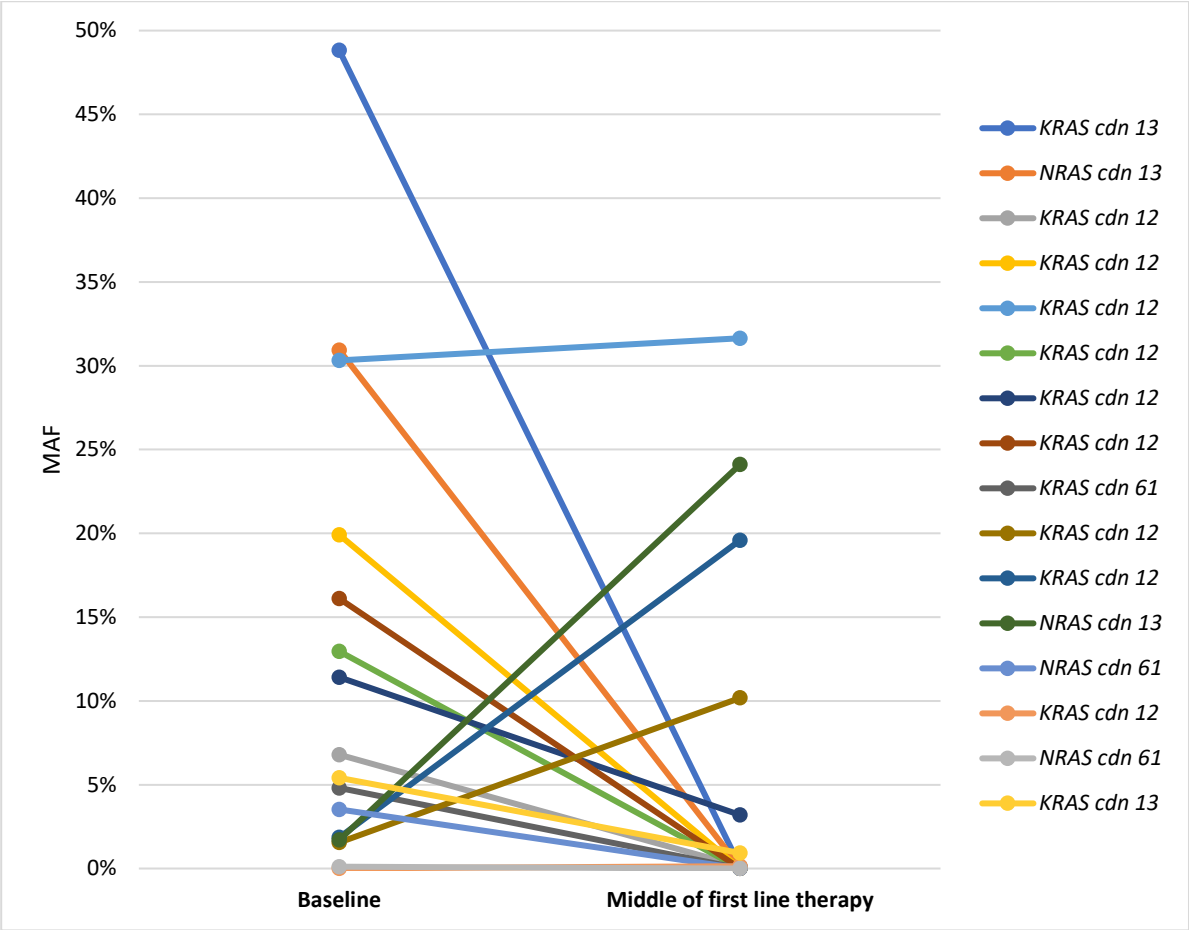

Figure 17. Changes in *RAS* MAF levels from baseline to middle of first line therapy for the 16 mutations detected in blood samples from patients with a *RAS* mutation at baseline (*N*=14).

3.10. *RAS* status at first progression

RAS status was available for 28 patients who progressed on first-line treatment. *RAS* mutation was found in 17 patients (60.7%) and no mutation was found in the remaining 11 patients (39.3%) (Table 9). More specifically, in the patients with *RAS* mutation, 10 patients (58.8%) had a mutation in *KRAS*, one patient (5.9%) had a mutation in *NRAS*, while six patients (35.3%) had mutations in both genes. Overall, 94% of patients had a *KRAS* mutation and 41% had a mutation in *NRAS*.

Table 9. *RAS* mutation status in ctDNA of patients who progressed on first-line treatment (N=28).

	N	**%**
KRAS mutation only	10	35.7
KRAS and NRAS mutation	6	21.4
NRAS mutation only	1	3.33
No mutation detected	11	39.3
Total	28	100

Although emergence of *RAS* mutated subclones is indicative of tumour evolution, the clinically relevant question is whether the *RAS* status changes in the course of the disease. Patients with initially wild-type tumours may develop *RAS* mutated subclones which can cause resistance to anti-EGFR. On the other hand, in patients with initially *RAS* mutated tumours, the mutated subclones may be effaced over time, suggesting that these patients may respond to anti-EGFR.

Thus, we examined *RAS* status change at baseline and first progression in these 28 patients. As compared to baseline, *RAS* mutational status was preserved for 22 of the 28 patients with paired data (78.6%; 11 without mutations at baseline and first progression and 11 carriers at both timepoints), whereas in six patients (21.4%) with no mutations detected at baseline, *RAS* mutations were detected at the time of the first progression (Fig. 18). Repeat

testing at first progression revealed six cases where the *RAS* status changed from wild type to mutated. This was not significantly associated with prior anti-EGFR therapeutic blockade in this cohort (p>0.05), likely due to the small subgroup size.

In two patients with *KRAS* only mutations at baseline and in two patients with *NRAS* only mutations at baseline, mutations in both *KRAS* and *NRAS* were detected at the time of first progression. Notably, the presence of mutations in both *KRAS* and *NRAS* at first progression compared to baseline was found to be statistically significant (p=0.01), as there was an increase from 5% to 21%.

Therefore, this leads to a total of 10 out of 28 patients (35.7%) with appearance of a new mutation at first progression, with the difference in *RAS* emergence rates between baseline and first progression being statistically significant (p=0.014).

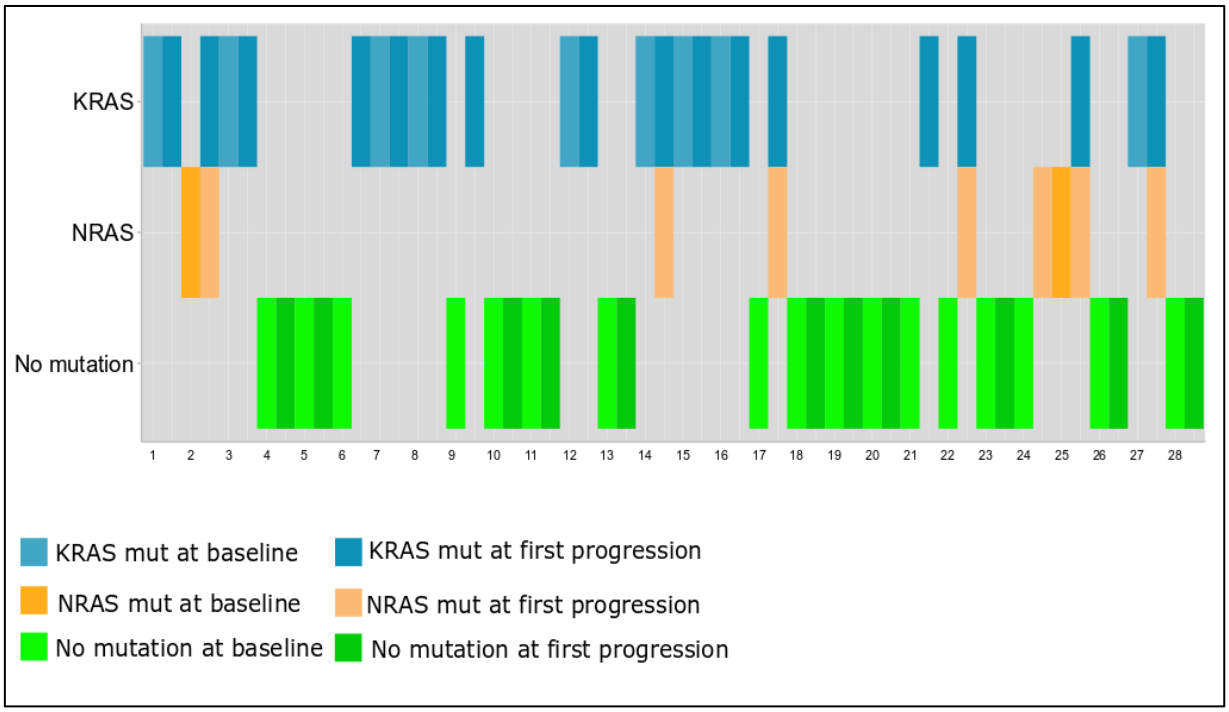

Figure 18. Plasma *RAS* mutational status at baseline and first disease progression. The graph depicts *RAS* mutational status at the 2 timepoints for each of the patients with available samples in both timepoints (N=28).

Regarding the treatments that patients received before the emergence of *RAS* mutations at first progression, three had received oxaliplatin-based doublet chemotherapy and one patient had received antiangiogenic triplet as first-line therapy. In addition, out of the six patients with wild-type *RAS* at baseline and *RAS* mutations at the time of first progression, four had been treated with oxaliplatin-based first-line chemotherapy, one with irinotecan-based chemotherapy and one patient had received first line cetuximab.

No statistically significant association was detected between the type of first-line chemotherapy and the appearance of new mutations at the time of first progression (*p*=0.744) (Table 10).

Table 10. Association of the type of first-line therapy with the emergence of new *RAS* mutations at disease progression on first-line treatment (PD1).

	Emergence of new mutations at PD1		
	No (*N*=18)	**Yes (*N*=10)**	***p*-value**
Scheme			0.744
Fluoropyrimidine monotherapy	1 (5.6)	0 (0.0)	
Irinotecan-based doublet	3 (16.7)	1 (10.0)	
Oxaliplatin-based doublet	13 (72.2)	7 (70.0)	
Triplet	1 (5.6)	1 (10.0)	
Other	0 (0.0)	1 (10.0)	
Targeted agent			0.42
Anti-EGFR	6 (33.3)	5 (50.0)	
Antiangiogenic	8 (44.4)	5 (50.0)	
None	4 (22.2)	0 (0.0)	

As previously mentioned, treatment selection is based on baseline tissue *RAS* status and as re-biopsy is neither feasible nor standard practice, no further insight is gained about the *RAS* status at the time of disease progression. Therefore, a change in *RAS* status, which is

not captured prior to second line treatment initiation, may negatively affect treatment outcomes, as treatment is not based on the updated *RAS* status, which as previously shown can change with emergence of new mutations.

Thus, we defined the median Time to Progression (TTP) among patients with preserved *RAS* status and patients with a change in *RAS* status. In the 22 patients with preserved *RAS* status from baseline to first progression, median TTP was 8.3 months (95% CI 3.8-10.4), which was numerically shorter than that of six patients with change in *RAS* status, who had a median TTP of 10.3 months (95% CI 9.6-14.7). However, due to the small number of patients with a change in *RAS* status between the two timepoints no formal statistical comparison was performed.

3.11. *RAS* status at the time of second progression

RAS status at second disease progression was available for eight out of 15 patients (53.3%) with disease progression at second line. Six out of eight patients had mutated *RAS* at second progression (75%; five with *KRAS* only mutations and one with *NRAS* mutation) (Table 11).

Table 11. *RAS* mutation status in ctDNA of patients who progressed on second-line treatment (*N*=8).

	N	**%**
KRAS only mutation	5	62.5
NRAS only mutation	1	12.5
No mutation detected	2	25
Total	8	100

It is of note that all six patients with mutations at the time of second progression also had *RAS* mutations at first progression, and the *RAS* status did not change from the first to the second progression for the two patients with wild-type *RAS*.

However, it should be mentioned that in one patient with a *KRAS* codon 12 mutation at first progression an additional *NRAS* mutation was identified at second progression. Additionally, in one patient carrying both *KRAS* and *NRAS* mutations at first progression only a *KRAS* mutation was detected at second progression. Both patients had wild-type *RAS* at baseline according to BEAMing Digital PCR results. As compared to baseline, *RAS* status was preserved for five of the eight patients (62.5%), whereas three patients (37.5%) without mutations in ctDNA at baseline had *RAS* mutations at second progression (Fig. 19).

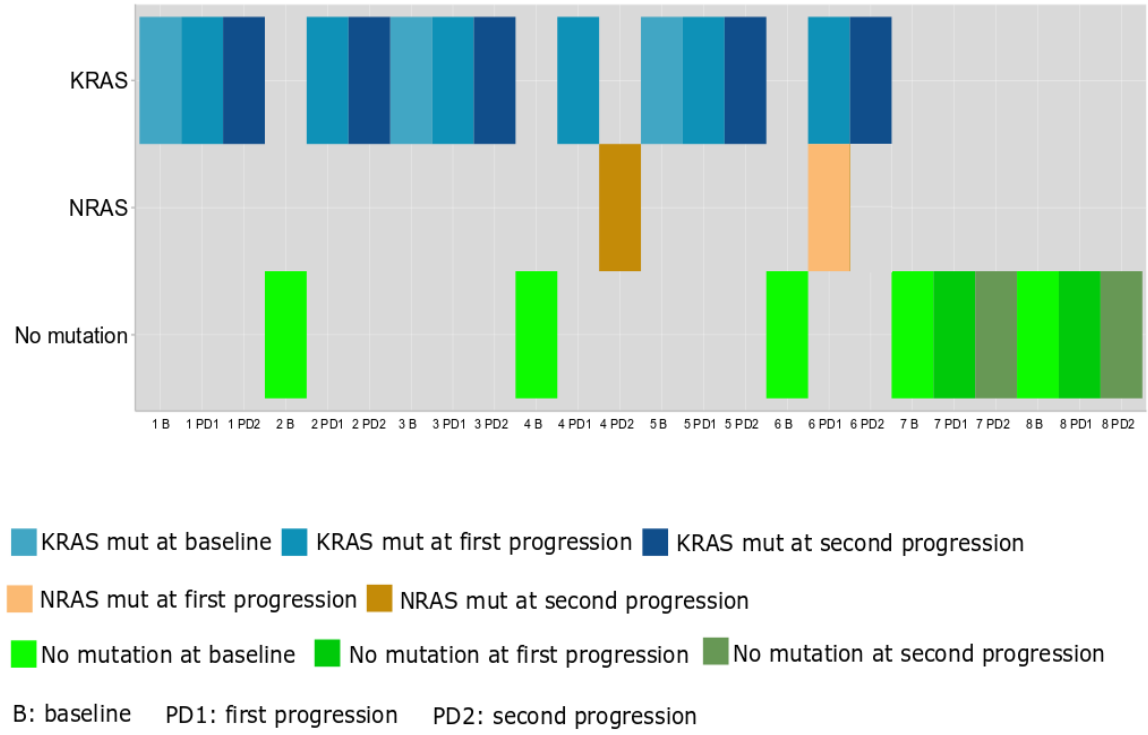

Figure 19. Plasma *RAS* mutational status at baseline, first and second disease. The graph depicts *RAS* mutational status at the 3 timepoints for each of the patients with available samples in the progression in the three timepoints (*N*=8).

4. DISCUSSION

To the best of our knowledge, this study is based on one of the largest mCRC patient cohorts in the Greek population and constitutes one of the few studies on the clinical application of a high-sensitivity liquid biopsy technique in Greece.

In our cohort, the prevalence of *RAS* mutations in tissue was 51.5%, with the remaining 48.5% of patients having no *RAS* mutations. The breakdown of these mutations is as follows: *KRAS* mutations in 92.2% and *NRAS* mutations in 8.8%, which is comparable with the rates reported in the literature (55).

As far as ctDNA is concerned, *RAS* mutations were detected in 47.8% of patients at baseline. Mutations were located in *KRAS* in 84.3% of patients and in *NRAS* in 6.3% of patients, while the remaining 9.4% of patients had both a *KRAS* and an *NRAS* mutation. Overall, 94% of patients with a *RAS* mutation in ctDNA were mutated in *KRAS* and only 6% in *NRAS*. These findings are comparable to known detection rates and *RAS* mutation distribution in tissue (55), as well as in cfDNA based on the study of a cohort of 1,397 patients with advanced CRC (56).

After defining the prevalence of *RAS* mutations in both tissue and ctDNA in our cohort, we compared ctDNA with tissue to define concordance rates between the two. Our results showed overall percent agreement of 72.3% for *RAS* status in tissue and plasma. This is similar to the concordance of 78.3% reported in the CAPRI-GOIM trial (57), while other studies using BEAMing Digital PCR report concordance reaching 89.0%, although in a much larger sample of 236 patients (54). Of interest, this study confirmed that higher concordance in patients who had metastases at diagnosis and had the primary *in situ* at the time of blood collection, which was not proved in our study likely due to the smaller sample size (54).

Concordance for *NRAS* status was 100%, despite the small number of patients (*N*=3), highlighting that when BEAMing Digital PCR identifies *NRAS* mutations it accurately reflects tissue status. This contrasts with the concordance for *KRAS* mutations which was only 64.5%. It also suggests that the non-detection of *RAS* mutations in ctDNA should be interpreted with caution, and it is more likely to correspond to a “missed” *KRAS* rather than *NRAS* mutation.

Subsequently, we performed analysis of several clinico-pathological features known to affect ctDNA release in order to explain the not very high *RAS* overall percent agreement (72.3%). We found a statistically significant correlation of the hepatic metastases with high concordance of tissue and ctDNA *RAS* status. The liver is a common site of metastasis in colorectal cancer and patients with liver involvement are known to have higher levels of MAF (58). ctDNA is thought to be easily released into the circulation from the liver, irrespective of the size or number of metastases (58), suggesting higher positive percent agreement and overall concordance in the presence of liver disease, which has been confirmed in a large multicentre study (54). This finding suggests that the presence of liver metastases may be a positive predictor of concordance of ctDNA *RAS* status with that of tissue, thus aiding clinicians to interpret liquid biopsy results more reliably in mCRC.

We also found a near statistically significant correlation (p=0.092) of the concordance with the sensitivity of the method used in tissue testing. This implies that tissue-ctDNA discordance may partly arise from the low sensitivity of standard tissue testing methods that are routinely available such as PCR and pyrosequencing.

Although liver involvement and sensitivity of tissue testing affects tissue-ctDNA concordance, cancer stage at diagnosis and primary tumour resection were not found to impact on ctDNA release, as there was no statistically significant association.

Regarding the discordant cases with tissue *RAS* wild-type and ctDNA *RAS* mutant status, we performed case-by-case analysis. This suggested that the detection of *RAS* mutant ctDNA could have a role in predicting treatment response and disease progression, as it likely represents a subclone of the tumour with the potential to impact on the response to treatment.

Another goal of the current study was to assess the utility of repeat *RAS* testing with liquid biopsy to aid therapeutic decisions in mCRC. To this end, after establishing that our method could reliably detect *RAS* mutations, we used it for testing in additional timepoints where tissue re-biopsy is not possible, but *RAS* mutation testing is relevant, either for qualitative analysis (status) or quantitative (levels of *RAS* mutated ctDNA, i.e. *RAS* MAF).

Regarding middle of first-line therapy, *RAS* MAF mean of 16 mutations detected in 14 patients decreased from 12.27% to 5.63% in the middle of first-line therapy in a non-statistically significant way (p=0.147), possibly due to the small number of samples. Of note, in 10 out of 14 patients (28.6%), ctDNA levels were undetectable in the middle of first-line therapy, leading to a statistically significant difference (p=0.046) in the *RAS* mutational status between baseline and middle of first-line therapy. These findings show that *RAS* MAF can be effectively monitored with BEAMing Digital PCR.

At the time of first disease progression, *RAS* mutations were identified in 60.7% of patients. In total, 58.8% were in *KRAS*, which was decreased from 84.3% at baseline, 5.9% in *NRAS*, which was similar to 6.3% at baseline, and 35.3% in both genes, showing an almost 4-fold increase from 9.4% at baseline. These differences show that a significant proportion of patients with *KRAS* mutations at baseline acquired *NRAS* mutations at first progression.

Overall, 94% of patients had a *KRAS* mutation and 41% had a *NRAS* mutation. Interestingly, the percentage of patients with a *NRAS* mutation increased from 15.6% when compared to baseline.

Overall, *RAS* status at first progression was preserved in 78.6% of patients and changed from baseline in 21.4% of patients. Of importance, in 35.7% of patients, a new mutation at first progression appeared, with a difference in *RAS* emergent rate that was found to be statistically significant (p=0.014).

As far as second disease progression is concerned, the *RAS* status was found to be preserved in 62.5% of patients in second progression as compared with baseline. Of note, 100% of patients preserved their *RAS* status from first to second disease progression. This supports that *RAS* mutation testing with liquid biopsy can be informative at first progression, but may not be necessary at second progression, although larger numbers of patients are required to support this further.

With regard to the limitations of our study, these include the small number of patients in the later timepoints. Also, although BEAMing Digital PCR is a highly sensitive technique, it only detects mutations in the *KRAS* and *NRAS* genes, which are both predictive biomarkers for the response to anti-EGFR therapy. However, other genes such as *BRAF*, *ERBB2, PIK3CA,*

MET, EGFR are currently under investigation for their role as biomarkers in mCRC. *BRAF* is a prognostic biomarker *PIK3CA* and *ERBB2* are potential targets for targeted therapies, whereas the remaining mutations are implicated in the development of resistance to treatment, mainly with anti-EGFR. In the near future, these genes may form part of the routine testing for mCRC, thus there is need for comprehensive testing of larger gene panels. This has become possible with the development of Next-Generation Sequencing (NGS) technologies that are able to detect low-frequency mutations with high sensitivity for applications in ctDNA testing.

Many studies are using NGS as a liquid biopsy in mCRC and a summary of these was provided in a review by our group (59). NGS in ctDNA holds promise to transform management of mCRC allowing for the detection of clinically actionable mutations with lower turnaround times and cost compared to conventional tissue testing or digital PCR-based liquid biopsy techniques.

However, although high sensitivity and a low limit of detection are prerequisites for the application of liquid biopsy technologies including BEAMing Digital PCR, questions arise as to the clinical significance of underrepresented subclones carrying specific mutations. Researchers have examined different cut-offs for mutation detection in liquid biopsies, with a recent study in mCRC by Elez *et al.* concluded that 5.8% MAF is a useful cut-off in ctDNA testing, which was found to correlate well with clinical responses.

Overall, this was a proof-of-concept study for the viability of serial liquid biopsies mCRC in a real-world setting. One of the strengths of our study is that it was based on one of the largest mCRC cohorts in Greece comprising over 100 samples from up to four timepoints for each patient in the course of their disease. Also, by using a high sensitivity ctDNA testing method for the detection of *RAS* mutations, with the ability to detect even very low frequencies of mutant ctDNA, we proved that it can be a valuable tool in the monitoring of patients for response to the administered treatment.

5. CONCLUSIONS

This study has generated original data on the use of liquid biopsies from a large real-world mCRC patient cohort, reaching the following conclusions:

- At baseline in tissue, the frequency of *RAS* mutations was 51.5%, of which 92.2% affected *KRAS* and only 8.8% *NRAS*.
- At baseline in ctDNA, the frequency of *RAS* mutations was 47.8%, of which 84.3% affected *KRAS*, 6.3% in *NRAS*, and 9.4% both *KRAS* and *NRAS*. Overall, 93.8% of patients had a mutation in *KRAS* and 15.6% in *NRAS*.
- Comparison of baseline *RAS* in paired plasma and tissue samples showed satisfactory overall percent agreement (OPA) of 72.3%, positive percent agreement (PPA) of 67.6% and negative percent agreement (NPA) of 77.4%.
- The PPA for *KRAS* was 64.5% and for *NRAS* was 100%, highlighting that identification of *NRAS* in ctDNA reflects *NRAS* status in tissue.
- Tissue-ctDNA concordance is affected by the presence of liver metastases (p=0.01) and the sensitivity of the method used for tissue testing with a near statistically significant result (p=0.092).
- The detection of *RAS* mutant ctDNA in tissue wild-type patients might have a role in predicting treatment response and/or disease progression.
- Dynamic changes of *RAS* MAF from baseline to the middle of first-line therapy are captured by BEAMing Digital PCR.
- *RAS* mutational status differs between baseline and the middle of first-line treatment (p=0.046), as in 28.6% of patients the *RAS* MAF levels were undetectable in middle of first-line treatment compared to baseline.
- Mean *RAS* MAF decreased from 12.27% at baseline to 5.63% in the middle of first-line therapy, with no statistically significant difference (p=0.147), possibly due to the small sample size.

- At first progression in ctDNA, the frequency of *RAS* mutations was 60.7%, of which 58.8% affected *KRAS* (versus 84.3% at baseline), 5.9% in *NRAS* (versus 6.3% at baseline) and 35.3% both *KRAS* and *NRAS* (versus 9.4% at baseline). Overall, 94% of patients had a mutation in *KRAS* (versus 93.8% at baseline) and 41% in *NRAS* (versus 15.6% at baseline).

- New *RAS* mutations emerged at first progression compared to baseline (p=0.014), with 35.7% of patients developing a new *RAS* mutation.

- At first progression, the overall *RAS* status was preserved in 78.6% of patients and changed from wild type to mutated in 21.4% of patients.

- At second progression in ctDNA, the frequency of *RAS* mutations was 75%, of which 83.3% were in *KRAS* (versus 84.3% at baseline and 58.8% at first progression), and 16.7% in *NRAS* (versus 6.3% at baseline and 5.9% at first progression), with no cases of mutation in both genes (versus 9.4% at baseline and 35.3% at first progression).

- At second progression, the overall *RAS* status was preserved in 62.5% of patients compared to baseline and in 100% compared to first progression.

In summary, we studied mCRC patients using ctDNA *RAS* testing with liquid biopsies, alongside baseline tissue *RAS* status, in four key timepoints: baseline, middle of first-line treatment, first and second disease progressions. We showed that liquid biopsy had a satisfactory concordance with baseline tissue and effectively detected even subtle differences both in *RAS* status and in *RAS* MAF at the above timepoints. This is of value, as tissue is only available at baseline and not in the middle of therapy or when the disease progresses. Therefore, capturing the dynamic *RAS* changes, we would be able to better understand when and why a patient with mCRC develops resistance to currently available treatments and eventually progresses. By using this validated and sufficiently tissue-concordant method for *RAS* ctDNA testing, we can create a personalised *RAS* timeline for every patient and base our treatment decision on the *RAS* trajectory. Thus, our study provides the data and framework to support further clinical trials to this end, not only for *KRAS* and *NRAS*, but also for other clinically relevant genes with an appropriate method.

Moreover, it sheds light on where our efforts should be focused on in the future, as we demonstrated that the proportion of patients with *RAS* mutations at disease progression increases and we urgently need better therapeutic strategies. Lastly, we strongly believe that this study is important, as it supports the feasibility of repeat liquid biopsies in the management of patients with mCRC in Greece, and that would benefit clinicians and patients to take better informed decisions about their oncological management.

ABSTRACT

Introduction: Metastatic colorectal cancer (mCRC) affects approximately 60% of patients with colorectal cancer and is associated with high mortality. *RAS* status is an important biomarker, as it guides treatment by predicting response to anti-EGFR monoclonal antibodies. However, *RAS* is routinely tested only on archival tumour tissue, and it is not possible to update the information on its status during the disease course, as in most patients rebiopsy is not possible or practical. Liquid biopsies are an emerging and promising alternative to tissue biopsy, as they provide an accurate real-time picture of the mutational landscape of the tumour.

Aim: The study aims to use and test the high-sensitivity liquid biopsy platform BEAMing Digital PCR for *RAS* mutation detection in a cohort of mCRC patients in a collaborating network of Oncology centres in Greece. Moreover, it aims to study the utility of repeat liquid biopsies, alongside standard tissue testing, for the customisation of therapeutic strategy in mCRC.

Methods: Clinical and molecular profiling data were prospectively collected from mCRC patient records. Plasma samples were collected from mCRC patients at the University Hospital of Ioannina, University Hospital of Larissa and the EUROMEDICA General Clinic in Thessaloniki, Greece. BEAMing Digital PCR was used to define the *RAS* status on ctDNA at four key timepoints: baseline, middle of first line therapy, first and second disease progressions.

Results: Sixty-eight patients with mCRC were recruited between January 2018 and October 2019 with a median follow up of 13.3 months. *RAS* mutations were detected in tissue and ctDNA in 51.5% and 47.8% of patients, respectively, with overall percent agreement of 72.3%. Tissue-ctDNA concordance was positively associated with the presence of liver metastases (p=0.010) and negatively associated with the low sensitivity of tissue testing method (p=0.092). *RAS* mutational status differed between baseline and the middle of first-line treatment (p=0.046), as *RAS* MAF became undetectable in 28.6% of patients, and the mean *RAS* MAF decreased from 12.27% at baseline to 5.63% in the middle of first-line therapy (p=0.147). At first progression, new *RAS* mutations emerged compared to baseline

(*p*=0.014), as 35.7% of patients developed a new *RAS* mutation, leading to a *RAS* status change from wild type to mutated in 21.4% of patients. At second progression, *RAS* status was preserved in 62.5% and in 100% of patients compared to baseline and first progression, respectively.

Conclusions: Plasma ctDNA analysis with BEAMing Digital PCR was shown to have satisfactory concordance with tissue testing and to effectively detect even subtle differences both in *RAS* status and in *RAS* MAF at the tested timepoints. Therefore, it can be used alongside baseline tissue testing and, by capturing the dynamic *RAS* changes, it can inform personalised therapeutic decisions for mCRC patients and the design of future clinical trials.

ΠΕΡΙΛΗΨΗ ΣΤΗΝ ΕΛΛΗΝΙΚΗ

Εισαγωγή: Ο μεταστατατικός ορθοκολικός καρκίνος (μΟΚΚ) αφορά περίπου 60% των ασθενών με ορθοκολικό καρκίνο και σχετίζεται με υψηλή θνητότητα. Η κατάσταση των γονιδίων *RAS* είναι ένας σημαντικός βιοδείκτης που καθοδηγεί τη θεραπεία προβλέποντας την ανταπόκριση στα μονοκλωνικά αντισώματα έναντι του υποδοχέα του επιδερμιδικού αυξητικού παράγοντα (epidermal growth factor receptor, EGFR). Ωστόσο, η εξέταση των γονιδίων *RAS* πραγματοποιείται παραδοσιακά μόνο σε αρχειακό υλικό ιστού από τον όγκο, χωρίς να παρέχει ανανεωμένη πληροφορία για την κατάσταση *RAS* στη διάρκεια της νόσου, καθώς στους περισσότερους ασθενείς η επαναβιόψηση δεν είναι δυνατή ή πρακτική. Οι υγρές βιοψίες είναι μια αναδυόμενη και υποσχόμενη εναλλακτική στη βιοψία ιστού, καθώς παρέχουν με ακρίβεια μια εικόνα του μοριακού προφίλ του όγκου σε πραγματικό χρόνο.

Σκοπός: Η μελέτη στοχεύει στη χρήση και τον έλεγχο της υψηλής ευαισθησίας πλατφόρμας υγρής βιοψίας ψηφιακής PCR BEAMing για την ανίχνευση μεταλλάξεων *RAS* σε μια κόορτη ασθενών με μΟΚΚ σε ένα συνεργαζόμενο δίκτυο ογκολογικών κέντρων στην Ελλάδα. Επιπρόσθετα, στοχεύει στη μελέτη της χρησιμότητας των επαναλαμβανόμενων υγρών βιοψιών, παράλληλα με τον standard έλεγχο ιστού, για την εξατομίκευση της θεραπευτικής στρατηγικής στον μΟΚΚ.

Μέθοδοι: Κλινικά δεδομένα και δεδομένα μοριακής ανάλυσης συλλέχθηκαν προοπτικά από τον φάκελο των ασθενών με μΟΚΚ. Δείγματα πλάσματος συλλέχθηκαν από ασθενείς με μΟΚΚ στο Πανεπιστημιακό Γενικό Νοσοκομείο Ιωαννίνων, το Πανεπιστημιακό Γενικό Νοσοκομείο Λάρισας και τη EUROMEDICA Γενική Κλινική Θεσσαλονίκης, στην Ελλάδα. Η ψηφιακή PCR BEAMing χρησιμοποιήθηκε για τον ορισμό της κατάστασης *RAS* στο κυκλοφορούν DNA (circulating tumour DNA, ctDNA) του όγκου σε τέσσερα στιγμιότυπα-«κλειδιά»: διάγνωση, μέσον της θεραπείας πρώτης γραμμής, πρώτη και δεύτερη επιδείνωση.

Αποτελέσματα: Εξήντα οκτώ ασθενείς με μΟΚΚ εντάχθηκαν στη μελέτη μεταξύ Ιανουαρίου 2018 και Οκτωβρίου 2019 με διάμεσο χρόνο παρακολούθησης 13.3 μήνες. Μεταλλάξεις *RAS* ανιχνεύτηκαν στον ιστό και στο ctDNA σε 51.5% και 47.8% των ασθενών,

αντίστοιχα, με ολική ποσοστιαία συμφωνία 72.3%. Η συμφωνία ιστού-ctDNA σχετιζόταν θετικά με την ύπαρξη ηπατικών μεταστάσεων (p=0.010) και αρνητικά με τη χαμηλή ευαισθησία της μεθόδου ελέγχου ιστού (p=0.092). Η κατάσταση *RAS* διέφερε μεταξύ της διάγνωσης και του μέσου της θεραπείας πρώτης γραμμής (p=0.046), καθώς το κλάσμα μεταλλαγμένων αλληλομόρφων *RAS* (*RAS* mutant allele fraction, *RAS* MAF) έγινε μη ανιχνεύσιμο σε 28.6% των ασθενών, και ο μέσος όρος του *RAS* μειώθηκε από 12.27% στη διάγνωση σε 5.63% στο μέσο της θεραπείας πρώτης γραμμής (p=0.147). Στην πρώτη επιδείνωση, νέες μεταλλάξεις *RAS* αναδύθηκαν σε σύγκριση με τη διάγνωση (p=0.014), καθώς 35.7% των ασθενών ανέπτυξαν νέα μετάλλαξη *RAS*, οδηγώντας σε μεταβολή της κατάστασης *RAS* από φυσιολογική σε μεταλλαγμένη στο 21.4% των ασθενών. Στη δεύτερη επιδείνωση, η κατάσταση *RAS* διατηρήθηκε στο 62.5% και στο 100% των ασθενών σε σύγκριση με τη διάγνωση και την πρώτη επιδείνωση, αντίστοιχα.

Συμπεράσματα: Η ανάλυση του ctDNA πλάσματος με την ψηφιακή PCR BEAMing βρέθηκε να έχει ικανοποιητική συμφωνία με τον ιστό και να ανιχνεύει αποτελεσματικά ακόμα και μικρές διαφορές τόσο στην κατάσταση *RAS* όσο και στο *RAS* MAF στα στιγμιότυπα που ελέγχθηκαν. Συνεπώς, μπορεί να χρησιμοποιηθεί παράλληλα με τον έλεγχο του ιστό στη διάγνωση και, αποτυπώνοντας τις δυναμικές αλλαγές *RAS*, μπορεί να υποβοηθήσει τη λήψη εξατομικευμένων θεραπευτικών αποφάσεων για τους ασθενείς με μΟΚΚ και τον σχεδιασμό μελλοντικών κλινικών δοκιμών.

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