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Review

Liquid Biopsy in Advanced Colorectal Cancer: Clinical Applications of Different Analytes

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Abstract: Colorectal cancer is one of the most prevalent cancers nowadays. In the metastatic setting, diagnosis and treatment have relied on tumor tissue analysis. However, the different limitations of this approach have recently opened the door to the introduction of liquid biopsy in the clinical setting. Liquid biopsy provides real-time information about the tumor and its heterogeneity in a simple, non-invasive, and repeatable way. There are several analytes that can be sought: exosomes, circulating tumor cells, and circulating tumor DNA, showing promising results in the areas of early detection, minimal residual disease, prognosis, or response to treatment. Here, we review the clinical applications of liquid biopsy in advanced colorectal cancer patients, focusing on metastatic diagnosis, prognostic assessment, drug sensitivity, treatment response, and acquired resistance monitoring.

Keywords: colorectal cancer; metastatic; liquid biopsy; exosomes; circulating tumor cells; circulating tumor DNA

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide, with 1.1 million new cases per year [1]. About 20% of patients present an advanced stage at the time of diagnosis, and about 50% of those with localized disease will develop metastases over time [2]. The overall survival (OS) at 5-year in patients with stage IV disease is estimated to be around 15%, with a median overall survival (mOS) of approximately 30–40 months [3]. To date, the treatment algorithm of advanced CRC patients is based on performance status and molecular features assessed on surgical samples or tumor tissue biopsy. In particular, the mutational status of the Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*), Neuroblastoma Rat Sarcoma Viral Oncogene Homolog (*NRAS*), v-Raf Murine Sarcoma Viral Oncogene Homolog B (*BRAF*), and Human Epidermal Growth Factor Receptor 2 (*ERBB2*) genes, together with the Microsatellite Instability (*MSI*) status, are routinely assessed [2,4]. In recent years, new molecular biomarkers, such as Tumor Mutational Burden (*TMB*) and Neurotrophic Tyrosine Receptor Kinase (*NTRK*) 1–3 translocations, have emerged [5]. Intratumoral heterogeneity and emerging genomic changes due to disease spread and treatments limit tissue sample reliability in providing real-time biological disease information. In addition, tissue samples may be degraded or may be difficult to repeat during disease history due to patient compliance or a lack of technical feasibility. In order to overcome the different limitations of tissue

biopsy, liquid biopsy is becoming increasingly important in clinical practice. Indeed, this is a non-invasive and repeatable approach, making it possible to provide a real-time picture of tumor heterogeneity to clinicians. Liquid biopsy can be performed from numerous body fluids, such as blood, saliva, stool, urine, cephalorachidian fluid, pleural fluid, or peritoneal fluid. Several analytes can be sought using this strategy; in particular, it is possible to search for Circulating Tumor Cells (CTCs), exosomes, and Circulating Tumor DNA (ctDNA). Each of these markers has been shown to play a role in early diagnosis, prognosis prediction, recurrence monitoring, treatment response assessment, and precision treatment guidance. Based on the prospective PRESEPT trial, the Food and Drug Administration (FDA) approved methylated Septin 9 (*SEPT9*) DNA detection by ctDNA in the CRC screening setting, with a sensitivity of 50% and specificity of 90% [6]. The overexpression of miR-17a and miR-130a analyzed at the level of exosomes has been shown to play a role in the early detection and prognosis of CRC [7]. In the case of early/localized disease and neoadjuvant therapies, thymidylate synthase+/RAD23 homolog B+ (*Tyms+/RADS23B+*) CTCs were found in up to 83% of patients without a clinicopathological response after neoadjuvant treatment; however, these CTCs were not detectable at baseline in patients with a complete or partial pathological response [8]. ctDNA has been shown to predict the risk of disease recurrence in both stage II and stage III patients [9,10]. Recently, Tie et al. conducted a prospective study in radically operated stage II patients, showing that a ctDNA-guided approach reduced the use of adjuvant chemotherapy without compromising the risk of recurrence compared with the standard strategy based on clinico-pathological high-risk characteristics [11]. In the metastatic setting, liquid biopsy may have important applications, both as a diagnostic and prognostic tool. Moreover, this strategy plays a role in the assessment of the treatment response, being able to predict it more effectively than known serum tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9). The role of liquid biopsy in metastatic CRC patients is not only limited to the evaluation of the response, thus leading to the early discontinuation of drugs that may be considered non-functional, but also in the assessment of primary and acquired resistance mutations that may direct the choice of first-line or subsequent-line treatment. In this context, there are several lines of evidence confirming a high concordance between the molecular result obtained by liquid and tissue biopsy in metastatic CRC patients across different treatment lines. Furthermore, Parikh et al. showed, in a prospective cohort of 42 patients with gastrointestinal cancers and acquired resistance to targeted therapies, that circulating free DNA (cfDNA) better identifies clinically relevant resistance mutations and other multiple mechanisms of resistance in 78% of cases than single-tissue biopsy [12].

In our review, we describe the potential roles of different liquid biopsy analytes (CTCs, exosomes, and ctDNA) in the management of metastatic colorectal cancer (mCRC) patients, analyzing their methodology and limitations, but focusing mainly on their clinical applications (Figures 1 and 2).

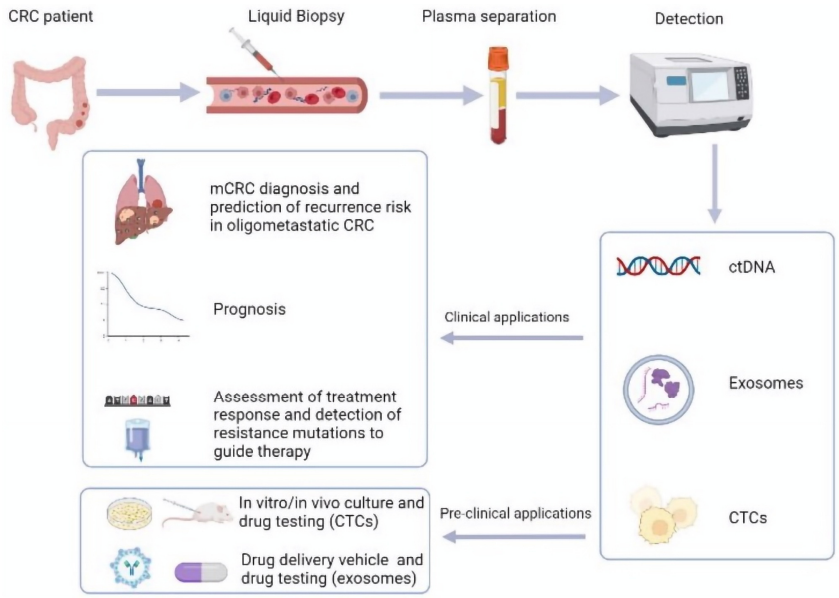

Figure 1. Liquid biopsy in mCRC. Liquid biopsy allows several analytes to be detected by blood sampling and plasma isolation: circulating tumor DNA (ctDNA), exosomes, and circulating tumor cells (CTCs). In a metastatic colorectal cancer (mCRC) patient, these analytes have several clinical applications: diagnosis of mCRC or assessment of the risk of recurrence in an oligometastatic patient, prognosis and assessment of response to treatment, and search for possible resistance mutations that may guide a clinician in the choice of therapy. In addition, these analytes may have pre-clinical applications: CTCs and exosomes can be used in drug testing using in vivo/in vitro cell cultures or by using the drug delivery vehicle mechanism, respectively.

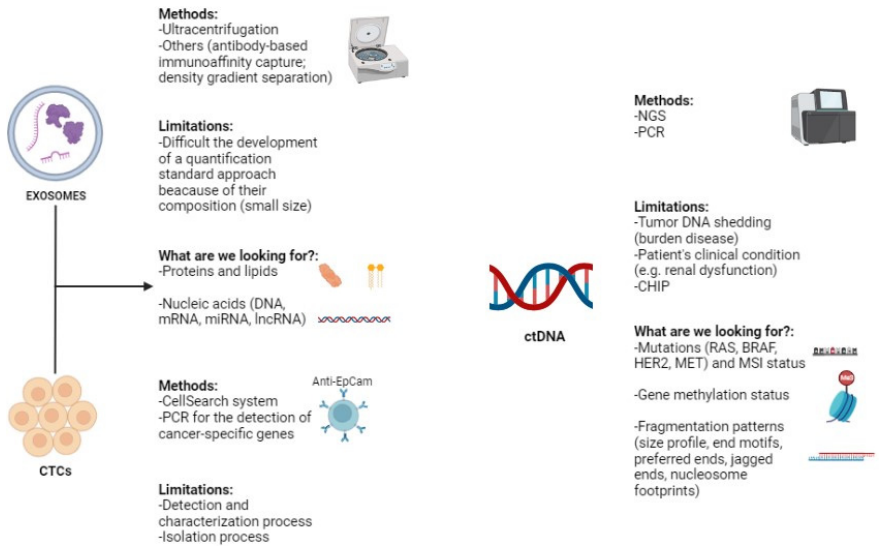

Figure 2. Liquid biopsy in mCRC (methods, limitations, and markers). Exosomes are isolated mainly by ultracentrifugation. Other techniques include antibody-based immunoaffinity capture and density gradient separation. The main limitation is the difficulty in the standard quantification approach. CTCs are isolated mainly with the CellSearch system. PCR is used for the detection of specific genes. The main limitations are the process of detection, characterization, and isolation. With both exosomes and CTCs, proteins, lipids, and nucleic acids (DNA, mRNA, miRNA, and lncRNA) are searched as markers. CtDNA is studied by NGS and PCR. The main limitations are tumor DNA shedding, the patient's clinical condition, and CHIP. With ctDNA, mutations, such as RAS, BRAF, HER2, and MET, and MSI status are searched for; in addition, gene methylation status and fragmentation patterns can also be studied.

2. Exosomes

2.1. Introduction, Methodology, and Limitations

Exosomes are extracellular vesicles of nanometric size (50–130 nm) enclosed in a lipid bilayer that protects them from degradation and makes them stable [13]. These small vesicles are secreted by various cell types and are present in almost all body fluids (blood, urine, cerebrospinal fluid, breast milk, saliva, etc.) [14]. Exosomes contain a variable spectrum of biologically active molecules, including nucleic acids (DNA, mRNA, microRNA, and long non-coding RNA), proteins, and lipids, reflecting the composition of the cells from which they originate, including cancer cells [13,15]. Several studies have shown that exosomes, by mediating communication between cells, play a role in tumor growth processes, metastatic niche formation, and immune system evasion [16]. The expression of proteins involved in altered cellular metabolic processes has been widely reported in exosomes isolated from patients with CRC [17]. In several studies, the concordance between molecular biomarkers detected in exosomes and tumor tissues has also been demonstrated [18–20]. These characteristics make exosomes very interesting analytes in the field of liquid biopsy.

Exosomes can be isolated by various methods, such as ultracentrifugation, density gradient separation, and antibody-based immunoaffinity capture, and it is also possible to analyze the expression levels of intra-exosomal non-coding RNAs [21,22]. Among these methods, ultracentrifugation is the gold standard for the isolation of exosomes, but requires a large amount of starting material, expensive equipment, and a lot of work. Commercial kits are available, but they are not standardized and involve the use of buffers that digest exosomal membranes, limiting the quantity and quality of analyses. Ultracentrifugation can also lead to the formation of particle clusters, limiting the characterization of individual vesicles [23]. Furthermore, their composition, but especially their small size, makes it difficult to develop a standard quantification approach [24].

2.2. Diagnosis, Prognosis, and Therapeutic Response Evaluation

Exosomes are being extensively researched for their possible role in the clinical management of mCRC patients, particularly for non-invasive diagnosis, tumor heterogeneity assessment, prognosis estimation, and therapeutic response monitoring (Table 1).

Exosomal surface proteins, such as Quiescin Sulfhydryl Oxidase 1 (*QSOX1*) [25], Glypican-1 [26], and Copine3 [26,27], have been identified as promising biomarkers for the non-invasive diagnosis of CRC, as they have been found at much higher levels in the exosomes of CRC patients than in those healthy subjects. Similarly, several exosomal microRNAs (miRNAs) have been found to be differentially expressed in CRC patients and identified as potential diagnostic biomarkers [28–31]. In a study conducted by Tsukamoto on 326 CRC patients, the expression levels of exosomal miR-21 were significantly correlated with those detected in tumor tissue samples [32]. Similar correlations were reported in subsequent studies for miR-122 [33] and miR-25-3p [34].

Silva et al. identified a miRNA signature for the diagnosis of CRC by analyzing more than 100 plasma samples obtained from subjects with either localized or metastatic CRC, as well as from non-oncological patients [30]. A classifier based on four miRNAs (miR-28-3p, let-7e-5p, miR-106a-5p, and miR-542-5p) was shown to discriminate cancer from non-cancer cases among the analyzed plasma samples. The overexpression of these miRNAs was confirmed by reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) and the data were externally validated. The sensitivity, specificity, and accuracy of the model were maintained when applied to tumor tissue samples [30].

The expression of miR-122 could help to specifically differentiate patients with and without liver metastases [33], and the overexpression of miR-320d proved to be useful in differentiating metastatic from non-metastatic CRC cases [35]. An Italian group monitored, for the first time, the mutational status of *KRAS* in CRC patients at different stages using exosomal DNA [36]. The expression level of exosomes was correlated with the extent of the disease. The resection of the primary tumor was correlated with a reduction in the *KRAS*

G12V/D copy number, while CEA expression and the presence of liver metastases were correlated with a higher copy number [36].

Table 1. Summary of the biological functions and detection modes of nucleic acids and exosomal proteins in metastatic colorectal cancer patients.

Exosomal	Molecule Type	Sample Type	Detection Technique	Trend	Application
QSOX1	protein	plasma	ultracentrifugation	downregulated	diagnostic
Copine3	protein	plasma	ELISA	upregulated	diagnostic prognostic
miR-21	miRNA	plasma	ultracentrifugation	upregulated	diagnostic prognostic
miR-122	miRNA	serum	Invitrogen Exosome Isolation Kit	upregulated	diagnostic prognostic
miR-25-3p	miRNA	serum	ultracentrifugation	upregulated	prognostic
miR-28-3p let-7e-5p miR-106a-5p miR-542-5p	miRNA	plasma	TaqMan Low-Density Array	upregulated	diagnostic
KRAS mut	ExoDNA	plasma	ultracentrifugation ddPCR	upregulated	diagnostic prognostic predictive
UCA1	lncRNAs	serum	ultracentrifugation ExoQuick	downregulated	diagnostic
CRNDE-h	lncRNAs	serum	ExoQuick	upregulated	diagnostic prognostic
miR-17-92a	miRNA	serum	ultracentrifugation	upregulated	prognostic
miR-320d	miRNA	serum	ultracentrifugation	upregulated	diagnostic
miR-203	miRNA	serum	ultracentrifugation	upregulated	prognostic
miR-17-5p miR-92a-3p	miRNA	serum	ultracentrifugation	upregulated	diagnostic
hsa-circ0004771	circRNA	serum	Invitrogen Exosome Isolation Kit	upregulated	diagnostic prognostic
miR-196b-5p	miRNA	serum	exoRNeasy Kit	upregulated	predictive (5-fluorouracil resistance)
miR-21-5p miR-1246 miR-1229-5p miR-96-5p	miRNA	serum	ultracentrifugation	upregulated	predictive (oxaliplatin/5- fluorouracil resistance)
miR-222	miRNA	serum	ultracentrifugation	upregulated	diagnostic prognostic
UCA1	lncRNAs	serum	ultracentrifugation ExoQuick	Upregulated	predictive (cetuximab resistance)

Abbreviations: ELISA: Enzyme-Linked ImmunoSorbent Assay; miRNA: microRNA; ddPCR: droplet-digital polymerase chain reaction; ExoDNA: exosome DNA; lncRNAs: long non-coding RNAs; circRNA: circular RNA; *UCA1*: urothelial cancer-associated lncRNA 1; *CRNDE-h*: colorectal neoplasia differentially expressed-h.

Long non-coding RNAs (lncRNAs) could also find application in the non-invasive diagnosis of CRC, as they have been reported to be differentially expressed in the exosomes of CRC patients [37]. For instance, urothelial cancer-associated lncRNA 1 (*UCA1*) was downregulated in CRC [38], whereas the differentially expressed lncRNA of colorectal neoplasm-h (*CRNDE-h*) was upregulated compared with normal controls. In particular, the *CRNDE-h* levels were significantly associated with metastatic disease ($p = 0.003$) [39], and the exosomal circular RNA hsa-circ0004771 was also correlated with the presence of distant metastases [40].

As reported by several studies, exosomes are involved in the progression of CRC due to their role in mediating intercellular cross talk [41,42]. The exosomal load in mCRC, therefore, has prognostic importance.

Chen and colleagues extracted exosomes from the serum of CRC patients and identified 36 upregulated proteins involved in pro-metastatic modulation processes of the tumor microenvironment and 22 downregulated proteins involved in cell growth and survival [43]. The study showed that exosomes in CRC patients played a central role in promoting tumor invasiveness [43].

Exosomal dipeptidyl peptidase IV (*DPP4*) was identified as a potent inducer of angiogenesis through the activation of the Small Mother Against Decapentaplegic (*SMAD*) signaling pathway [44]. When *DPP4* was inhibited, tumor growth was suppressed in in vivo models. In a study on 5-fluorouracil (5-FU)-resistant CRC, exosomal *DPP4* concentrations were proposed as a useful prognostic marker, as they were correlated with the metastatic process [44].

The overexpression of exosomal miRNAs has recently been correlated with tumor progression and the development of liver metastases in CRC. For instance, the expression of the miR-17-92a cluster was associated with CRC recurrence and poorer prognosis if miR-17-92a was expressed at higher levels [45].

miR-25-3p miRNA can be transferred from CRC cells to endothelial cells via exosomes, promoting vascular permeability and angiogenesis [34]. It has been reported that exosomal miR-25-3p facilitates the formation of liver and lung metastases in mouse models of CRC and that the expression level of exosomal miR-25-3p in the serum of patients with mCRC was significantly higher than that in patients without metastases [34].

Another molecule that has been identified as a mediator of signaling between tumor cells and monocytes in patients with mCRC is miR-203, which promotes the differentiation of monocytes into tumor-associated M2 macrophages and has been associated with the development of liver metastases [46]. miR-203 has also been established as an independent negative prognostic factor in patients with CRC [46].

Microarray analysis of miRNAs showed the upregulation of miR-21 in exosomes, primary tumor tissue, and liver metastases [32]. Significant correlations were demonstrated between the exosomal miR-21 levels, TNM staging, and prognosis of CRC patients. The expression level of miR-21 was identified as an independent prognostic factor for OS and disease-free survival (DFS) in patients with stage II or III CRC and for OS in stage IV patients [32].

Similar data were reported for the molecules miR-17-5p and miR-92a-3p [47].

High levels of miR-222 at 24 weeks after the start of bevacizumab treatment have been shown to be associated with shorter survival in patients with mCRC [48].

Several studies have been conducted to assess a possible correlation between exosomal load and chemotherapy resistance in patients with CRC [49,50]. Exosomes could be an important element in the assessment of the treatment response and could represent biomarkers of treatment sensitivity and resistance [51]. Cancer Stem Cells (CSCs) are intrinsically resistant to chemotherapy [52]. Exosome-mediated communication with stromal fibroblasts can induce the de-differentiation of tumor cells into stem cells, promoting chemoresistance in CRC [53]. The main regulator of fibroblast-mediated reprogramming is the exosomal Wingless-related Integration Site (*Wnt*); thus, interfering with exosomal *Wnt* signaling could result in chemoresistance reduction [53].

The activation of the Extracellular Signal-Regulated Kinase/Protein–Kinase B (*ERK/AKT*) pathway by exosomes derived from CRC-associated fibroblasts appears to promote oxaliplatin resistance [54]. miR-196b-5p stimulates the maintenance of stem cell properties and the resistance of CRC cells to 5-FU via activation of the Signal Transducer and Activator of Transcription (*STAT3*) signaling pathway [55].

Recently, a panel of exosomal serum miRNAs containing miR-21-5p, miR-1246, miR-1229-5p, and miR-96-5p was developed to effectively distinguish 5-FU chemotherapy-resistant CRC cell lines from the chemo-sensitive control group [56]. High plasma levels of exosomal *UCA1* have been shown to correlate with an inferior response to cetuximab treatment [57].

In addition to diagnostics and treatment monitoring, exosomes can also be used for therapeutic purposes to efficiently deliver drugs into various types of cancer cells, acting as vectors with low immunogenicity and high biocompatibility. Liang and colleagues used engineered exosomes to simultaneously deliver 5-FU and a miR-21 inhibitor oligonucleotide (miR-21i) to a 5-FU-resistant CRC cell line [58]. The engineered co-delivery system was able to efficiently facilitate the cellular uptake of the delivered molecules, reversing chemoresis-

tance and significantly lowering miR-21 expression in cell lines and ultimately reducing tumor growth [58]. In another recent study, exosomes isolated from A33-positive cells were loaded with doxorubicin, generating an A33 antibody complex (A33Ab-US-Exo/Dox) aiming to target A33-positive CRC cell lines [59]. The A33Ab-US-Exo/Dox complex was shown to have excellent tumor-targeting and growth-inhibiting capacity for the target cancer cells [59].

Exosomes, therefore, perform a wide range of biological functions by transporting signaling molecules that regulate various cellular processes. Exosomes also contribute to CRC development and tumor invasiveness. Their easy extraction from a wide variety of biological fluids makes exosomes an attractive means to detect diagnostic, predictive, and prognostic information in mCRC patients. However, although preclinical data seem very promising, large studies are needed to assess the repeatability of the method and the applicability of exosomes as biomarkers for clinical management and therapeutic decisions.

3. Circulating Tumor Cells

3.1. Introduction, Methodology, and Limitations

CTCs are epithelial cancer cells that shed from the primary tumor and, after extravasation from the blood vessels through the epithelial–mesenchymal transition (EMT), acquire migration capacities and thus are considered one of the primary triggers of the metastasis process [60–62]. CTCs carry information from their primary tumor and newly gained phenotypic and genotypic characteristics that confer survival capacity in the circulating microenvironment that protects them from the immune system recognition and attack. These changes also provide them with interaction capacities with adhesion molecules and epithelial cells, as well as secondary metastatic niche formation potential [61,62]. CTC dynamics are also driven by their interaction with circulating proteins and growth factors, such as Fibroblast Growth Factor (*FGF*) and Vascular Endothelial Growth Factor (*VEGF*), generated by macrophages in the tumor microenvironment [62,63].

Conversely, the role of other immune cells and circulating components in CTC physiology has been described. Platelets play a fundamental role in CTCs' protection and survival, helping them to evade cancer-associated fibroblast attack and promoting neutrophil recruitment at tumor sites, functioning as a shield against immunity [61,62,64]. Likewise, platelets can produce transforming growth factor beta (*TGF-β*), which facilitates EMT and vascular permeability [62,65]. In the case of neutrophils, their interaction with CTCs occurs through the upregulation of cell adhesion proteins, such as cadherin, integrin, and glycoproteins, as well as the release of NETS (Neutrophil Extracellular Traps), which are complexes of histone DNA and proteins that capture and promote CTC intravasation [61,66].

Detection techniques generally require the recognition, isolation, and separation of CTCs from other components by physical and biological differentiation (cell density, size, deformability, and polarity), followed by subsequent enrichment steps [67]. Among such techniques, those based on immune-affinity positive cell selection allow the recognition of epithelial or tumor surface antigens, such as cytokeratin (CK) 18, 19, and 20, and Epithelial Cell Adhesion Molecules (EpCAMs, CEA, and ERBB2). There are also immune-affinity negative selection techniques, which exclude circulating non-CTC cells based on the detection of leukocyte surface antigens usually not present on CTC membranes (i.e., CD45 and CD61) [60]. To date, the CellSearch system has been one of the most used techniques for recognizing CTCs, which is based on a positive selection through epithelial cell adhesion molecule antibody (EpCAM-based antigen detection) recognition, immunostaining for cytokeratin and epithelial cell markers, and CD45 leukocyte marker cell exclusion [67–69]. Other techniques use the Polymerase Chain Reaction (PCR) for the detection of cancer-specific genes in the selected cells without prior enrichment [60].

The use of CTCs in clinical practice is limited by technical difficulties regarding the detection and characterization processes due to their low frequency and counts in peripheral blood samples (1–10 CTCs/mL of whole blood) compared with 10^7–10^8 in circulating leukocytes [67,70], which is mainly related to their elimination by the immune

system. Other limitations regarding the isolation process from other circulating cellular components are mainly related to their short half-life and the downregulation of CTC-associated markers [37,71], such as those of the epithelial lineage (EpCAM) usually detected by standard screening platforms, in favor of a mesenchymal phenotype [60,62].

3.2. Metastatic Diagnosis, Prognosis, and Therapeutic Response Evaluation

Multiple studies have focused on the diagnostic, prognostic, and predictive potential of CTCs and their role in early and advanced diagnosis, as well as dynamic change monitoring in response to treatment. Particularly in the case of CRC, the quantification of CTCs has served as a differential marker for discerning between benign and malignant diagnoses. Tsai et al. conducted a study to assess the diagnostic role of CTCs. This prospective trial quantified CTCs in patients with premalignant lesions, as well as colon tumors at different stages, in comparison with clinical diagnostic tests (i.e., enteroscopy). The results showed 88% accuracy, with a false positive rate of only 3.3% in healthy controls, as well as a false negative rate of 16% in patients with malignant lesions [72]. Healthy patients (2.11 CTCs) could be distinguished from those with adenomas (5.83 CTCs; $p = 0.0001$) or CRC (16.99 CTCs; $p = 0.0001$); furthermore, the mean CTC count for patients with advanced CRC (III-IV stage) was significantly different from the mean CTC count for patients with early stage CRC ($p = 0.015$) and adenomas ($p = 0.016$) [72].

A prospective study by Cohen et al. investigated a potential relationship between CTCs and radiological imaging response parameters, and prognosis in terms of Progression-Free Survival (PFS) and OS in mCRC patients, with a CTC cutoff of $\geq$3 CTC/7 mL. The results showed that CTC counts were significantly related to the disease's aggressiveness in terms of poor clinical–radiological prognosis. Indeed 20% of CRC patients with radiological progression and 27% of deceased patients had unfavorable CTC counts in both study cutoffs (3 and 5 weeks after treatment start), compared with only 7% of the not-progressing subgroup [73]. The study also reported a sensitivity and specificity of 27% and 93%, respectively, in terms of concordance with radiological findings. Likewise, predictive and prognostic relationships were found, with worse median Progressive-Free Survival (mPFS) and mOS in both patients with poor baseline CTCs (mPFS 4.5 months vs. 7.9 months; $p = 0.0002$; mOS 9.4 months vs. 18.5 months; $p < 0.0001$) and those with persistently elevated CTCs throughout treatment [73]. Another study by Camera et al., focusing on the dynamic evolution of biomarkers (cfDNA, CEA, and CTC), as well as radiological changes as prognostic factors of OS in mCRC patients, failed to show a significant association with outcomes, while only baseline elevation of such biomarkers, especially CTC, predicted worse OS (HR 6.5) [37,74].

Conversely, CTC counts and their characterization can help to predict disease recurrences in CRC patients. In particular, CTC counts before and after tumor resection can predict the risk of metastasis, with both baseline and one-month-after high CTC loads anticipating poor DFS and OS in patients with metastatic, surgically resected liver metastasis [60,75]. Pan et al. also showed that CTC counts were correlated with tumor T, N, and M staging, with a directly proportional relationship between higher stage and CTC counts in both right- and left-side disease [76]. This correlation between higher CTC counts and worse prognosis was also confirmed in a meta-analysis by Tan et al., taking into account 15 studies and >3000 patients, showing that the presence of CTCs was correlated with worse OS (HR 2.28) and PFS (HR 1.53). Conversely, the multivariate analysis in the same study showed that the worse OS and PFS were independent of CTC detection timepoints (baseline or along treatment) [77].

Concerning the detection of the *KRAS* mutation, Fabbri et al. identified a 50% concordance between tissue sample and CTC molecular analyses in patients with mCRC using a dielectrophoresis-based platform able to detect and sort pure CTCs [78].

The role of CTCs as a dynamic monitoring tool and treatment response predictor has also been largely investigated. The study by Delgado-Urena et al. showed a concordant

decrease in CTC counts throughout treatment in patients with mCRC experiencing a favorable clinical response or at least stable disease by RECIST [79].

In addition, CTC counts may also play a role in treatment tailoring. In this context, the VISNU1-Trial investigated the potential role of poor CTC counts ($\geq$3) as a biomarker guiding treatment decisions. After comparing triple versus double therapy (FOLFOXIRI vs. FOLFOX), the study found that the former was associated with better mPFS (12.4 months vs. 9.3 months; HR 0.64, 95% CI 0.49–0.82; $p = 0.0006$) in patients with poor baseline CTC counts; ECOG 1, *RAS*, and *BRAF* mutations with a CTC count of $\geq$20 were considered as independent negative prognostic factors in this patient cohort [80].

A prospective study conducted in mCRC *KRAS* Wild-Type (WT) patients, mostly (90%) receiving an anti-Epidermal Growth Factor Receptor (*EGFR*) antibody during systemic treatment, evaluated the response-predicting and prognostic role of CTCs. Patients who achieved favorable kinetics, with CTCs below the established median, had a better mPFS than those with unfavorable kinetics (14.7 months vs. 9.4 months; $p = 0.02$) [81].

Finally, the role of CTCs in predicting treatment resistance has been considered. In this way, it has been seen that, throughout treatment, CTCs express resistance traits derived from the overexpression of genes related to the mammalian Target of Rapamycin (*mTOR*) and PhosphatidylInositol 3-Kinase (*PI3K*)/*AKT* cascades, which are usually associated with cell growth, proliferation, and chemotherapy resistance [82]. Similarly, the overexpression of CTCs characterized by upregulated xenobiotic metabolism enzymes and enhanced cytochrome p450 activity, responsible for detoxifying and metabolizing drugs, justifies the lower effect and final resistance to chemotherapy (ChT) [82,83]. In this sense, a study by Grillet et al. showed a lower response to in vitro FOLFIRI ChT schemes in CTCs compared with primary tumor cells ($p = 0.0109$), which was likely related to higher expression of irinotecan-resistance genes, such as UDP-glucuronosyltransferase 1-1 (*UGT1A1*) and ATP-binding cassette super-family G member 2 (*ABCG2*) in CTCs [83].

CTCs can be isolated from individual patients and grafted into mice (CTC-Derived Xenografts, CDX) in order to create a patient-specific model on which specific therapies can be tested, biomarkers of response can be evaluated, and resistance mechanisms can be detected [84]. Grillet et al., based on the hypothesis that some CTCs can develop the CSC phenotype, generated multiple cell lines of CTCs from mCRC patients. They observed how the CTC lines expressed CSC markers with multilineage differentiation ability, both in vitro and in vivo, with the ability to produce liver metastasis after intrasplenic injection in xenografts [83].

4. Circulating Tumor DNA

4.1. Introduction, Methodology, and Limitations

cfDNA were first detected by Mandel and Metais in 1948 in healthy patients [85]. However, only in 1989 did Stroun first report that some ctDNA in oncological patients was derived from tumor cells [86].

cfDNA consists of single- and double-stranded DNA fragments ($\leq$200 pb) released into bodily fluids by apoptotic or necrotic cells. While cfDNA is mostly derived from normal healthy leukocytes and stromal cells, in the context of malignancies, cfDNA can also incorporate DNA released by tumor cells, known as ctDNA. ctDNA accounts for 0.01 to 90% of the total cfDNA, depending on the tumor type, biological behavior, and cancer stage [87]. Furthermore, CRC is a solid tumor shedding the highest amount of ctDNA into the bloodstream [88,89].

In recent years, several technologies have emerged to detect ctDNA, including PCR-based approaches and next-generation sequencing (NGS). In particular, NGS allows the detection of multiple genes in terms of copy number or fusions at higher cost and lower sensitivity. On the other hand, digital PCR (dPCR) presents the highest quantitative sensitivity with restricted gene coverage.

In general, cfDNA is used to discover mutations, copy number aberrations, microsatellite alterations, differential cfDNA lengths, and methylation status [90]. At present, it is

believed that ctDNA may be useful for risk classification, early recurrence detection in CRC, and as a predictive biomarker of responses to systemic treatment (for adjuvant and metastatic therapy), as we are going to analyze below.

Despite the advantages of ctDNA over a tissue-based approach, logistical and biological limitations are restricting its clinical application. From a biological point of view, the first and the most relevant limitation is tumor DNA shedding, which is known to correlate with tumor burden and with the localization of tumor metastasis, ultimately affecting sensitivity and the false negative rate [91–93]. Furthermore, cfDNA clearance is dependent on renal function; thus, ctDNA analyses in certain patients with renal dysfunction warrants caution [94]. Another important limitation is the CHIP (Clonal Hematopoiesis of Indeterminate Potential) mutations, aberrations derived from the DNA of hematopoietic stem cells that can also affect genes implicated in solid tumors (e.g., Tumor Protein P 53, *KRAS*) and hematological diseases (e.g., Tet Methylcytosine Dioxygenase 2, Janus Kinase 2), causing an increase in the false positive rate [95].

4.2. Metastatic Diagnosis and Prognosis

There is increasing evidence supporting a strong concordance between metastatic disease diagnosis and high detection sensitivity of ctDNA analysis in CRC patients due to the great amount of shedding of circulating tumor fragments (including cells, DNA, methylation markers, etc.) [96]. Variability in the detection of this analyte is known to change according to tumoral burden: liver disease is associated with higher detection rates compared with either peritoneal or lung metastases. Despite certain limitations, such as the presence of barriers (ascites, pleural effusion, etc.) or clonal heterogeneity, there is evidence confirming that it is a useful tool with high sensitivity and specificity, both in diagnostic and prognostic CRC scenarios [37,96]. The positive association between the ctDNA levels and stage IV disease has been demonstrated in different studies, suggesting that a higher ctDNA concentration correlates with disseminated disease and increased tumor burden [97,98]. Olmedillas-López et al. performed whole-exome sequencing of ctDNA in metastatic and non-metastatic patients, showing a higher level in the former group, although it was not statistically significant; in addition, a "Differential Presence of Exone (DPE)" was identified, allowing the distinction between the two groups [99]. Another study reported ctDNA levels according to tumor stage: patients with stage IV had significantly higher values than those with stage I ($p = 0.0149$), with the increase being directly proportional to the size of the tumor [100].

Regarding the prognosis of mCRC patients, a meta-analysis including 1076 patients from ten studies showed a favorable OS for patients with low median ctDNA levels (HR 2.39, 95% CI 2.03–2.82; $p < 0.0001$). Overall, cfDNA levels above the median value were significantly associated with poor prognosis (HR 2.01, 95% CI 1.54–2.62; $p < 0.0001$) [101]. A post hoc analysis of the NORDIC-VII study showed in mCRC patients that those with a cfDNA above the predefined normal limit had a worse mOS than those with a lower value (16.6 months vs. 25.9 months, HR 1.83, 95% CI 1.51–2.21; $p < 0.001$) [102]. ctDNA was also found to play a prognostic role in oligometastatic operated patients. In a study of 54 patients with Resectable Colorectal Liver Metastases (CRLMs), those with detectable ctDNA in the postoperative setting had significantly worse Recurrence-Free Survival (RFS) (HR 6.3; 95% CI 2.58–15.2; $p < 0.001$) and mOS (HR 4.2; 95% CI 1.5–11.8; $p < 0.001$) than patients with undetectable ctDNA [103]. A more recent prospective study confirmed this trend. Of 96 patients with resected liver metastases, those with postoperative ctDNA or undergoing adjuvant chemotherapy had a lower RFS than those without ctDNA (HR 4.5, $p < 0.0001$ and HR 8.4, $p < 0.0001$). Furthermore, the ctDNA status proved to be a stronger predictor of recurrence than standard clinical risk factors and CEA levels [104].

A diagnostic and prognostic role in mCRC has also been studied with the hypermethylation of genes identified by ctDNA analysis [105]. Some methylated ctDNA markers have been reported as predictors of disease prognosis of mortality, such as ranched Chain Amino Acid Transaminase 1 (*BCAT1*), IKAROS Family Zinc Finger 1I (*KZF1*), and Interferon Regu-

latory Factor 4 (*IRF4*) [106]. Picardo et al. demonstrated how downregulated expression and hypermethylation of the Beta-1,4-Galactosyltransferase 1 (*B4GALT1*) promoter has a negative prognostic impact on mCRC [107]. Philipp et al. detected a lower OS in mCRC patients with the methylation of helicase-like transcription factor (*HLTF*) or hyperplastic polyposis 1 (*HPP1*), or elevated levels of CEA ($p < 0.0001$ for all) [108].

4.3. Therapeutic Response Evaluation

Several studies have shown that early changes in ctDNA levels can predict the radiological response to standard chemotherapy in mCRC. Tie et al. demonstrated, in 53 patients treated with first-line chemotherapy, that a fold reduction of $\geq$10 in ctDNA at two weeks of treatment predicted the clinical response at a CT scan performed at eight weeks ($p = 0.016$). The decline in this analyte from the pre-treatment period to the second cycle of therapy was the best predictor of the radiological response ($p = 0.004$ vs. $p = 0.253$ of absolute ctDNA) [109]. In the prospective PLACOL trial, mCRC patients treated with a first- or second-line treatment who had a ctDNA reduction of $\geq$80% achieved a better Objective Response Rate (ORR) (47.1% vs. 0%; $p = 0.03$), better mPFS (8.5 months vs. 2.4 months; HR 0.19, 95% CI 0.09–0.40; $p < 0.0001$), and mOS (27.1 months vs. 11.2 months; HR 0.25, 95% CI 0.11–0.57; $p < 0.001$) [110]. Studies were also performed to assess the response to regorafenib and TAS-102 by ctDNA analysis. Patients who had an early decline in this analyte in the plasma experienced better PFS in contrast with those with a minimal change or increase in the ctDNA levels [111–113].

In addition, ctDNA liquid biopsy has been shown to play an important role in evaluating responses to immunotherapy and, in particular, in assessing pseudoprogression. This event could manifest at the beginning of immunotherapy treatment in some solid tumors and not be related to disease progression [114]. There are some prospective trials that will hopefully provide more knowledge regarding the optimal use of ctDNA in those cases. In melanoma patients treated with anti-PD-1 antibodies, ctDNA showed a sensitivity of 90% (95% CI, 68–99%) and a specificity of 100% (95% CI, 60–100%) in predicting pseudoprogression [115]. In a study evaluating treatment with regorafenib and nivolumab or pembrolizumab in 18 pre-treated Microsatellite-Stable (*MSS*) mCRC patients, the increase in ctDNA level at 4 weeks predicted progression at 2 months, and the decline predicted disease stability [116].

Aberrant hypermethylation also plays an important role in measuring ctDNA and the treatment response in mCRC. There are also some reports [110] describing a correlation between decreasing levels of methylated genes and response to treatment, even before radiological findings (RECIST criteria). Furthermore, hypermethylated neuropeptide Y circulating tumor DNA (meth-NPY) has recently been studied as an early indicator for a loss of treatment effect and early indicator of progression in mCRC patients in the FOLFOXIRI-Toco trial [37,117].

4.4. Detection of RAS Mutations

KRAS (exon 2, 3, or 4) and *NRAS* (exon 2, 3, or 4) mutations are detected in approximately 44% and 4% of patients with mCRC [4]. These are known to be negative predictive biomarkers for anti-*EGFR* antibody therapies, as already demonstrated in numerous prospective and retrospective trials [118,119]. Several studies have demonstrated the possibility of determining *RAS* mutations by ctDNA using new technologies. Taly et al. investigated the utility of multiplex dPCR to screen for the seven most-common mutations in codons 12 and 13 of *KRAS* from the plasma of mCRC patients. Out of 50 patients, 19 were identified to have these alterations by ctDNA analysis and 14 coincided with tissue alterations; conversely, 2 *KRAS* mutations were identified among those who were *KRAS* WT by tissue analysis [120]. In a prospective study of 106 patient samples, a quantitative PCR-based method to detect the seven point mutations of *KRAS* showed a specificity of 98% and a sensitivity of 92%, with a concordance value of 96% [121]. Bettegowda et al. demonstrated a ctDNA sensitivity and specificity of 87.2% and 92.2%, respectively, in

determining *KRAS* mutations in advanced CRC patients [88]. Vidal et al. conducted an OncoBEAM analysis of *RAS* alterations in tissue and plasma samples from 115 mCRC patients and found 93% overall agreement; the authors identified the site of metastasis (peritoneal and lung), mucinous histology, and prior treatment as factors negatively impacting *RAS* detection in ctDNA [122]. Two other analyses showed a concordance of 97% and 91.8%, respectively [123,124], while Grasselli et al. identified a 90% agreement between standard of care (SoC) PCR techniques and dPCR (BEAMing) for plasma molecular analysis [125]. A prospective trial conducted on 425 chemotherapy-naive patients with liver metastases showed an accuracy in determining *RAS* status of 93.5% with NGS and 97% with NGS plus methylated biomarkers [126]. The high concordance rates from these studies may allow clinicians to use the analysis of the plasma RAS status to select patients who are WT and thus sensitive to anti-*EGFR* treatment. In the CAPRI-GOIM trial, the status of *RAS* mutations in ctDNA was retrospectively investigated in *KRAS* WT patients who received first-line treatment with FOLFIRI plus cetuximab; WT patients had better PFS and OS than those with mutations considering both tissue and liquid biopsy [127]. ctDNA is also effective in detecting primary resistance mutations to anti-*EGFR* antibody therapies. Siravegna et al. retrospectively analyzed the ctDNA of WT patients refractory to anti-*EGFR* agents with an NGS panel of 226 genes. Molecular alterations related to intrinsic resistance to cetuximab or panitumumab, such as aberrations in *EGFR*, Fms-like Tyrosine Kinase 3 (*FLT3*), Mitogen-activated Protein Kinase Kinase 1 (*MAP2K1*), and *ERBB2*, were detected in about 50% of the study population [123].

The change in the *RAS* mutation status in ctDNA could also play a predictive role in predicting the therapeutic response in CRC patients. In a study conducted by Sunakawa in 62 mutated *RAS* patients, the FOLFOXIRI + bevacizumab triplet exhibited an mPFS of 12.1 months, an mOS of 30.2 months, and an ORR of 75.8%. In 78% of patients with pre-treatment ctDNA *RAS*-positive disease, post-treatment ctDNA analysis at 8 weeks showed that *RAS* mutations disappeared, predicting better outcomes than those with *RAS* mutation persistence on liquid biopsy (mOS 16 months vs. 27.2 months, respectively; HR 0.53, 95% CI 0.23–1.21, $p = 0.12$) [128]. The *KRASp.G12C* mutation accounts for around 10% of *KRAS* mutations in mCRC, and several specific inhibitors have demonstrated efficacy in terms of response [129,130]. In a 5-year retrospective analysis conducted by Thein et al., a 3.5% rate of *KRASp.G12C* mutation was found in the ctDNA of mCRC patients, with a concordance of 100% and 62% to tissue molecular analysis, considering times between the two approaches of less or more than 6 months, respectively [131].

4.5. Rechallenge with Anti-EGR Antibodies

It is known that mCRC *RAS* and *BRAF* WT patients are sensitive to the *EGFR* blockade, but often, despite an initial response to treatment, acquired resistance mutations arise. These aberrations are mostly activating mutations of *RAS* and point mutations of the extracellular domain of *EGFR* (ECD), although amplifications of Mesenchymal-epithelial Transition Tyrosine Kinase Receptor (*MET*) and *ERBB2* are sometimes detected [123,132,133]. It has been shown that *KRAS* and *EGFR* ECD mutations may be non-mutually exclusive [132]. In this context, ctDNA can easily and effectively identify multiple mechanisms of resistance in the same patient. Recently, Topham et al. identified a panel of 12 genes via ctDNA with an increased frequency of mutations after anti-*EGFR* therapy [134]. In a series of 24 patients, approximately 40% of them developed *KRAS* mutations after panitumumab monotherapy; the median time of onset of these aberrations was between 5 and 6 months following treatment, indicating that they were present in expanded subclones before therapy [135]. Van Burgher et al. showed that patients who developed *EGFR* ECD variants had a longer and greater response than those with the emergence of *RAS* mutations, who experienced limited responses and worse PFS. Furthermore, the detection of *RAS* aberrations prior to exposure to anti-*EGFR* antibody therapy compared with *EGFR* ECD mutations indicates that the latter are de novo resistance mutations [136].

Therefore, under the selective pressure of anti-*EGFR* antibody treatment, pre-existing resistant subclones may expand and de novo mutations may develop, leading to resistance and disease progression. In the meantime, subsequent therapy could reduce the proliferation of resistance clones, potentially restoring anti-*EGFR* therapy sensitivity (122). Parseghian et al. pointed out that, after the discontinuation of panitumumab or cetuximab, mutant *RAS* and *EGFR* clones decay with a half-life of 4.4 months [137]. The early identification of resistance mutations by ctDNA could allow the discontinuation of an ineffective treatment, complementing radiological assessments. In addition, numerous studies have retrospectively and prospectively evaluated the role of ctDNA-guided rechallenge with anti-*EGFR* antibodies in CRC patients (Table 2). The CAPRI-GOIM trial showed that *KRAS*, *NRAS*, *BRAF*, and *PIK3K* WT patients, from tissue biopsy at baseline, benefited in terms of PFS from rechallenge with cetuximab [138]. Santini et al. prospectively studied the efficacy of a rechallenge with cetuximab plus irinotecan-based therapy in 39 heavily pre-treated patients. The mPFS was 6.6 months and the ORR was 53.8%, of which CR accounted for 5.1% and PR accounted for 48.7% [139]. These results were confirmed by another phase II trial where third-line rechallenge with irinotecan plus cetuximab demonstrated a PFS and OS of 2.4 and 8.2 months, respectively [140]. The role of ctDNA in guiding the rechallenge strategy was first demonstrated in the CRICKET trial. The 28 mCRC *RAS* and *BRAF* WT patients enrolled achieved 21% ORR and 54% disease control rate (DCR) with a third-line treatment of irinotecan plus cetuximab. No *RAS* mutations were identified with ctDNA in the patients who obtained a PR. Furthermore, WT ctDNA patients had a better PFS than those with *RAS*-mutated ctDNA (4 months vs. 1.9 months; HR 0.44, 95% CI 0.18–0.98; $p = 0.03$) [141]. The CAVE trial demonstrated the efficacy of the combination of cetuximab and avelumab as a rechallenge. *RAS/BRAF* WT ctDNA patients had an OS of 17.3 months compared with 10.4 months in ctDNA-mutated patients (HR 0.49, 95% CI 0.27–0.90; $p = 0.02$); this advantage was also confirmed in terms of PFS (4.1 months vs. 3 months; HR 0.42, 95% CI 0.23–0.75; $p = 0.004$) [142]. A phase II study demonstrated the efficacy of Sym004, a combination of two anti-*EGFR* antibodies, futuximab and modotuximab, in mCRC patients who had progressed to either panitunumab or cetuximab and maintained a triple WT status for *RAS*, *BRAF*, and *EGFR* ECDs [143]. The first study to investigate the prospective role of ctDNA in guiding the rechallenge strategy was the CHRONOS trial. *RAS/BRAF* WT mCRC patients in tumor tissue analysis, with a PS ECOG 0–2, should have obtained a PR or CR with an anti-*EGFR*-based first-line treatment. After progression to a second anti-*EGFR*-free regimen, patients underwent interventional ctDNA-based screening, and those without acquired resistance mutations received treatment with panitumumab. Of 52 patients, 16 (31%) were excluded because they had at least one alteration that conferred resistance to anti-*EGFR* therapy. Twenty-seven WT patients were enrolled, and 30% achieved a PR, with a DCR of 63%; a PFS and OS of 16 and 55 weeks were also recorded, respectively [144]. Data from the phase 2 BEYOND study have recently been published. Thirty-one WT mCRC *RAS* patients who received first-line treatment with FOLFOX plus panitumumab and who persisted WT in ctDNA after progression received second-line treatment with FOLFIRI plus panitumumab (arm A) versus FOLFIRI alone (arm B). Despite closure for inadequate recruitment, mPFS of 11 months versus 4 months in favor of the anti-*EGFR* arm (HR 0.58, 95% CI 0.25–1.3) and an ORR of 33% and 7.7%, respectively, were observed; however, serious adverse events were more frequent in arm A (44% vs. 23%). At progression, *RAS* mutations were detected by liquid biopsy in 36% and 20% of patients, respectively [145].

Table 2. Principal rechallenge studies with anti-*EGFR* antibodies in mCRC.

Trial Name and Author	Study Type	Rechallenge Treatment	Line	N	OS	PFS	ORR	ctDNA Selection
Santini et al.	Retrospective	Irinotecan + cetuximab	≥3rd	39	NR	6.6 m	53.8%	None
Liu et al.	Retrospective	Cetuximab ± erlotinib	≥2nd	89	NR	4.9 m for prior responder vs. 2.5 m for prior no-responder ($p = 0.064$)	NR	None
Tanioka et al.	Retrospective	Irinotecan + cetuximab	≥3rd	14	NR	4.4 m	21.4%	None
Rossini et al.	Retrospective	FOLFIRI + cetuximab/FOLFOX + panitumumab/CapIri + cetuximab/Irinotecan + panitumumab/Irinotecan + cetuximab/Cetuximab/Panitumumab	≥3rd	86	10.2 m	3.8 m	19.8%	None
Karani et al.	Retrospective	Cetuximab ± CT	≥3rd	17	7.5 m	3.3 m	18%	None
Chong et al.	Retrospective	Anti-*EGFR* ± CT	≥2nd	22	7.7 m	4.1 m	4.5%	None
CAPRI-GOIM (Ciardello et al.)	Prospective	FOLFOX + cetuximab vs. FOLFOX (PD after FOLFIRI + cetuximab in WT pt)	2nd	66 (pt WT in retrospective NGS analysis)	23.7 m vs. 19.8 m (HR 0.57, 95% CI 0.32–1.02; $p = 0.056$)	6.9 m vs. 5.3 m (HR 0.56, 95% CI 0.33–0.94; $p = 0.025$)	29.4% vs. 9.4%	None
CRICKET (Cremolini et al.)	Prospective	Irinotecan + cetuximab	3rd	28	9.8 m (12.5 m for ctDNA WT vs. 5.2 m for ctDNA M; HR 0.58, 95% CI 0.22–1.52; $p = 0.24$)	3.4 m (4 m for ctDNA WT vs. 1.9 m for ctDNA M;HR 0.44, 95% CI 0.18–0.98; $p = 0.03$)	14% (all ctDNA WT)	Retrospective analysis
PACER (Piccirillo et al.)	Prospective	Panitumumab	≥2nd	41 (all WT before rechallenge)	6.8 m	2.1 m	7.3%	None
JACCRO CC-08 (Masuishi et al.)	Prospective	Irinotecan + cetuximab	3rd	34 (all baseline WT)	8.2 m	2.4 m	2.9%	None
JACCRO CC-09 (Tsuji et al.)	Prospective	Irinotecan + panitumumab	3rd	25 (all baseline WT)	8.9 m	3.1 m	8.3%	None
Sunakawa et al.	Retrospective(post-hoc analysis of JACCRO CC-08 and CC-09)	Irinotecan + anti-*EGFR*	3rd	16 (all baseline WT)	8.9 m (3.8 m for ctDNA M vs. 16 m for ctDNA WT; HR 12.4, 95% CI 2.7–87.7; $p = 0.0028$)	3.1 m (2.3 m for ctDNA M vs. 4.7 m for ctDNA WT; HR 6.2, 95% CI 1.6–30.5; $p = 0.013$)	0%	Retrospective analysis
CAVE (Martinelli et al.)	Prospective	Avelumab + cetuximab	3rd	77 (all baseline WT)	11.6 m (17.3m for ctDNA WT vs. 10.4 m for ctDNA M; HR 0.49, 95% CI 0.27–0.90; $p = 0.02$)	3.6 m (4.1 m for ctDNA WT vs. 3 m for ctDNA M;HR 0.42, 95% CI 0.23–0.75; $p = 0.004$)	7.8% (8.5% for WT vs. 5.1% for M)	Retrospective analysis
BEYOND (Aparicio et al.)	Prospective	FOLFIRI + panitumumab vs. FOLFIRI	2nd	31 (all WT ctDNA before retreatment)	13 m vs. 10 m (HR 0.55, 95% CI 0.2–1.48)	11 m vs. 4 m (HR 0.58, 95% CI 0.25–1.3)	33% vs. 7.7%	Interventional
CHRONOS (Sartore-Bianchi et al.)	Prospective	Panitumumab	≥3rd	27 (all WT ctDNA before rechallenge)	55 wks	16.4 wks	30%	Interventional
E-RECHALLANGE (Nakamura et al.)	Prospective	Irinotecan + cetuximab	≥3rd	33 (all WT at baseline)	8.6 m	2.9 m (7 m for ctDNA WT vs. 2.9 m for ctDNA M)	15.6% (50% in ctDNA WT)	Retrospective analysis
Montagut et al.	Prospective	Sym004 (futuximab + modotuximab) 12 mg/Kg (arm A) vs. Sym004 6 mg/Kg (arm B) vs. SoC (arm C)	≥3rd	254 (all WT at baseline and acquired resistance to prior anti-EGFR therapy)	7.9 m vs. 10.3 m vs. 9.6 m (HR 1.31, 95% CI 0.92–1.87 for A vs. C; HR 0.97, 95% CI 0.68–1.4 for B vs. C) In ctDNA WT: 10.6m vs. 12.8m vs. 7.3m	2.8 m vs. 2.7 m vs. 2.6 m	14.1% vs. 9.6% vs. 2.9%	Retrospective analysis

Table 2. *Cont.*

Trial Name and Author	Study Type	Rechallenge Treatment	Line	N	OS	PFS	ORR	ctDNA Selection
Mariani et al.	Retrospective	Irinotecan + cetuximab or cetuximab	≥3rd	26 (all WT ctDNA before rechallenge)	5 m	3.5 m	25%	Retrospective analysis
D'Onofrio et al.	Prospective	CT + anti-*EGFR*	≥3rd	10 (all WT ctDNA before rechallenge)	NR	11.3 m	70%	Interventional

There are several ongoing trials investigating the efficacy of rechallenge with anti-*EGFR* compared with standard treatment, with some of those involving the use of ctDNA in an interventional manner. In the multicenter phase II CAPRI 2 GOIM study (NCT05312398), 200 *RAS/BRAF* WT mCRC patients will be treated with FOLFIRI plus cetuximab in the first-line treatment. At progression, those with acquired resistance mutations in ctDNA will receive second-line with FOLFOX plus bevacizumab; conversely, patients with persistent WT status will continue to receive anti-*EGFR* combined with FOLFOX. Finally, at later disease progression, the third line will consist of regorafenib or trifluridine/tipiracil for ctDNA-mutated patients and irinotecan plus cetuximab for ctDNA WT patients. (Table 3).

Table 3. Ongoing trials with interventional use of ctDNA in mCRC.

Name (NCT Number)	Phase	Setting	Line	Treatment Arms	N (Actual/Estimated Enrollment)	Primary Endpoints	Status
PULSE (NCT03992456)	2	Rechallenge	3rd	Panitumumab vs. regorafenib or trifluridine/tipiracil	120	OS	Active, not recruiting
PARERE (NCT04787341)	2	Rechallenge	3rd–4th (sequence strategy)	Panitumumab followed by regorafenib vs. regorafenib followed by panitumumab	214	OS	Recruiting
CAPRI 2 GOIM (NCT05312398)	2	Rechallenge/reintroduction	2nd–3rd (1L FOLFIRI + Cetuximab)	2L FOLFOX + cetuximab (ctDNA WT) or FOLFOX + bevacizumab (ctDNA M) 3L irinotecan + cetuximab (ctDNA WT) vs. regorafenib or trifluridine/tipiracil (ctDNA M)	200	ORR	Recruiting
NCT04775862	2	Rechallenge	3rd	Anti-*EGFR* (ctDNA WT) or SoC (ctDNA M)	60	ORR and PFS	Recruiting
CITRIC (EudraCT Number:2020-000443-31)	2	Rechallenge	3rd	Irinotecan + cetuximab vs. regorafenib or trifluridine/tipiracil	66	ORR	Recruiting
PURSUIT (jRCTs031190096)	2	Rechallenge	3rd	Irinotecan + panitumumab	50	ORR	Not yet recruiting
NCT04509635	3	Rechallenge	3rd (non-resectable liver metastases)	Cetuximab + CT vs. CT	50	DCR	Not yet recruiting
NCT03844620	2	Response to treatment	≥3rd	Regorafenib or trifluridine/tipiracil	100	Early change in ctDNA as a predictor of radiological progression; safety	Recruiting
NCT04831528	2	Target therapy	Progression at cetuximab-based therapy	2L target therapy according to ctDNA analysis	100	ORR	Not yet recruiting
FOLICOLOR (NCT04735900)	NA	Response to treatment	1 L (WT)	FOLFOX/FOLFIRI + panitumumab	60	To evaluate response and progression by *NPY* methylation(ctDNA)	Recruiting
LIBImAb (NCT04776655)	3	Efficacy of treatment	1 L (WT on solid tumor biopsy but M at liquid biopsy)	Bevacizumab + FOLFIRI vs. cetuximab + FOLFIRI	280	PFS	Recruiting

Table 3. *Cont.*

Name (NCT Number)	Phase	Setting	Line	Treatment Arms	N (Actual/Estimated Enrollment)	Primary Endpoints	Status
COPERNIC (NCT05487248)	NA	Response to treatment	≥3rd	SoC	103	To select timepoint and cut-off value for early on-treatment ctDNA changes	Not yet recruiting
OPTIMISE (NCT04680260)	2	Treatment selection and follow up	Oligometastatic CRC treated with local therapy (escalation or de-escalation CT/observation)	ctDNA-guided treatment approach vs. SoC	350	Recurrence-free rate	Recruiting
NCT05495672	NA	Treatment selection and follow up	mCRC with metastatic small pulmonary nodules (local therapy or observation)	ctDNA-guided treatment approach	100	PFS	Recruiting
NCT03436563, cohort D	1b/2	Treatment selection	Oligometastatic CRC MSI with positive ctDNA following resection of liver metastases	Anti-PD-L1/*TGFbetaRII* fusion protein M7824	NA	Clearance ctDNA	Active, not recruiting
NCT04555369	NA	Response to treatment	mCRC receiving CT	ctDNA testing	300	ORR	Recruiting
NCT05141721	2/3	Response to treatment	Maintenance therapy in mCRC pt after SoC	GRT-C901/GRT-R902 (neoantigen vaccine) + ipilimumab + atezolizumab + fluoropyrimidine + bevacizumab vs. fluoropyrimidine + bevacizumab	665	Antitumor activity by number of pt with ≥50% decrease from baseline in ctDNA; PFS	Recruiting

Abbreviations: mCRC: metastatic colorectal cancer; ctDNA: circulating tumor DNA; OS: overall survival; PFS: progression-free survival; ORR: objective response rate; DCR: disease control rate; NA: not available; *MSI*: microsatellite instability; *NPY*: neuropeptide Y; PD-L1:programmed cell death ligand 1; *TGF*: transforming growth factor; WT: wild type; M: mutated; pt: patients; CT: chemotherapy; FOLFOX: 5-fluorouracilo + oxaliplatino; FOLFIRI: 5-fluorouracilo + irinotecan; FOLFOXIRI: 5-fluorouracilo + oxaliplatino + irinotecan; SoC: standard of care; *EGFR*: epidermal growth factor receptor.

4.6. Detection of Other Mutations (MSI, BRAF, MET, and ERBB2)

4.6.1. *MSI*

Microsatellites are repetitive DNA sequences ranging in length from one to six bases spread throughout the whole human genome, and they are prone to DNA replication errors. During the process of DNA replication, deletions or insertions are introduced at these sites, most of which are usually corrected by the DNA Mismatch Repair System (MMR). If the MMR is damaged, these deletions or insertions are accumulated, producing length polymorphisms of microsatellites known as *MSI*. Depending on their frequency, tumors are divided into three subtypes: high-*MSI* (>30%), low-*MSI* (0–30%), and microsatellite-stable (*MSS*) [146]. Low-*MSI* and *MSS* tumors are classified as a single type in clinics [147]. Nowadays, 12–20% of CRCs are explained by *MSI-H*. Its incidence is higher in the early stages (20% in stages I and II, and 12% in stage III), and a lower incidence in stage IV (about 5%) [73,148]. During the last years, the *MSI* has shown implications in prognosis and as a predictive biomarker of the response to immune checkpoint inhibitors [149–151]. Currently, *MSI* detection is being carried out on tumoral tissue by immunohistochemistry (IHC) and PCR, with the most widely used commercial kit being the "pentaplex assay" [152,153]. Owing to the current trend toward the systematic detection of *MSI* in patients with mCRC and the limitations of these techniques in tissue, non-invasive diagnostic methods are being developed using bodily fluids. Furthermore, the use of droplet-digital PCR (ddPCR) has increased, by at least two times, the detection threshold of the gold standard (pentaplex assay) [154–156]. In 2020, a study demonstrated that *MSI* detected by ddPCR in blood samples (ctDNA) from colorectal cancer patients showed a clinical specificity and accuracy of 100% [154].

On the other hand, NGS allows the examination of microsatellites at thousands of loci simultaneously, while also obtaining the mutational profile across targeted regions in a single assay [157]. Furthermore, the NGS may quantify *MSI* reaching a sensitivity down to 0.05%. In 2019, another study performed ctDNA testing with a Guardant360 NGS kit. In patients, the test detected 87% (71/82) of tissue *MSI-H* and 99.5% (863/867) of *MSS* for an overall accuracy of 98.4% (934/949) and a positive predictive value of 95%. Moreover, the concordance of ctDNA *MSI* with tissue PCR and NGS was significantly higher than that of IHC [158].

At present, one observational prospective trial is aiming to determine the concordance between the electrophoretic mobility profiles of microsatellite biomarkers in cfDNA versus primary tumor tissues in patients with *MSI* CRC (NCT0359448).

4.6.2. *BRAF*

BRAF is a protein kinase downstream of *RAS* in the *RAS-RAF-MEK-ERK* kinase pathway. Mutations in *BRAF* are present in approximately 10% of patients with mCRC [159].

Nowadays, in metastatic CRC assessment, the *BRAF* status is mandatory before applying systemic treatment due to its therapeutic implications. The *BRAF V600E* mutation is described as a biomarker of poor prognosis in CRC and a significant predictor of resistance to *EGFR* treatment [160,161]. However, non-*V600E BRAF* mutations occur in about 2% of all patients with mCRC and mostly define a clinically distinct subtype of CRC with good prognosis [162].

Related to the treatment of *BRAF*-mutant CRC tumors, the Beacon trial demonstrated that patients with the *BRAF V600E* mutation were highly sensitive to doublet or triplet therapy with *EGFR* and *MAPK* kinase pathway inhibitors over the standard-of-care chemotherapy [163].

In 2014, the analysis of the *BRAF V600E* mutation in ctDNA was validated, showing nearly 100% specificity and sensitivity for this specific mutation [121]. Conversely, a phase I/II study showed that the *BRAF V600E* mutant fraction burden in ctDNA was more markedly reduced by week 4 in responder patients than in non-responder patients when they received dabrafenib, trametinib, and panitumumab combination therapy [164]. Another phase IB trial by Hong et al. in patients treated with vemurafenib, cetuximab, and irinotecan confirmed the role of *BRAF V600E* ctDNA allele fraction reduction in predicting the radiological response to these therapies [165]. In a study of *V600E*-mutated mCRC patients treated with vemurafenib + cetuximab + irinotecan, ctDNA liquid biopsy identified resistance-related genetic alterations, such as the *MAPK* (*KRAS*, *NRAS*, and *ERBB4*), *PI3K* (*PIK3CA* and *PIK3R2*), and receptor tyrosine kinase (Platelet-Derived Growth Factor, *PDGFRB*) pathways; moreover, resistance mutations were also found in TGF-β pathways (*TGFBR2* and *SMAD4*) [166].

4.6.3. *MET*

MET or *c-MET* belongs to the family of receptor tyrosine kinases (*RTKs*) that is encoded by the *MET* proto-oncogene located on human chromosome 7 (7q21-31) [167]. The *cMET*-hepatocyte growth factor/scatter factor (*HGF/SF*) pathway plays a crucial role in several biological activities, such as motility, proliferation, cell survival, embryogenesis, angiogenesis, and wound healing [168].

A meta-analysis suggested that patients diagnosed with stage III and IV illness had higher *MET* expression levels compared with those diagnosed with stage I–II [169]. However, in a large cohort of mCRC, the frequency of *MET* amplifications was detected in 1.7% (10/590) of tumor tissue biopsies, without differences between primary and metastatic lesions [170]. These results were consistent with the data generated by The Cancer Genome Atlas [171].

Despite the low prevalence of de novo *MET* amplification in mCRC, acquired *MET* amplification was detected in up to 23% of *RAS* WT patients who had been treated with anti-*EGFR* treatments and showed disease progression [170]. This detection was performed in ctDNA.

In the future, *MET* amplification could be used as a potential biomarker to identify acquired resistance in patients treated with anti-*EGFR* therapy in mCRC [170,172]. This approach may drive the early initiation of *MET* inhibitors in patients who respond to cetuximab and panitumumab and do not display the emergence of *KRAS* mutations in blood tests during anti-*EGFR* therapy. In a phase 1b study published in 2020, the combination of anti-*EGFR* and *MET* inhibitor showed disease control in up to 46% of patients [173].

4.6.4. *ERBB2* (HER2)

ERBB2 is a part of the family of epidermal growth factor receptors (*ERBB*); this family represents a group of *RTKs*. The best-known pathogenic mechanisms involved in *ERBB2* aberrant activation are overexpression and activating mutations, both described in CRC.

The reported rates of *ERBB2* positivity have varied widely in different studies due to differences in antibody clone selection, scoring criteria, staining platform, and cohort composition. *ERBB2* amplifications have been observed in approximately 3% of patients with mCRC [174]. Recently, a validated scoring system was developed, HERACLES. In these diagnostic criteria, the pattern of expression, intensity of staining, and percentage of positive cells are used to define positivity. Furthermore, in equivocal cases, which are defined by moderate expression in $\geq$50% or 3 + *ERBB2* in more than 10%, but less than 50% of tumor cells, they require in situ hybridization (FISH) to define *ERBB2* overexpression [174]. On the other hand, NGS can also provide information on *ERBB2* and other drivers. Some authors suggest that a Copy Number Variant (CNV) of $\geq$5.0 found in NGS can be diagnosed as *ERBB2*+. However, if the CNV is between 4.0 and 4.9, the diagnosis should be confirmed by IHQ or FISH [175].

Related to ctDNA detection, in the HERACLES trial, ctDNA sequencing by the Guardant360 assay correctly identified 96.6% of samples as *ERBB2*-amplified. Furthermore, to improve the diagnosis in ctDNA, the authors developed an adjusted plasma copy number (apCN) in order to correct for variations in the plasma tumor fraction between samples. This apCN showed a strong correlation with the tissue *ERBB2* copy number [176]. These positive correlations between tissue and ctDNA could suggest serial determinations of ctDNA to monitor the response to treatment and to elucidate resistance mechanisms.

4.7. Cell-Free DNA Fragmentomics

One of the new frontiers in liquid biopsy is cell-free DNA fragmentomics. cfDNA fragments are DNA molecules that are released into the bloodstream by the apoptosis or necrosis of cells. In 2015, Ivanov identified the non-random fragmentation of cfDNA as a possible representation of epigenetic regulation [177]. Subsequently, Cristiano et al. highlighted how different fragmentation profiles may differ between individuals with cancer and healthy individuals [178]. Different fragmentomic markers are being studied: the size profile, preferred end, jagged end, end motif, and nucleosome footprint. Jiang et al. discovered that the length of cfDNA fragments in cancer cells is shorter than that in normal cells (dominant peaks of 143 bp and 167, respectively) [179]. The preferred ends represent genomic coordinates where cfDNA cleavage preferentially occurs, being able to be useful in the detection of early stage neoplasms. End motifs constitute the sequence of nucleotides proximal to the 5′ end of DNA, correlating with nuclease-specific activity. For example, the CCCA end motif has been shown to be at low levels in hepatocarcinoma patients compared with healthy subjects [180]. This is a phenomenon that could be related to the low expression of DNAES1L3 nuclease, whose downregulation has also been identified in other neoplasms, including colorectal cancer [180]. Another fragmentation pattern is nucleosome footprints; in fact, the disposition of nucleosomes can provide information about the origin of the tissue [181].

There are several studies that have shown how these features, which reflect different fragmentation processes, can be used in early cancer detection [182–184].

Wang et al. evaluated the utility of cfDNA fragmentomics in predicting the pathologic response after neoadjuvant chemoradiation therapy in patients with locally advanced rectal

cancer. The model based on the 5′-end motif profile plus MRI-based tumor regression grade (mrTRG) achieved the highest cross-validation AUC (0.92, 95% CI, 0.91–0.93) [185].

In the metastatic setting, Sanchez et al. compared the fragmentomic cfDNA size profile obtained by quantitative-PCR of samples from seven healthy individuals with those of seven from mCRC patients. Although both cohorts peak at 166 bp, mCRC patients had a higher proportion of fragments between 40 and 150 bp and less between 150 and 250 bp. Furthermore, these differences directly increased with increasing mutant allele frequency (MAF). All of this indicates that the cfDNA from tumor cells has a higher degree of fragmentation and nuclease activity than that from healthy individuals [186].

5. Future Perspectives and Conclusions

The different liquid biopsy analytes (exosomes, CTCs, and ctDNA) could play an important role as a diagnostic, prognostic, and predictive tool in guiding the choice of the most appropriate therapy in the field of precision oncology for mCRC patients.

We have seen the importance of using ctDNA and CTCs for predicting prognosis in oligometastatic CRC patients [75,103,104]. This application could be considered to assess the resectability of patients with a high risk of recurrence. Similarly, there is also emerging evidence on the use of ctDNA in patients with peritoneal metastases [187], with a randomized trial evaluating ctDNA as a marker of diagnosis, early intervention, and prognosis for peritoneal metastases in mCRC compared with conventional radiological techniques currently ongoing (NCT04752930).

ctDNA could be crucial to assess the response to standard chemotherapy, as it can facilitate the early discontinuation of ineffective therapies, especially considering the last lines where preserving the patient's quality of life is crucial [111–113]. In the meantime, it could provide information on the possible mechanisms of resistance in particular when using anti-*EGFR*s to guide clinicians in the choice of rechallenge [144]. Some data are already available about the conversion of mutated *RAS* status after treatment with bevacizumab-based therapy, which could generate a "WT *RAS* window" that would allow the subsequent use of an anti-*EGFR* [128,188]. The future of ctDNA in mCRC patients will be likely developed in the context of detecting less frequent molecular alterations with an already real clinical implication, such as *ERBB2*, *MSI*, and *KRASp.G12C*. The ongoing KRYSTAL-10 trial is investigating the efficacy of the combination of adagrasib + cetuximab compared with a standard second-line treatment in *KRASp.G12C*-mutated mCRC patients, with mutation detection also being performed on ctDNA (NCT04793958). Other studies testing the use of ctDNA in the mCRC setting are described in Table 3. The new frontier of liquid biopsy in mCRC will be detecting predictive biomarkers of the response to both target therapy and immunotherapy. It will be fundamental in introducing its application to clinical practice where there is already evidence, such as in the case of anti-*EGFR* rechallenge; this could also promote a gradual standardization of sample collection and subsequent detection techniques. Finally, a new frontier in the future of liquid biopsy in mCRC patients could be the study of cfDNA fragmentomics.

In addition, it will be essential to expand liquid biopsy use in prospective clinical trials and employ it to support preclinical research in transcriptomics and proteomics to better understand the complexity of tumor heterogeneity.

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Abbreviations

miRNA: microRNA; lncRNA: long non-coding RNA; PCR: polymerase chain reaction; NGS: next-generation sequencing; CHIP: clonal hematopoiesis of indeterminate potential; RAS: Rat Sarcoma Viral Oncogene Homolog; BRAF: v-Raf Murine Sarcoma Viral Oncogene Homolog B; HER2: Human Epidermal Growth Factor Receptor 2; MET: Tyrosine-protein Kinase Met; MSI: Microsatellite Instability; EpCam: Epithelial Cell Adhesion Molecule.

References

1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249. [CrossRef] [PubMed]
2. Cervantes, A.; Adam, R.; Roselló, S.; Arnold, D.; Normanno, N.; Taïeb, J.; Seligmann, J.; De Baere, T.; Osterlund, P.; Yoshino, T.; et al. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann. Oncol.* **2023**, *34*, 10–32. [CrossRef] [PubMed]
3. National Cancer Institute Surveillance, Epidemiology, and End Results Program. Cancer Stat Facts: Colorectal Cancer. Available online: https://seer.cancer.gov/statfacts/html/colorect.html (accessed on 28 January 2021).
4. Mosele, F.; Remon, J.; Mateo, J.; Westphalen, C.B.; Barlesi, F.; Lolkema, M.P.; Normanno, N.; Scarpa, A.; Robson, M.; Meric-Bernstam, F.; et al. Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: A report from the ESMO Precision Medicine Working Group. *Ann. Oncol.* **2020**, *31*, 1491–1505. [CrossRef] [PubMed]
5. Wang, H.; Li, Z.W.; Ou, Q.; Wu, X.; Nagasaka, M.; Shao, Y.; Ou, S.I.; Yang, Y. NTRK fusion positive colorectal cancer is a unique subset of CRC with high TMB and microsatellite instability. *Cancer Med.* **2022**, *11*, 2541–2549. [CrossRef]
6. Church, T.R.; Wandell, M.; Lofton-Day, C.; Mongin, S.J.; Burger, M.; Payne, S.R.; Castaños-Vélez, E.; Blumenstein, B.A.; Rösch, T.; Osborn, N.; et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* **2014**, *63*, 317–325. [CrossRef]
7. Liu, X.; Pan, B.; Sun, L.; Chen, X.; Zeng, K.; Hu, X.; Xu, T.; Xu, M.; Wang, S. Circulating Exosomal miR-27a and miR-130a Act as Novel Diagnostic and Prognostic Biomarkers of Colorectal Cancer. *Cancer Epidemiol. Biomark. Prev.* **2018**, *27*, 746–754. [CrossRef] [PubMed]
8. Troncarelli Flores, B.C.; Souza E Silva, V.; Ali Abdallah, E.; Mello, C.A.L.; Gobo Silva, M.L.; Gomes Mendes, G.; Camila Braun, A.; Aguiar Junior, S.; Thomé Domingos Chinen, L. Molecular and Kinetic Analyses of Circulating Tumor Cells as Predictive Markers of Treatment Response in Locally Advanced Rectal Cancer Patients. *Cells* **2019**, *8*, 641. [CrossRef]
9. Tie, J.; Wang, Y.; Tomasetti, C.; Li, L.; Springer, S.; Kinde, I.; Silliman, N.; Tacey, M.; Wong, H.L.; Christie, M.; et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci. Transl. Med.* **2016**, *8*, 346ra92. [CrossRef]
10. Tie, J.; Cohen, J.D.; Wang, Y.; Christie, M.; Simons, K.; Lee, M.; Wong, R.; Kosmider, S.; Ananda, S.; McKendrick, J.; et al. Circulating Tumor DNA Analyses as Markers of Recurrence Risk and Benefit of Adjuvant Therapy for Stage III Colon Cancer. *JAMA Oncol.* **2019**, *5*, 1710–1717. [CrossRef]
11. Tie, J.; Cohen, J.D.; Lahouel, K.; Lo, S.N.; Wang, Y.; Kosmider, S.; Wong, R.; Shapiro, J.; Lee, M.; Harris, S.; et al. Circulating Tumor DNA Analysis Guiding Adjuvant Therapy in Stage II Colon Cancer. *N. Engl. J. Med.* **2022**, *386*, 2261–2272. [CrossRef]
12. Parikh, A.R.; Leshchiner, I.; Elagina, L.; Goyal, L.; Levovitz, C.; Siravegna, G.; Livitz, D.; Rhrissorrakrai, K.; Martin, E.E.; Van Seventer, E.E.; et al. Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nat. Med.* **2019**, *25*, 1415–1421. [CrossRef] [PubMed]
13. Liu, J.; Ren, L.; Li, S.; Li, W.; Zheng, X.; Yang, Y.; Fu, W.; Yi, J.; Wang, J.; Du, G. The biology, function, and applications of exosomes in cancer. *Acta Pharm. Sin. B* **2021**, *11*, 2783–2797. [CrossRef] [PubMed]
14. Jia, S.; Zhang, R.; Li, Z.; Li, J. Clinical and biological significance of circulating tumor cells, circulating tumor DNA, and exosomes as biomarkers in colorectal cancer. *Oncotarget* **2017**, *8*, 55632–55645. [CrossRef] [PubMed]
15. Cai, X.; Janku, F.; Zhan, Q.; Fan, J.B. Accessing Genetic Information with Liquid Biopsies. *Trends Genet.* **2015**, *31*, 564–575. [CrossRef] [PubMed]
16. Halvaei, S.; Daryani, S.; Eslami-S, Z.; Samadi, T.; Jafarbeik-Iravani, N.; Bakhshayesh, T.O.; Majidzadeh-A, K.; Esmaeili, R. Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. *Mol. Ther. Nucleic Acids* **2018**, *10*, 131–141. [CrossRef]
17. Eylem, C.C.; Yilmaz, M.; Derkus, B.; Nemutlu, E.; Camci, C.B.; Yilmaz, E.; Turkoglu, M.A.; Aytac, B.; Ozyurt, N.; Emregul, E. Untargeted multi-omic analysis of colorectal cancer-specific exosomes reveals joint pathways of colorectal cancer in both clinical samples and cell culture. *Cancer Lett.* **2020**, *469*, 186–194. [CrossRef]
18. Galbiati, S.; Damin, F.; Burgio, V.; Brisci, A.; Soriani, N.; Belcastro, B.; Di Resta, C.; Gianni, L.; Chiari, M.; Ronzoni, M.; et al. Evaluation of three advanced methodologies, COLD-PCR, microarray and ddPCR, for identifying the mutational status by liquid biopsies in metastatic colorectal cancer patients. *Clin. Chim. Acta* **2019**, *489*, 136–143. [CrossRef]

19. Kang, J.K.; Heo, S.; Kim, H.P.; Song, S.H.; Yun, H.; Han, S.W.; Kang, G.H.; Bang, D.; Kim, T.Y. Liquid biopsy-based tumor profiling for metastatic colorectal cancer patients with ultra-deep targeted sequencing. *PLoS ONE* **2020**, *15*, e0232754. [CrossRef]
20. Yu, H.; Han, L.; Yuan, J.; Sun, Y. Circulating tumor cell free DNA from plasma and urine in the clinical management of colorectal cancer. *Cancer Biomark.* **2020**, *27*, 29–37. [CrossRef]
21. Baassiri, A.; Nassar, F.; Mukherji, D.; Shamseddine, A.; Nasr, R.; Temraz, S. Exosomal Non Coding RNA in LIQUID Biopsies as a Promising Biomarker for Colorectal Cancer. *Int. J. Mol. Sci.* **2020**, *21*, 1398. [CrossRef]
22. Greening, D.W.; Gopal, S.K.; Mathias, R.A.; Liu, L.; Sheng, J.; Zhu, H.J.; Simpson, R.J. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. *Semin. Cell Dev. Biol.* **2015**, *40*, 60–71. [CrossRef]
23. Minciacchi, V.R.; Zijlstra, A.; Rubin, M.A.; Di Vizio, D. Extracellular vesicles for liquid biopsy in prostate cancer: Where are we and where are we headed? *Prostate Cancer Prostatic Dis.* **2017**, *20*, 251–258. [CrossRef] [PubMed]
24. Maas, S.L.; de Vrij, J.; van der Vlist, E.J.; Geragousian, B.; van Bloois, L.; Mastrobattista, E.; Schiffelers, R.M.; Wauben, M.H.; Broekman, M.L.; Nolte-'t Hoen, E.N. Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *J. Control. Release* **2015**, *200*, 87–96. [CrossRef] [PubMed]
25. Ganig, N.; Baenke, F.; Thepkaysone, M.L.; Lin, K.; Rao, V.S.; Wong, F.C.; Polster, H.; Schneider, M.; Helm, D.; Pecqueux, M.; et al. Proteomic Analyses of Fibroblast- and Serum-Derived Exosomes Identify QSOX1 as a Marker for Non-invasive Detection of Colorectal Cancer. *Cancers* **2021**, *13*, 1351. [CrossRef] [PubMed]
26. Li, J.; Chen, Y.; Guo, X.; Zhou, L.; Jia, Z.; Peng, Z.; Tang, Y.; Liu, W.; Zhu, B.; Wang, L.; et al. GPC1 exosome and its regulatory miRNAs are specific markers for the detection and target therapy of colorectal cancer. *J. Cell. Mol. Med.* **2017**, *21*, 838–847. [CrossRef] [PubMed]
27. Sun, B.; Li, Y.; Zhou, Y.; Ng, T.K.; Zhao, C.; Gan, Q.; Gu, X.; Xiang, J. Circulating exosomal CPNE3 as a diagnostic and prognostic biomarker for colorectal cancer. *J. Cell. Physiol.* **2019**, *234*, 1416–1425. [CrossRef] [PubMed]
28. Lai, P.S.; Chang, W.M.; Chen, Y.Y.; Lin, Y.F.; Liao, H.F.; Chen, C.Y. Circulating microRNA-762 upregulation in colorectal cancer may be accompanied by Wnt-1/β-catenin signaling. *Cancer Biomark.* **2021**, *32*, 111–122. [CrossRef]
29. Radwan, E.; Shaltout, A.S.; Mansor, S.G.; Shafik, E.A.; Abbas, W.A.; Shehata, M.R.; Ali, M. Evaluation of circulating microRNAs-211 and 25 as diagnostic biomarkers of colorectal cancer. *Mol. Biol. Rep.* **2021**, *48*, 4601–4610. [CrossRef]
30. Silva, C.M.S.; Barros-Filho, M.C.; Wong, D.V.T.; Mello, J.B.H.; Nobre, L.M.S.; Wanderley, C.W.S.; Lucetti, L.T.; Muniz, H.A.; Paiva, I.K.D.; Kuasne, H.; et al. Circulating let-7e-5p, miR-106a-5p, miR-28-3p, and miR-542-5p as a Promising microRNA Signature for the Detection of Colorectal Cancer. *Cancers* **2021**, *13*, 1493. [CrossRef]
31. Karimi, N.; Ali Hosseinpour Feizi, M.; Safaralizadeh, R.; Hashemzadeh, S.; Baradaran, B.; Shokouhi, B.; Teimourian, S. Serum overexpression of miR-301a and miR-23a in patients with colorectal cancer. *J. Chin. Med. Assoc.* **2019**, *82*, 215–220. [CrossRef]
32. Tsukamoto, M.; Iinuma, H.; Yagi, T.; Matsuda, K.; Hashiguchi, Y. Circulating exosomal MicroRNA-21 as a biomarker in each tumor stage of colorectal cancer. *Oncology* **2017**, *92*, 360–370. [CrossRef] [PubMed]
33. Sun, L.; Liu, X.; Pan, B.; Hu, X.; Zhu, Y.; Su, Y.; Guo, Z.; Zhang, G.; Xu, M.; Xu, X.; et al. Serum exosomal miR-122 as a potential diagnostic and prognostic biomarker of colorectal cancer with liver metastasis. *J. Cancer* **2020**, *11*, 630–637. [CrossRef] [PubMed]
34. Zeng, Z.; Li, Y.; Pan, Y.; Lan, X.; Song, F.; Sun, J.; Zhou, K.; Liu, X.; Ren, X.; Wang, F.; et al. Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis. *Nat. Commun.* **2018**, *9*, 5395. [CrossRef] [PubMed]
35. Tang, Y.; Zhao, Y.; Song, X.; Song, X.; Niu, L.; Xie, L. Tumor-derived exosomal miRNA-320d as a biomarker for metastatic colorectal cancer. *J. Clin. Lab. Anal.* **2019**, *33*, e23004. [CrossRef] [PubMed]
36. Lucchetti, D.; Zurlo, I.V.; Colella, F.; Ricciardi-Tenore, C.; Di Salvatore, M.; Tortora, G.; De Maria, R.; Giuliante, F.; Cassano, A.; Basso, M.; et al. Mutational status of plasma exosomal KRAS predicts outcome in patients with metastatic colorectal cancer. *Sci. Rep.* **2021**, *11*, 22686. [CrossRef]
37. Raza, A.; Khan, A.Q.; Inchakalody, V.P.; Mestiri, S.; Yoosuf, Z.S.K.M.; Bedhiafi, T.; El-Ella, D.M.A.; Taib, N.; Hydrose, S.; Akbar, S.; et al. Dynamic liquid biopsy components as predictive and prognostic biomarkers in colorectal cancer. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 99. [CrossRef]
38. Barbagallo, C.; Brex, D.; Caponnetto, A.; Cirnigliaro, M.; Scalia, M.; Magnano, A.; Caltabiano, R.; Barbagallo, D.; Biondi, A.; Cappellani, A.; et al. LncRNA UCA1, Upregulated in CRC Biopsies and Downregulated in Serum Exosomes, Controls mRNA Expression by RNA-RNA Interactions. *Mol. Ther. Nucleic Acids* **2018**, *12*, 229–241. [CrossRef]
39. Liu, T.; Zhang, X.; Gao, S.; Jing, F.; Yang, Y.; Du, L.; Zheng, G.; Li, P.; Li, C.; Wang, C. Exosomal long noncoding RNA CRNDE-h as a novel serum-based biomarker for diagnosis and prognosis of colorectal cancer. *Oncotarget* **2016**, *7*, 85551–85563. [CrossRef]
40. Pan, B.; Qin, J.; Liu, X.; He, B.; Wang, X.; Pan, Y.; Sun, H.; Xu, T.; Xu, M.; Chen, X.; et al. Identification of Serum Exosomal hsa-circ-0004771 as a Novel Diagnostic Biomarker of Colorectal Cancer. *Front. Genet.* **2019**, *10*, 1096. [CrossRef]
41. Cekaite, L.; Eide, P.W.; Lind, G.E.; Skotheim, R.I.; Lothe, R.A. MicroRNAs as growth regulators, their function and biomarker status in colorectal cancer. *Oncotarget* **2016**, *7*, 6476–6505. [CrossRef]
42. Gao, W.; Chen, Y.; Yang, J.; Zhuo, C.; Huang, S.; Zhang, H.; Shi, Y. Clinical Perspectives on Liquid Biopsy in Metastatic Colorectal Cancer. *Front. Genet.* **2021**, *12*, 634642. [CrossRef] [PubMed]
43. Chen, Y.; Xie, Y.; Xu, L.; Zhan, S.; Xiao, Y.; Gao, Y.; Wu, B.; Ge, W. Protein content and functional characteristics of serum-purified exosomes from patients with colorectal cancer revealed by quantitative proteomics. *Int. J. Cancer* **2017**, *140*, 900–913. [CrossRef] [PubMed]

44. Zheng, X.; Liu, J.; Li, X.; Tian, R.; Shang, K.; Dong, X.; Cao, B. Angiogenesis is promoted by exosomal DPP4 derived from 5-fluorouracil-resistant colon cancer cells. *Cancer Lett.* **2021**, *497*, 190–201. [CrossRef] [PubMed]
45. Matsumura, T.; Sugimachi, K.; Iinuma, H.; Takahashi, Y.; Kurashige, J.; Sawada, G.; Ueda, M.; Uchi, R.; Ueo, H.; Takano, Y.; et al. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br. J. Cancer* **2015**, *113*, 275–281. [CrossRef]
46. Takano, Y.; Masuda, T.; Iinuma, H.; Yamaguchi, R.; Sato, K.; Tobo, T.; Hirata, H.; Kuroda, Y.; Nambara, S.; Hayashi, N.; et al. Circulating exosomal microRNA-203 is associated with metastasis possibly via inducing tumor-associated macrophages in colorectal cancer. *Oncotarget* **2017**, *8*, 78598–78613. [CrossRef]
47. Fu, F.; Jiang, W.; Zhou, L.; Chen, Z. Circulating Exosomal miR-17-5p and miR-92a-3p Predict Pathologic Stage and Grade of Colorectal Cancer. *Transl. Oncol.* **2018**, *11*, 221–232. [CrossRef]
48. De Miguel Pérez, D.; Rodriguez Martínez, A.; Ortigosa Palomo, A.; Delgado Ureña, M.; Garcia Puche, J.L.; Robles Remacho, A.; Exposito Hernandez, J.; Lorente Acosta, J.A.; Ortega Sánchez, F.G.; Serrano, M.J. Extracellular vesicle-miRNAs as liquid biopsy biomarkers for disease identification and prognosis in metastatic colorectal cancer patients. *Sci. Rep.* **2020**, *10*, 3974. [CrossRef] [PubMed]
49. Hu, J.L.; Wang, W.; Lan, X.L.; Zeng, Z.C.; Liang, Y.S.; Yan, Y.R.; Song, F.Y.; Wang, F.F.; Zhu, X.H.; Liao, W.J.; et al. CAFs secreted exosomes promote metastasis and chemotherapy resistance by enhancing cell stemness and epithelial-mesenchymal transition in colorectal cancer. *Mol. Cancer* **2019**, *18*, 91. [CrossRef]
50. Huber, V.; Fais, S.; Iero, M.; Lugini, L.; Canese, P.; Squarcina, P.; Zaccheddu, A.; Colone, M.; Arancia, G.; Gentile, M.; et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: Role in immune escape. *Gastroenterology* **2005**, *128*, 1796–1804. [CrossRef]
51. Mannavola, F.; Salerno, T.; Passarelli, A.; Tucci, M.; Internò, V.; Silvestris, F. Revisiting the Role of Exosomes in Colorectal Cancer: *Where Are We Now? Front. Oncol.* **2019**, *9*, 521. [CrossRef]
52. Mohammad, I.S.; He, W.; Yin, L. Understanding of human ATP binding cassette superfamily and novel multidrug resistance modulators to overcome MDR. *Biomed. Pharmacother.* **2018**, *100*, 335–348. [CrossRef] [PubMed]
53. Hu, Y.B.; Yan, C.; Mu, L.; Mi, Y.L.; Zhao, H.; Hu, H.; Li, X.L.; Tao, D.D.; Wu, Y.Q.; Gong, J.P.; et al. Exosomal Wnt-induced dedifferentiation of colorectal cancer cells contributes to chemotherapy resistance. *Oncogene* **2019**, *38*, 1951–1965. [CrossRef] [PubMed]
54. Bhome, R.; Goh, R.W.; Bullock, M.D.; Pillar, N.; Thirdborough, S.M.; Mellone, M.; Mirnezami, R.; Galea, D.; Veselkov, K.; Gu, Q.; et al. Exosomal microRNAs derived from colorectal cancer-associated fibroblasts: Role in driving cancer progression. *Aging* **2017**, *9*, 2666–2694. [CrossRef] [PubMed]
55. Ren, D.; Lin, B.; Zhang, X.; Peng, Y.; Ye, Z.; Ma, Y.; Liang, Y.; Cao, L.; Li, X.; Li, R.; et al. Maintenance of cancer stemness by miR-196b-5p contributes to chemoresistance of colorectal cancer cells via activating STAT3 signaling pathway. *Oncotarget* **2017**, *8*, 49807–49823. [CrossRef] [PubMed]
56. Jin, G.; Liu, Y.; Zhang, J.; Bian, Z.; Yao, S.; Fei, B.; Zhou, L.; Yin, Y.; Huang, Z. A panel of serum exosomal microRNAs as predictive markers for chemoresistance in advanced colorectal cancer. *Cancer Chemother. Pharmacol.* **2019**, *84*, 315–325. [CrossRef]
57. Yang, Y.N.; Zhang, R.; Du, J.W.; Yuan, H.H.; Li, Y.J.; Wei, X.L.; Du, X.X.; Jiang, S.L.; Han, Y. Predictive role of UCA1-containing exosomes in cetuximab-resistant colorectal cancer. *Cancer Cell Int.* **2018**, *18*, 164. [CrossRef]
58. Liang, G.; Zhu, Y.; Ali, D.J.; Tian, T.; Xu, H.; Si, K.; Sun, B.; Chen, B.; Xiao, Z. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *J. Nanobiotechnol.* **2020**, *18*, 10. [CrossRef]
59. Li, Y.; Gao, Y.; Gong, C.; Wang, Z.; Xia, Q.; Gu, F.; Hu, C.; Zhang, L.; Guo, H.; Gao, S. A33 antibody-functionalized exosomes for targeted delivery of doxorubicin against colorectal cancer. *Nanomedicine* **2018**, *14*, 1973–1985. [CrossRef]
60. Jiang, M.; Jin, S.; Han, J.; Li, T.; Shi, J.; Zhong, Q.; Li, W.; Tang, W.; Huang, Q.; Zong, H. Detection and clinical significance of circulating tumor cells in colorectal cancer. *Biomark. Res.* **2021**, *9*, 85. [CrossRef]
61. Lin, D.; Shen, L.; Luo, M.; Zhang, K.; Li, J.; Yang, Q.; Zhu, F.; Zhou, D.; Zheng, S.; Chen, Y.; et al. Circulating tumor cells: Biology and clinical significance. *Signal Transduct. Target Ther.* **2021**, *6*, 404. [CrossRef]
62. De Renzi, G.; De Marco, G.; De Meo, M.; Del Rosso, E.; Gazzaniga, P.; Nicolazzo, C. In vitro cultures of circulating tumor cells: A potential tool to unravel drug sensitivity. *Cancer Drug Resist.* **2022**, *5*, 245–260. [CrossRef]
63. Micalizzi, D.S.; Maheswaran, S.; Haber, D.A. A conduit to metastasis: Circulating tumor cell biology. *Genes Dev.* **2017**, *31*, 1827–1840. [CrossRef]
64. Garrido-Navas, C.; de Miguel-Perez, D.; Exposito-Hernandez, J.; Bayarri, C.; Amezcua, V.; Ortigosa, A.; Valdivia, J.; Guerrero, R.; Garcia Puche, J.L.; Lorente, J.A.; et al. Cooperative and Escaping Mechanisms between Circulating Tumor Cells and Blood Constituents. *Cells* **2019**, *8*, 1382. [CrossRef] [PubMed]
65. Tsubakihara, Y.; Moustakas, A. Epithelial-Mesenchymal Transition and Metastasis under the Control of Transforming Growth Factor β. *Int. J. Mol. Sci.* **2018**, *19*, 3672. [CrossRef] [PubMed]
66. Masucci, M.T.; Minopoli, M.; Del Vecchio, S.; Carriero, M.V. The Emerging Role of Neutrophil Extracellular Traps (NETs) in Tumor Progression and Metastasis. *Front. Immunol.* **2020**, *11*, 1749. [CrossRef] [PubMed]
67. Patelli, G.; Vaghi, C.; Tosi, F.; Mauri, G.; Amatu, A.; Massihnia, D.; Ghezzi, S.; Bonazzina, E.; Bencardino, K.; Cerea, G.; et al. Liquid Biopsy for Prognosis and Treatment in Metastatic Colorectal Cancer: Circulating Tumor Cells vs Circulating Tumor DNA. *Target Oncol.* **2021**, *16*, 309–324. [CrossRef] [PubMed]

68. Riethdorf, S.; Fritsche, H.; Müller, V.; Rau, T.; Schindlbeck, C.; Rack, B.; Janni, W.; Coith, C.; Beck, K.; Jänicke, F.; et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: A validation study of the CellSearch system. *Clin. Cancer Res.* **2007**, *13*, 920–928. [CrossRef]
69. Sastre, J.; Maestro, M.L.; Puente, J.; Veganzones, S.; Alfonso, R.; Rafael, S.; García-Saenz, J.A.; Vidaurreta, M.; Martín, M.; Arroyo, M.; et al. Circulating tumor cells in colorectal cancer: Correlation with clinical and pathological variables. *Ann. Oncol.* **2008**, *19*, 935–938. [CrossRef]
70. Allard, W.J.; Matera, J.; Miller, M.C.; Repollet, M.; Connelly, M.C.; Rao, C.; Tibbe, A.G.; Uhr, J.W.; Terstappen, L.W. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* **2004**, *10*, 6897–6904. [CrossRef]
71. Pantel, K.; Alix-Panabières, C. Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol. Med.* **2010**, *16*, 398–406. [CrossRef]
72. Tsai, W.S.; You, J.F.; Hung, H.Y.; Hsieh, P.S.; Hsieh, B.; Lenz, H.J.; Idos, G.; Friedland, S.; Yi-Jiun Pan, J.; Shao, H.J.; et al. Novel Circulating Tumor Cell Assay for Detection of Colorectal Adenomas and Cancer. *Clin. Transl. Gastroenterol.* **2019**, *10*, e00088. [CrossRef] [PubMed]
73. Cohen, S.J.; Punt, C.J.; Iannotti, N.; Saidman, B.H.; Sabbath, K.D.; Gabrail, N.Y.; Picus, J.; Morse, M.; Mitchell, E.; Miller, M.C.; et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **2008**, *26*, 3213–3221. [CrossRef] [PubMed]
74. Camera, S.; Akin Telli, T.; Woff, E.; Vandeputte, C.; Kehagias, P.; Guiot, T.; Critchi, G.; Wissam, Y.; Bregni, G.; Trevisi, E.; et al. Prognostic Value of the Pace of Tumor Progression as Assessed by Serial ^{18}F-FDG PET/CT Scan and Liquid Biopsy in Refractory Colorectal Cancer: The CORIOLAN Trial. *Cancers* **2020**, *12*, 2752. [CrossRef] [PubMed]
75. Arrazubi, V.; Mata, E.; Antelo, M.L.; Tarifa, A.; Herrera, J.; Zazpe, C.; Teijeira, L.; Viudez, A.; Suárez, J.; Hernández, I.; et al. Circulating Tumor Cells in Patients Undergoing Resection of Colorectal Cancer Liver Metastases. Clinical Utility for Long-Term Outcome: A Prospective Trial. *Ann. Surg. Oncol.* **2019**, *26*, 2805–2811. [CrossRef] [PubMed]
76. Pan, R.J.; Hong, H.J.; Sun, J.; Yu, C.R.; Liu, H.S.; Li, P.Y.; Zheng, M.H. Detection and Clinical Value of Circulating Tumor Cells as an Assisted Prognostic Marker in Colorectal Cancer Patients. *Cancer Manag. Res.* **2021**, *13*, 4567–4578. [CrossRef]
77. Tan, Y.; Wu, H. The significant prognostic value of circulating tumor cells in colorectal cancer: A systematic review and meta-analysis. *Curr. Probl. Cancer* **2018**, *42*, 95–106. [CrossRef]
78. Fabbri, F.; Carloni, S.; Zoli, W.; Ulivi, P.; Gallerani, G.; Fici, P.; Chiadini, E.; Passardi, A.; Frassineti, G.L.; Ragazzini, A.; et al. Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. *Cancer Lett.* **2013**, *335*, 225–231. [CrossRef]
79. Delgado-Ureña, M.; Ortega, F.G.; de Miguel-Pérez, D.; Rodriguez-Martínez, A.; García-Puche, J.L.; Ilyine, H.; Lorente, J.A.; Exposito-Hernandez, J.; Garrido-Navas, M.C.; Delgado-Ramirez, M.; et al. Circulating tumor cells criteria (CyCAR) versus standard RECIST criteria for treatment response assessment in metastatic colorectal cancer patients. *J. Transl. Med.* **2018**, *16*, 251. [CrossRef]
80. Aranda, E.; Viéitez, J.M.; Gómez-España, A.; Gil Calle, S.; Salud-Salvia, A.; Graña, B.; Garcia-Alfonso, P.; Rivera, F.; Quintero-Aldana, G.A.; Reina-Zoilo, J.J.; et al. Spanish Cooperative Group for the Treatment of Digestive Tumors (TTD). FOLFOXIRI plus bevacizumab versus FOLFOX plus bevacizumab for patients with metastatic colorectal cancer and ≥3 circulating tumour cells: The randomised phase III VISNÚ-1 trial. *ESMO Open* **2020**, *5*, e000944. [CrossRef]
81. Souza E Silva, V.; Chinen, L.T.; Abdallah, E.A.; Damascena, A.; Paludo, J.; Chojniak, R.; Dettino, A.L.; de Mello, C.A.; Alves, V.S.; Fanelli, M.F. Early detection of poor outcome in patients with metastatic colorectal cancer: Tumor kinetics evaluated by circulating tumor cells. *Onco Targets Ther.* **2016**, *9*, 7503–7513. [CrossRef]
82. Cayrefourcq, L.; Thomas, F.; Mazard, T.; Assenat, E.; Assou, S.; Alix-Panabières, C. Selective treatment pressure in colon cancer drives the molecular profile of resistant circulating tumor cell clones. *Mol. Cancer* **2021**, *20*, 30. [CrossRef] [PubMed]
83. Grillet, F.; Bayet, E.; Villeronce, O.; Zappia, L.; Lagerqvist, E.L.; Lunke, S.; Charafe-Jauffret, E.; Pham, K.; Molck, C.; Rolland, N.; et al. Circulating tumour cells from patients with colorectal cancer have cancer stem cell hallmarks in ex vivo culture. *Gut* **2017**, *66*, 1802–1810. [CrossRef]
84. Vasseur, A.; Kiavue, N.; Bidard, F.C.; Pierga, J.Y.; Cabel, L. Clinical utility of circulating tumor cells: An update. *Mol. Oncol.* **2021**, *15*, 1647–1666. [CrossRef] [PubMed]
85. MANDEL, P.; METAIS, P. Les acides nucléiques du plasma sanguin chez l'homme [Nuclear Acids In Human Blood Plasma]. *C. R. Seances Soc. Biol. Fil.* **1948**, *142*, 241–243.
86. Stroun, M.; Anker, P.; Maurice, P.; Lyautey, J.; Lederrey, C.; Beljanski, M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* **1989**, *46*, 318–322. [CrossRef] [PubMed]
87. Schwarzenbach, H.; Stoehlmacher, J.; Pantel, K.; Goekkurt, E. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. *Ann. N. Y. Acad. Sci.* **2008**, *1137*, 190–196. [CrossRef]
88. Bettegowda, C.; Sausen, M.; Leary, R.J.; Kinde, I.; Wang, Y.; Agrawal, N.; Bartlett, B.R.; Wang, H.; Luber, B.; Alani, R.M.; et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* **2014**, *6*, 224ra24. [CrossRef]
89. Nakamura, Y.; Taniguchi, H.; Ikeda, M.; Bando, H.; Kato, K.; Morizane, C.; Esaki, T.; Komatsu, Y.; Kawamoto, Y.; Takahashi, N.; et al. Clinical utility of circulating tumor DNA sequencing in advanced gastrointestinal cancer: SCRUM-Japan GI-SCREEN and GOZILA studies. *Nat. Med.* **2020**, *26*, 1859–1864. [CrossRef]

90. Vymetalkova, V.; Cervena, K.; Bartu, L.; Vodicka, P. Circulating Cell-Free DNA and Colorectal Cancer: A Systematic Review. *Int. J. Mol. Sci.* **2018**, *19*, 3356. [CrossRef]
91. Ou, S.I.; Nagasaka, M.; Zhu, V.W. Liquid Biopsy to Identify Actionable Genomic Alterations. *Am. Soc. Clin. Oncol. Educ. Book* **2018**, *38*, 978–997. [CrossRef]
92. Bando, H.; Nakamura, Y.; Taniguchi, H.; Shiozawa, M.; Yasui, H.; Esaki, T.; Ohta, T.; Denda, T.; Satoh, T.; Yamazakiet, K.; et al. Impact of a metastatic site on circulating tumor DNA (ctDNA) analysis in patients (pts) with metastatic colorectal cancer (mCRC). *J. Clin. Oncol.* **2021**, *39* (Suppl. 15), 3554. [CrossRef]
93. Sefrioui, D.; Beaussire, L.; Gillibert, A.; Blanchard, F.; Toure, E.; Bazille, C.; Perdrix, A.; Ziegler, F.; Gangloff, A.; Hassine, M.; et al. CEA, CA19-9, circulating DNA and circulating tumour cell kinetics in patients treated for metastatic colorectal cancer (mCRC). *Br. J. Cancer* **2021**, *125*, 725–733. [CrossRef] [PubMed]
94. Kirsch, C.; Weickmann, S.; Schmidt, B.; Fleischhacker, M. An improved method for the isolation of free-circulating plasma DNA and cell-free DNA from other body fluids. *Ann. N. Y. Acad. Sci.* **2008**, *1137*, 135–139. [CrossRef]
95. Severson, E.A.; Riedlinger, G.M.; Connelly, C.F.; Vergilio, J.A.; Goldfinger, M.; Ramkissoon, S.; Frampton, G.M.; Ross, J.S.; Fratella-Calabrese, A.; Gay, L.; et al. Detection of clonal hematopoiesis of indeterminate potential in clinical sequencing of solid tumor specimens. *Blood* **2018**, *131*, 2501–2505. [CrossRef]
96. Dasari, A.; Morris, V.K.; Allegra, C.J.; Atreya, C.; Benson, A.B., 3rd; Boland, P.; Chung, K.; Copur, M.S.; Corcoran, R.B.; Deming, D.A.; et al. ctDNA applications and integration in colorectal cancer: An NCI Colon and Rectal-Anal Task Forces whitepaper. *Nat. Rev. Clin. Oncol.* **2020**, *17*, 757–770. [CrossRef] [PubMed]
97. Reinert, T.; Schøler, L.V.; Thomsen, R.; Tobiasen, H.; Vang, S.; Nordentoft, I.; Lamy, P.; Kannerup, A.S.; Mortensen, F.V.; Stribolt, K.; et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut* **2016**, *65*, 625–634. [CrossRef]
98. Sato, K.A.; Hachiya, T.; Iwaya, T.; Kume, K.; Matsuo, T.; Kawasaki, K.; Abiko, Y.; Akasaka, R.; Matsumoto, T.; Otsuka, K.; et al. Individualized Mutation Detection in Circulating Tumor DNA for Monitoring Colorectal Tumor Burden Using a Cancer-Associated Gene Sequencing Panel. *PLoS ONE* **2016**, *11*, e0146275. [CrossRef]
99. Olmedillas-López, S.; García-Olmo, D.C.; García-Arranz, M.; Peiró-Pastor, R.; Aguado, B.; García-Olmo, D. Liquid biopsy by NGS: Differential presence of exons (DPE) in cell-free DNA reveals different patterns in metastatic and nonmetastatic colorectal cancer. *Cancer Med.* **2018**, *7*, 1706–1716. [CrossRef]
100. Yang, Y.C.; Wang, D.; Jin, L.; Yao, H.W.; Zhang, J.H.; Wang, J.; Zhao, X.M.; Shen, C.Y.; Chen, W.; Wang, X.L.; et al. Circulating tumor DNA detectable in early- and late-stage colorectal cancer patients. *Biosci. Rep.* **2018**, *38*, BSR20180322. [CrossRef]
101. Spindler, K.G.; Boysen, A.K.; Pallisgård, N.; Johansen, J.S.; Tabernero, J.; Sørensen, M.M.; Jensen, B.V.; Hansen, T.F.; Sefrioui, D.; Andersen, R.F.; et al. Cell-Free DNA in Metastatic Colorectal Cancer: A Systematic Review and Meta-Analysis. *Oncologist* **2017**, *22*, 1049–1055. [CrossRef]
102. Hamfjord, J.; Guren, T.K.; Dajani, O.; Johansen, J.S.; Glimelius, B.; Sorbye, H.; Pfeiffer, P.; Lingjærde, O.C.; Tveit, K.M.; Kure, E.H.; et al. Total circulating cell-free DNA as a prognostic biomarker in metastatic colorectal cancer before first-line oxaliplatin-based chemotherapy. *Ann. Oncol.* **2019**, *30*, 1088–1095. [CrossRef] [PubMed]
103. Tie, J.; Wang, Y.; Cohen, J.; Li, L.; Hong, W.; Christie, M.; Wong, H.L.; Kosmider, S.; Wong, R.; Thomson, B.; et al. Circulating tumor DNA dynamics and recurrence risk in patients undergoing curative intent resection of colorectal cancer liver metastases: A prospective cohort study. *PLoS Med.* **2021**, *18*, e1003620. [CrossRef] [PubMed]
104. Øgaard, N.; Reinert, T.; Henriksen, T.V.; Frydendahl, A.; Aagaard, E.; Ørntoft, M.W.; Larsen, M.Ø.; Knudsen, A.R.; Mortensen, F.V.; Andersen, C.L. Tumour-agnostic circulating tumour DNA analysis for improved recurrence surveillance after resection of colorectal liver metastases: A prospective cohort study. *Eur. J. Cancer* **2022**, *163*, 163–176. [CrossRef] [PubMed]
105. Wallner, M.; Herbst, A.; Behrens, A.; Crispin, A.; Stieber, P.; Göke, B.; Lamerz, R.; Kolligs, F.T. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. *Clin. Cancer Res.* **2006**, *12*, 7347–7352. [CrossRef]
106. Young, G.P.; Symonds, E.L.; Nielsen, H.J.; Ferm, L.; Christensen, I.J.; Dekker, E.; van der Vlugt, M.; Mallant-Hent, R.C.; Boulter, N.; Yu, B.; et al. Evaluation of a panel of tumor-specific differentially-methylated DNA regions in IRF4, IKZF1 and BCAT1 for blood-based detection of colorectal cancer. *Clin. Epigenetics* **2021**, *13*, 14. [CrossRef] [PubMed]
107. Picardo, F.; Romanelli, A.; Muinelo-Romay, L.; Mazza, T.; Fusilli, C.; Parrella, P.; Barbazán, J.; Lopez-López, R.; Barbano, R.; De Robertis, M.; et al. Diagnostic and Prognostic Value of *B4GALT1* Hypermethylation and Its Clinical Significance as a Novel Circulating Cell-Free DNA Biomarker in Colorectal Cancer. *Cancers* **2019**, *11*, 1598. [CrossRef] [PubMed]
108. Philipp, A.B.; Stieber, P.; Nagel, D.; Neumann, J.; Spelsberg, F.; Jung, A.; Lamerz, R.; Herbst, A.; Kolligs, F.T. Prognostic role of methylated free circulating DNA in colorectal cancer. *Int. J. Cancer* **2012**, *131*, 2308–2319. [CrossRef]
109. Tie, J.; Kinde, I.; Wang, Y.; Wong, H.L.; Roebert, J.; Christie, M.; Tacey, M.; Wong, R.; Singh, M.; Karapetis, C.S.; et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. *Ann. Oncol.* **2015**, *26*, 1715–1722. [CrossRef]
110. Garlan, F.; Laurent-Puig, P.; Sefrioui, D.; Siauve, N.; Didelot, A.; Sarafan-Vasseur, N.; Michel, P.; Perkins, G.; Mulot, C.; Blons, H.; et al. Early Evaluation of Circulating Tumor DNA as Marker of Therapeutic Efficacy in Metastatic Colorectal Cancer Patients (PLACOL Study). *Clin. Cancer Res.* **2017**, *23*, 5416–5425. [CrossRef]

111. Wong, A.L.; Lim, J.S.; Sinha, A.; Gopinathan, A.; Lim, R.; Tan, C.S.; Soh, T.; Venkatesh, S.; Titin, C.; Sapari, N.S.; et al. Tumour pharmacodynamics and circulating cell free DNA in patients with refractory colorectal carcinoma treated with regorafenib. *J. Transl. Med.* **2015**, *13*, 57. [CrossRef]
112. Tabernero, J.; Lenz, H.J.; Siena, S.; Sobrero, A.; Falcone, A.; Ychou, M.; Humblet, Y.; Bouché, O.; Mineur, L.; Barone, C.; et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: A retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol.* **2015**, *16*, 937–948. [CrossRef] [PubMed]
113. Pereira, A.A.L.; Lam, M.; Kanikarla-Marie, P.; Raghav, K.P.S.; Morris, V.K.; Brown, H.; Windham, J.; Duose, D.Y.; Overman, M.J.; Sanchez, E.V.; et al. Circulating tumor DNA (ctDNA) as an early marker to monitor clinical benefit of regorafenib and TAS-102 in patients with metastatic colorectal cancer (mCRC). *J. Clin. Oncol.* **2018**, *36* (Suppl. 15), 3533. [CrossRef]
114. Colle, R.; Radzik, A.; Cohen, R.; Pellat, A.; Lopez-Tabada, D.; Cachanado, M.; Duval, A.; Svrcek, M.; Menu, Y.; André, T. Pseudoprogression in patients treated with immune checkpoint inhibitors for microsatellite instability-high/mismatch repair-deficient metastatic colorectal cancer. *Eur. J. Cancer* **2021**, *144*, 9–16. [CrossRef] [PubMed]
115. Lee, J.H.; Long, G.V.; Menzies, A.M.; Lo, S.; Guminski, A.; Whitbourne, K.; Peranec, M.; Scolyer, R.; Kefford, R.F.; Rizos, H.; et al. Association Between Circulating Tumor DNA and Pseudoprogression in Patients With Metastatic Melanoma Treated With Anti-Programmed Cell Death 1 Antibodies. *JAMA Oncol.* **2018**, *4*, 717–721. [CrossRef] [PubMed]
116. Wang, C.; Chevalier, D.; Saluja, J.; Sandhu, J.; Lau, C.; Fakih, M. Regorafenib and Nivolumab or Pembrolizumab Combination and Circulating Tumor DNA Response Assessment in Refractory Microsatellite Stable Colorectal Cancer. *Oncologist* **2020**, *25*, e1188–e1194. [CrossRef]
117. Raunkilde, L.; Hansen, T.F.; Andersen, R.F.; Havelund, B.M.; Thomsen, C.B.; Jensen, L.H. *NPY* Gene Methylation in Circulating Tumor DNA as an Early Biomarker for Treatment Effect in Metastatic Colorectal Cancer. *Cancers* **2022**, *14*, 4459. [CrossRef]
118. Douillard, J.Y.; Oliner, K.S.; Siena, S.; Tabernero, J.; Burkes, R.; Barugel, M.; Humblet, Y.; Bodoky, G.; Cunningham, D.; Jassem, J.; et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N. Engl. J. Med.* **2013**, *369*, 1023–1034. [CrossRef]
119. Van Cutsem, E.; Lenz, H.J.; Köhne, C.H.; Heinemann, V.; Tejpar, S.; Melezínek, I.; Beier, F.; Stroh, C.; Rougier, P.; van Krieken, J.H.; et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J. Clin. Oncol.* **2015**, *33*, 692–700. [CrossRef]
120. Taly, V.; Pekin, D.; Benhaim, L.; Kotsopoulos, S.K.; Le Corre, D.; Li, X.; Atochin, I.; Link, D.R.; Griffiths, A.D.; Pallier, K.; et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. *Clin. Chem.* **2013**, *59*, 1722–1731. [CrossRef]
121. Thierry, A.R.; Mouliere, F.; El Messaoudi, S.; Mollevi, C.; Lopez-Crapez, E.; Rolet, F.; Gillet, B.; Gongora, C.; Dechelotte, P.; Robert, B.; et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat. Med.* **2014**, *20*, 430–435. [CrossRef]
122. Vidal, J.; Muinelo, L.; Dalmases, A.; Jones, F.; Edelstein, D.; Iglesias, M.; Orrillo, M.; Abalo, A.; Rodríguez, C.; Brozos, E.; et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. *Ann. Oncol.* **2017**, *28*, 1325–1332. [CrossRef] [PubMed]
123. Siravegna, G.; Mussolin, B.; Buscarino, M.; Corti, G.; Cassingena, A.; Crisafulli, G.; Ponzetti, A.; Cremolini, C.; Amatu, A.; Lauricella, C.; et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat. Med.* **2015**, *21*, 795–801. [CrossRef] [PubMed]
124. Schmiegel, W.; Scott, R.J.; Dooley, S.; Lewis, W.; Meldrum, C.J.; Pockney, P.; Draganic, B.; Smith, S.; Hewitt, C.; Philimore, H.; et al. Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: Concordance of results from circulating tumor DNA and tissue-based RAS testing. *Mol. Oncol.* **2017**, *11*, 208–219. [CrossRef] [PubMed]
125. Grasselli, J.; Elez, E.; Caratù, G.; Matito, J.; Santos, C.; Macarulla, T.; Vidal, J.; Garcia, M.; Viéitez, J.M.; Paéz, D.; et al. Concordance of blood- and tumor-based detection of RAS mutations to guide anti-EGFR therapy in metastatic colorectal cancer. *Ann. Oncol.* **2017**, *28*, 1294–1301. [CrossRef] [PubMed]
126. Bachet, J.B.; Bouché, O.; Taieb, J.; Dubreuil, O.; Garcia, M.L.; Meurisse, A.; Normand, C.; Gornet, J.M.; Artru, P.; Louafi, S.; et al. RAS mutation analysis in circulating tumor DNA from patients with metastatic colorectal cancer: The AGEO RASANC prospective multicenter study. *Ann. Oncol.* **2018**, *29*, 1211–1219. [CrossRef]
127. Normanno, N.; Esposito Abate, R.; Lambiase, M.; Forgione, L.; Cardone, C.; Iannaccone, A.; Sacco, A.; Rachiglio, A.M.; Martinelli, E.; Rizzi, D.; et al. CAPRI-GOIM Investigators. RAS testing of liquid biopsy correlates with the outcome of metastatic colorectal cancer patients treated with first-line FOLFIRI plus cetuximab in the CAPRI-GOIM trial. *Ann. Oncol.* **2018**, *29*, 112–118. [CrossRef]
128. Sunakawa, Y.; Satake, H.; Usher, J.; Jaimes, Y.; Miyamoto, Y.; Nakamura, M.; Kataoka, M.; Shiozawa, M.; Takagane, A.; Terazawa, T.; et al. Dynamic changes in RAS gene status in circulating tumour DNA: A phase II trial of first-line FOLFOXIRI plus bevacizumab for RAS-mutant metastatic colorectal cancer (JACCRO CC-11). *ESMO Open* **2022**, *7*, 100512. [CrossRef]
129. Hong, D.S.; Fakih, M.G.; Strickler, J.H.; Desai, J.; Durm, G.A.; Shapiro, G.I.; Falchook, G.S.; Price, T.J.; Sacher, A.; Denlinger, C.S.; et al. $KRAS^{G12C}$ Inhibition with Sotorasib in Advanced Solid Tumors. *N. Engl. J. Med.* **2020**, *383*, 1207–1217. [CrossRef]
130. Johnson, M.; Ou, S.; Barve, M.; Rybkin, I.; Papadopoulos, K.; Leal, T.; Velastegui, K.; Christensen, J.; Kheoh, T.; Chao, R.; et al. KRYSTAL-1: Activity and Safety of Adagrasib (MRTX849) in patients with Colorectal Cancer (CRC) and Other Solid Tumors Harboring a KRAS G12C Mutation. *Eur. J. Cancer* **2020**, *138* (Suppl. 2), S2. [CrossRef]

131. Thein, K.Z.; Biter, A.B.; Banks, K.C.; Duda, A.W.; Saam, J.; Roszik, J.; Janku, F.; Skoulidis, F.; Heymach, J.V.; Kopetz, S.; et al. Identification of *KRAS*^{G12C} Mutations in Circulating Tumor DNA in Patients With Cancer. *JCO Precis. Oncol.* **2022**, *6*, e2100547. [CrossRef]
132. Morelli, M.P.; Overman, M.J.; Dasari, A.; Kazmi, S.M.A.; Mazard, T.; Vilar, E.; Morris, V.K.; Lee, M.S.; Herron, D.; Eng, C.; et al. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. *Ann. Oncol.* **2015**, *26*, 731–736. [CrossRef] [PubMed]
133. Raghav, K.; Ou, F.S.; Venook, A.P.; Innocenti, F.; Sun, R.; Lenz, H.J.; Kopetz, S. Acquired Genomic Alterations on First-Line Chemotherapy With Cetuximab in Advanced Colorectal Cancer: Circulating Tumor DNA Analysis of the CALGB/SWOG-80405 Trial (Alliance). *J. Clin. Oncol.* **2023**, *41*, 472–478. [CrossRef]
134. Topham, J.T.; O'Callaghan, C.J.; Feilotter, H.; Kennecke, H.F.; Lee, Y.S.; Li, W.; Banks, K.C.; Quinn, K.; Renouf, D.J.; Jonker, D.J.; et al. Circulating Tumor DNA Identifies Diverse Landscape of Acquired Resistance to Anti-Epidermal Growth Factor Receptor Therapy in Metastatic Colorectal Cancer. *J. Clin. Oncol.* **2023**, *41*, 485–496. [CrossRef] [PubMed]
135. Diaz, L.A., Jr.; Williams, R.T.; Wu, J.; Kinde, I.; Hecht, J.R.; Berlin, J.; Allen, B.; Bozic, I.; Reiter, J.G.; Nowak, M.A.; et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* **2012**, *486*, 537–540. [CrossRef]
136. Van Emburgh, B.O.; Arena, S.; Siravegna, G.; Lazzari, L.; Crisafulli, G.; Corti, G.; Mussolin, B.; Baldi, F.; Buscarino, M.; Bartolini, A.; et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. *Nat. Commun.* **2016**, *7*, 13665. [CrossRef] [PubMed]
137. Parseghian, C.M.; Loree, J.M.; Morris, V.K.; Liu, X.; Clifton, K.K.; Napolitano, S.; Henry, J.T.; Pereira, A.A.; Vilar, E.; Johnson, B.; et al. Anti-EGFR-resistant clones decay exponentially after progression: Implications for anti-EGFR re-challenge. *Ann. Oncol.* **2019**, *30*, 243–249. [CrossRef] [PubMed]
138. Ciardiello, F.; Normanno, N.; Martinelli, E.; Troiani, T.; Pisconti, S.; Cardone, C.; Nappi, A.; Bordonaro, A.R.; Rachiglio, M.; Lambiase, M.; et al. CAPRI-GOIM investigators. Cetuximab continuation after first progression in metastatic colorectal cancer (CAPRI-GOIM): A randomized phase II trial of FOLFOX plus cetuximab versus FOLFOX. *Ann. Oncol.* **2016**, *27*, 1055–1061. [CrossRef]
139. Santini, D.; Vincenzi, B.; Addeo, R.; Garufi, C.; Masi, G.; Scartozzi, M.; Mancuso, A.; Frezza, A.M.; Venditti, O.; Imperatori, M.; et al. Cetuximab rechallenge in metastatic colorectal cancer patients: How to come away from acquired resistance? *Ann. Oncol.* **2012**, *23*, 2313–2318. [CrossRef]
140. Masuishi, T.; Tsuji, A.; Kotaka, M.; Nakamura, M.; Kochi, M.; Takagane, A.; Shimada, K.; Denda, T.; Segawa, Y.; Tanioka, H.; et al. Phase 2 study of irinotecan plus cetuximab rechallenge as third-line treatment in KRAS wild-type metastatic colorectal cancer: JACCRO CC-08. *Br. J. Cancer* **2020**, *123*, 1490–1495. [CrossRef]
141. Cremolini, C.; Rossini, D.; Dell'Aquila, E.; Lonardi, S.; Conca, E.; Del Re, M.; Busico, A.; Pietrantonio, F.; Danesi, R.; Aprile, G.; et al. Rechallenge for Patients With RAS and BRAF Wild-Type Metastatic Colorectal Cancer With Acquired Resistance to First-line Cetuximab and Irinotecan: A Phase 2 Single-Arm Clinical Trial. *JAMA Oncol.* **2019**, *5*, 343–350. [CrossRef]
142. Martinelli, E.; Martini, G.; Famiglietti, V.; Troiani, T.; Napolitano, S.; Pietrantonio, F.; Ciardiello, D.; Terminiello, M.; Borrelli, C.; Vitiello, P.P.; et al. Cetuximab Rechallenge Plus Avelumab in Pretreated Patients With RAS Wild-type Metastatic Colorectal Cancer: The Phase 2 Single-Arm Clinical CAVE Trial. *JAMA Oncol.* **2021**, *7*, 1529–1535. [CrossRef] [PubMed]
143. Montagut, C.; Argilés, G.; Ciardiello, F.; Poulsen, T.T.; Dienstmann, R.; Kragh, M.; Kopetz, S.; Lindsted, T.; Ding, C.; Vidal, J.; et al. Efficacy of Sym004 in Patients With Metastatic Colorectal Cancer With Acquired Resistance to Anti-EGFR Therapy and Molecularly Selected by Circulating Tumor DNA Analyses: A Phase 2 Randomized Clinical Trial. *JAMA Oncol.* **2018**, *4*, e175245. [CrossRef] [PubMed]
144. Sartore-Bianchi, A.; Pietrantonio, F.; Lonardi, S.; Mussolin, B.; Rua, F.; Crisafulli, G.; Bartolini, A.; Fenocchio, E.; Amatu, A.; Manca, P.; et al. Circulating tumor DNA to guide rechallenge with panitumumab in metastatic colorectal cancer: The phase 2 CHRONOS trial. *Nat. Med.* **2022**, *28*, 1612–1618. [CrossRef] [PubMed]
145. Aparicio, J.; Virgili Manrique, A.C.; Capdevila, J.; Muñoz Boza, F.; Galván, P.; Richart, P.; Oliveres, H.; Páez, D.; Hernando, J.; Serrano, S.; et al. Randomized phase II trial of FOLFIRI-panitumumab compared with FOLFIRI alone in patients with RAS wild-type circulating tumor DNA metastatic colorectal cancer beyond progression to first-line FOLFOX-panitumumab: The BEYOND study (GEMCAD 17-01). *Clin. Transl. Oncol.* **2022**, *24*, 2155–2165. [CrossRef]
146. Cicek, M.S.; Lindor, N.M.; Gallinger, S.; Bapat, B.; Hopper, J.L.; Jenkins, M.A.; Young, J.; Buchanan, D.; Walsh, M.D.; Le Marchand, L.; et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the Colon Cancer Family Registry. *J. Mol. Diagn.* **2011**, *13*, 271–281. [CrossRef]
147. Pawlik, T.M.; Raut, C.P.; Rodriguez-Bigas, M.A. Colorectal carcinogenesis: MSI-H versus MSI-L. *Dis. Markers* **2004**, *20*, 199–206. [CrossRef]
148. Vilar, E.; Gruber, S.B. Microsatellite instability in colorectal cancer-the stable evidence. *Nat. Rev. Clin. Oncol.* **2010**, *7*, 153–162. [CrossRef] [PubMed]
149. Marcus, L.; Lemery, S.J.; Keegan, P.; Pazdur, R. FDA Approval Summary: Pembrolizumab for the Treatment of Microsatellite Instability-High Solid Tumors. *Clin. Cancer Res.* **2019**, *25*, 3753–3758. [CrossRef]
150. Le, D.T.; Uram, J.N.; Wang, H.; Bartlett, B.R.; Kemberling, H.; Eyring, A.D.; Skora, A.D.; Luber, B.S.; Azad, N.S.; Laheru, D.; et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N. Engl. J. Med.* **2015**, *372*, 2509–2520. [CrossRef]

151. Le, D.T.; Durham, J.N.; Smith, K.N.; Wang, H.; Bartlett, B.R.; Aulakh, L.K.; Lu, S.; Kemberling, H.; Wilt, C.; Luber, B.S.; et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **2017**, *357*, 409–413. [CrossRef]
152. Shia, J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. *J. Mol. Diagn.* **2008**, *10*, 293–300. [CrossRef] [PubMed]
153. Bacher, J.W.; Flanagan, L.A.; Smalley, R.L.; Nassif, N.A.; Burgart, L.J.; Halberg, R.B.; Megid, W.M.; Thibodeau, S.N. Development of a fluorescent multiplex assay for detection of MSI-High tumors. *Dis. Markers* **2004**, *20*, 237–250. [CrossRef]
154. Silveira, A.B.; Bidard, F.C.; Kasperek, A.; Melaabi, S.; Tanguy, M.L.; Rodrigues, M.; Bataillon, G.; Cabel, L.; Buecher, B.; Pierga, J.Y.; et al. High-Accuracy Determination of Microsatellite Instability Compatible with Liquid Biopsies. *Clin. Chem.* **2020**, *66*, 606–613. [CrossRef]
155. Lee, H.S.; Kim, W.H.; Kwak, Y.; Koh, J.; Bae, J.M.; Kim, K.M.; Chang, M.S.; Han, H.S.; Kim, J.M.; Kim, H.W.; et al. Gastrointestinal Pathology Study Group of Korean Society of Pathologists, & Molecular Pathology Study Group of Korean Society of Pathologists. Molecular Testing for Gastrointestinal Cancer. *J. Pathol. Transl. Med.* **2017**, *51*, 103–121. [PubMed]
156. Ladas, I.; Yu, F.; Leong, K.W.; Fitarelli-Kiehl, M.; Song, C.; Ashtaputre, R.; Kulke, M.; Mamon, H.; Makrigiorgos, G.M. Enhanced detection of microsatellite instability using pre-PCR elimination of wild-type DNA homo-polymers in tissue and liquid biopsies. *Nucleic Acids Res.* **2018**, *46*, e74. [CrossRef]
157. Trabucco, S.E.; Gowen, K.; Maund, S.L.; Sanford, E.; Fabrizio, D.A.; Hall, M.J.; Yakirevich, E.; Gregg, J.P.; Stephens, P.J.; Frampton, G.M.; et al. A Novel Next-Generation Sequencing Approach to Detecting Microsatellite Instability and Pan-Tumor Characterization of 1000 Microsatellite Instability-High Cases in 67,000 Patient Samples. *J. Mol. Diagn.* **2019**, *21*, 1053–1066. [CrossRef] [PubMed]
158. Willis, J.; Lefterova, M.I.; Artyomenko, A.; Kasi, P.M.; Nakamura, Y.; Mody, K.; Catenacci, D.V.T.; Fakih, M.; Barbacioru, C.; Zhao, J.; et al. Validation of Microsatellite Instability Detection Using a Comprehensive Plasma-Based Genotyping Panel. *Clin. Cancer Res.* **2019**, *25*, 7035–7045. [CrossRef] [PubMed]
159. Davies, H.; Bignell, G.R.; Cox, C.; Stephens, P.; Edkins, S.; Clegg, S.; Teague, J.; Woffendin, H.; Garnett, M.J.; Bottomley, W.; et al. Mutations of the BRAF gene in human cancer. *Nature* **2002**, *417*, 949–954. [CrossRef]
160. Pietrantonio, F.; Mazzaferro, V.; Miceli, R.; Cotsoglou, C.; Melotti, F.; Fanetti, G.; Perrone, F.; Biondani, P.; Muscarà, C.; Di Bartolomeo, M.; et al. Pathological response after neoadjuvant bevacizumab- or cetuximab-based chemotherapy in resected colorectal cancer liver metastases. *Med. Oncol.* **2015**, *32*, 182. [CrossRef]
161. Bertotti, A.; Papp, E.; Jones, S.; Adleff, V.; Anagnostou, V.; Lupo, B.; Sausen, M.; Phallen, J.; Hruban, C.A.; Tokheim, C.; et al. The genomic landscape of response to EGFR blockade in colorectal cancer. *Nature* **2015**, *526*, 263–267. [CrossRef]
162. Jones, J.C.; Renfro, L.A.; Al-Shamsi, H.O.; Schrock, A.B.; Rankin, A.; Zhang, B.Y.; Kasi, P.M.; Voss, J.S.; Leal, A.D.; Sun, J.; et al. $^{\text{Non-V600}}$ BRAF Mutations Define a Clinically Distinct Molecular Subtype of Metastatic Colorectal Cancer. *J. Clin. Oncol.* **2017**, *35*, 2624–2630. [CrossRef] [PubMed]
163. Kopetz, S.; Grothey, A.; Yaeger, R.; Van Cutsem, E.; Desai, J.; Yoshino, T.; Wasan, H.; Ciardiello, F.; Loupakis, F.; Hong, Y.S.; et al. Encorafenib, Binimetinib, and Cetuximab in *BRAF* V600E-Mutated Colorectal Cancer. *N. Engl. J. Med.* **2019**, *381*, 1632–1643. [CrossRef] [PubMed]
164. Corcoran, R.B.; André, T.; Yoshino, T.; Bendell, J.C.; Atreya, C.E.; Schellens, J.H.M.; Ducreux, M.P.; McRee, A.; Siena, S.; Middleton, G.; et al. Efficacy and circulating tumor DNA (ctDNA) analysis of the BRAF inhibitor dabrafenib (D), MEK inhibitor trametinib (T), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E–mutated (BRAFm) metastatic colorectal cancer (mCRC). *Ann. Oncol.* **2016**, *27*, vi150. [CrossRef]
165. Hong, D.S.; Morris, V.K.; El Osta, B.; Sorokin, A.V.; Janku, F.; Fu, S.; Overman, M.J.; Piha-Paul, S.; Subbiah, V.; Kee, B.; et al. Phase IB Study of Vemurafenib in Combination with Irinotecan and Cetuximab in Patients with Metastatic Colorectal Cancer with BRAFV600E Mutation. *Cancer Discov.* **2016**, *6*, 1352–1365. [CrossRef]
166. Ye, L.F.; Huang, Z.Y.; Chen, X.X.; Chen, Z.G.; Wu, S.X.; Ren, C.; Hu, M.T.; Bao, H.; Jin, Y.; Wang, F.; et al. Monitoring tumour resistance to the BRAF inhibitor combination regimen in colorectal cancer patients via circulating tumour DNA. *Drug Resist. Updates* **2022**, *65*, 100883. [CrossRef]
167. Zhang, Y.; Xia, M.; Jin, K.; Wang, S.; Wei, H.; Fan, C.; Wu, Y.; Li, X.; Li, X.; Li, G.; et al. Function of the c-Met receptor tyrosine kinase in carcinogenesis and associated therapeutic opportunities. *Mol. Cancer* **2018**, *17*, 45. [CrossRef]
168. Birchmeier, C.; Birchmeier, W.; Gherardi, E.; Vande Woude, G.F. Met, metastasis, motility and more. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 915–925. [CrossRef]
169. Gao, H.; Guan, M.; Sun, Z.; Bai, C. High c-Met expression is a negative prognostic marker for colorectal cancer: A meta-analysis. *Tumour Biol.* **2015**, *36*, 515–520. [CrossRef]
170. Raghav, K.; Morris, V.; Tang, C.; Morelli, P.; Amin, H.M.; Chen, K.; Manyam, G.C.; Broom, B.; Overman, M.J.; Shaw, K.; et al. MET amplification in metastatic colorectal cancer: An acquired response to EGFR inhibition, not a de novo phenomenon. *Oncotarget* **2016**, *7*, 54627–54631. [CrossRef]
171. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **2012**, *487*, 330–337. [CrossRef]

172. Bardelli, A.; Corso, S.; Bertotti, A.; Hobor, S.; Valtorta, E.; Siravegna, G.; Sartore-Bianchi, A.; Scala, E.; Cassingena, A.; Zecchin, D.; et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. *Cancer Discov.* **2013**, *3*, 658–673. [CrossRef]
173. Delord, J.P.; Argilés, G.; Fayette, J.; Wirth, L.; Kasper, S.; Siena, S.; Mesia, R.; Berardi, R.; Cervantes, A.; Dekervel, J.; et al. A phase 1b study of the MET inhibitor capmatinib combined with cetuximab in patients with MET-positive colorectal cancer who had progressed following anti-EGFR monoclonal antibody treatment. *Investig. New Drugs* **2020**, *38*, 1774–1783. [CrossRef]
174. Valtorta, E.; Martino, C.; Sartore-Bianchi, A.; Penaullt-Llorca, F.; Viale, G.; Risio, M.; Rugge, M.; Grigioni, W.; Bencardino, K.; Lonardi, S.; et al. Assessment of a HER2 scoring system for colorectal cancer: Results from a validation study. *Mod. Pathol.* **2015**, *28*, 1481–1491. [CrossRef]
175. Fujii, S.; Magliocco, A.M.; Kim, J.; Okamoto, W.; Kim, J.E.; Sawada, K.; Nakamura, Y.; Kopetz, S.; Park, W.Y.; Tsuchihara, K.; et al. International Harmonization of Provisional Diagnostic Criteria for *ERBB2*-Amplified Metastatic Colorectal Cancer Allowing for Screening by Next-Generation Sequencing Panel. *JCO Precis. Oncol.* **2020**, *4*, 6–19. [CrossRef] [PubMed]
176. Siravegna, G.; Sartore-Bianchi, A.; Nagy, R.J.; Raghav, K.; Odegaard, J.I.; Lanman, R.B.; Trusolino, L.; Marsoni, S.; Siena, S.; Bardelli, A. Plasma HER2 (*ERBB2*) Copy Number Predicts Response to HER2-targeted Therapy in Metastatic Colorectal Cancer. *Clin. Cancer Res.* **2019**, *25*, 3046–3053. [CrossRef]
177. Ivanov, M.; Baranova, A.; Butler, T.; Spellman, P.; Mileyko, V. Non-random fragmentation patterns in circulating cell-free DNA reflect epigenetic regulation. *BMC Genom.* **2015**, *16*, S1. [CrossRef] [PubMed]
178. Cristiano, S.; Leal, A.; Phallen, J.; Fiksel, J.; Adleff, V.; Bruhm, D.C.; Jensen, S.Ø.; Medina, J.E.; Hruban, C.; White, J.R.; et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* **2019**, *570*, 385–389. [CrossRef] [PubMed]
179. Jiang, P.; Lo, Y.M.D. The Long and Short of Circulating Cell-Free DNA and the Ins and Outs of Molecular Diagnostics. *Trends Genet. TIG* **2016**, *32*, 360–371. [CrossRef]
180. Jiang, P.; Sun, K.; Peng, W.; Cheng, S.H.; Ni, M.; Yeung, P.C.; Heung, M.M.S.; Xie, T.; Shang, H.; Zhou, Z.; et al. Plasma DNA End-Motif Profiling as a Fragmentomic Marker in Cancer, Pregnancy, and Transplantation. *Cancer Discov.* **2020**, *10*, 664–673. [CrossRef] [PubMed]
181. Girirajan, S.; Campbell, C.; Eichler, E. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. *Physiol. Behav.* **2016**, *176*, 139–148.
182. Mathios, D.; Johansen, J.S.; Cristiano, S.; Medina, J.E.; Phallen, J.; Larsen, K.R.; Bruhm, D.C.; Niknafs, N.; Ferreira, L.; Adleff, V.; et al. Detection and characterization of lung cancer using cell-free DNA fragmentomes. *Nat. Commun.* **2021**, *12*, 5060. [CrossRef] [PubMed]
183. Bao, H.; Wang, Z.; Ma, X.; Guo, W.; Zhang, X.; Tang, W.; Chen, X.; Wang, X.; Chen, Y.; Mo, S.; et al. Letter to the Editor: An ultra-sensitive assay using cell-free DNA fragmentomics for multi-cancer early detection. *Mol. Cancer* **2022**, *21*, 129. [CrossRef] [PubMed]
184. Ma, X.; Chen, Y.; Tang, W.; Bao, H.; Mo, S.; Liu, R.; Wu, S.; Bao, H.; Li, Y.; Zhang, L.; et al. Multi-dimensional fragmentomic assay for ultrasensitive early detection of colorectal advanced adenoma and adenocarcinoma. *J. Hematol. Oncol.* **2021**, *14*, 175. [CrossRef]
185. Wang, Y.; Fan, X.; Bao, H.; Xia, F.; Wan, J.; Shen, L.; Wang, Y.; Zhang, H.; Wei, Y.; Wu, X.; et al. Utility of Circulating Free DNA Fragmentomics in the Prediction of Pathological Response after Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer. *Clin Chem.* **2023**, *69*, 88–99. [CrossRef] [PubMed]
186. Sanchez, C.; Roch, B.; Mazard, T.; Blache, P.; Dache, Z.A.A.; Pastor, B.; Pisareva, E.; Tanos, R.; Thierry, A.R. Circulating nuclear DNA structural features, origins, and complete size profile revealed by fragmentomics. *JCI Insight* **2021**, *6*, e144561. [CrossRef] [PubMed]
187. Van't Erve, I.; Rovers, K.P.; Constantinides, A.; Bolhuis, K.; Wassenaar, E.C.; Lurvink, R.J.; Huysentruyt, C.J.; Snaebjornsson, P.; Boerma, D.; van den Broek, D.; et al. Detection of tumor-derived cell-free DNA from colorectal cancer peritoneal metastases in plasma and peritoneal fluid. *J. Pathol. Clin. Res.* **2021**, *7*, 203–208. [CrossRef]
188. Nicolazzo, C.; Belardinilli, F.; Vestri, A.; Magri, V.; De Renzi, G.; De Meo, M.; Caponnetto, S.; Di Nicolantonio, F.; Cortesi, E.; Giannini, G.; et al. *RAS* Mutation Conversion in Bevacizumab-Treated Metastatic Colorectal Cancer Patients: A Liquid Biopsy Based Study. *Cancers* **2022**, *14*, 802. [CrossRef] [PubMed]