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Beneath the surface of colorectal cancer: Unmasking the evolving nature of (Neo)RAS

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ABSTRACT

Metastatic colorectal cancer (mCRC) remains a major clinical challenge, despite therapeutic advancements. Mutations in KRAS and NRAS (RAS) oncogenes drive resistance to anti-EGFR drugs, necessitating RAS mutational analysis prior to treatment. While tissue biopsy remains the gold standard for molecular profiling, it has limitations such as invasiveness, intra-tumoral heterogeneity, and delayed results. Liquid biopsy (LB), on the other hand, offers a non-invasive alternative by analyzing circulating tumor DNA (ctDNA) and it provides a dynamic view of molecular changes over time. Indeed, ctDNA analysis has expanded the understanding of the mCRC's molecular landscape, revealing that RAS mutated (MT) subclones undergo both a positive and a negative selection during treatment. This negative selection has been described as the "NeoRAS WT phenomenon." The temporary disappearance of RAS mutations opens "RAS WT windows," thus making potential candidates for anti-EGFR therapies even patients initially diagnosed as RAS MT. This review examines numerous studies investigating the clinical significance of the "NeoRas WT phenomenon" as a distinct pathological entity. It also highlights the key limitations arising from the variability in study designs, detecting methods and ctDNA shedding rates. The results of ongoing prospective trials are necessary to determine whether NeoRAS WT stands as a reliable marker for guiding anti-EGFR treatment strategies in RAS-mutated patients.

1. Introduction

Metastatic colorectal cancer (mCRC) remains a major clinical challenge. Advances in understanding its molecular complexity have led to significant improvements in clinical outcomes. *KRAS* and *NRAS* exon 2, 3, and 4 (*RAS*) oncogenic mutations occur in about 45 % of patients with mCRC, activating the *RAS/RAF/MEK/ERK* pathway and conferring resistance to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (moAbs) (Porru et al., 2018)., (Martinelli et al., 2020) For *RAS* mutant (*RAS* MT) mCRC, current clinical guidelines recommend first-line chemotherapy with either the cytotoxic FOLFOXIRI triplet or the FOLFOX/CAPOX/FOLFIRI doublets, in combination with anti-vascular endothelial growth factor (anti-VEGF) agents (Douillard et al., 2013; Bokemeyer et al., 2015; Cervantes et al., 2023; Benson et al., 2024). Conversely, patients with *RAS* and RAF wild type (WT) mCRC are eligible for either FOLFOX or FOLFIRI combined with anti-EGFR moAbs (i.e. Panitumumab or Cetuximab), particularly for left-sided tumors (Cervantes et al., 2023; Benson et al., 2024; Lenz et al., 2014; Stintzing et al., 2016). As a second-line strategy, anti-angiogenic agents (i.e. Bevacizumab, Aflibercept, or Ramucirumab) in combination with chemotherapy represent the standard of care option for both *RAS* MT and WT patients. In the presence of BRAF V600 gene mutations, a targeted approach with Encorafenib and Cetuximab has been approved (Cervantes et al., 2023; Benson et al., 2024). Among third-line treatment options, regorafenib, trifluridine/tipiracil, and fruquintinib have demonstrated limited efficacy in clinical trials, yielding modest objective response rates (ORR) and relatively short progression-free survival (PFS) and overall survival (OS) (Mayer et al., 2015; Prager et al., 2023;

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Dasari et al., 2023; Grothey et al., 2013). Given these limitations, ESMO guidelines recommend rechallenging pretreated *RAS* WT mCRC patients with anti-EGFR MoAbs, especially those who responded well initially (Cervantes et al., 2023). This approach, supported by liquid biopsy-driven selection, has shown promising results in small, non-randomized studies (Martinelli et al., 2021; Cremolini et al., 2019). In contrast, *RAS* MT patients are not eligible for the same strategy due to their well-established intrinsic resistance mechanisms (Cervantes et al., 2023). However, emerging evidence suggests that liquid biopsy (LB) may overcome these limitations (Gazzaniga et al., 2018). In response to treatment pressure and tumor microenvironment changes, mCRC's mutational landscape exhibits a dynamic and heterogeneous nature, underscoring the need for new targeting strategies (Nicolazzo et al., 2022). To address these challenges, this review explores *RAS* mutation dynamics in the era of LB, focusing on current and emerging evidence regarding anti-EGFR blockade in *RAS* MT patients.

1.1. The ctDNA revolution in CRC

In the era of precision medicine, the rapid evolution of targeted therapies demands advanced diagnostics to optimize patient selection. Despite being the gold standard for molecular profiling, tumor tissue biopsy carries notable limitations, including invasiveness and risks of severe complications (i.e. pain, bleeding, pneumothorax, pancreatitis, etc.) (Overman et al., 2013). Additionally, tumor cell yield may be insufficient for analysis, and sample preservation can be challenging (Diaz and Bardelli, 2014). Intratumoral heterogeneity, which varies spatially and temporally, further complicates tumor characterization, as single-site biopsies may miss distinct tumor subpopulations dispersed within a lesion, while the impracticality of serial sampling limits the

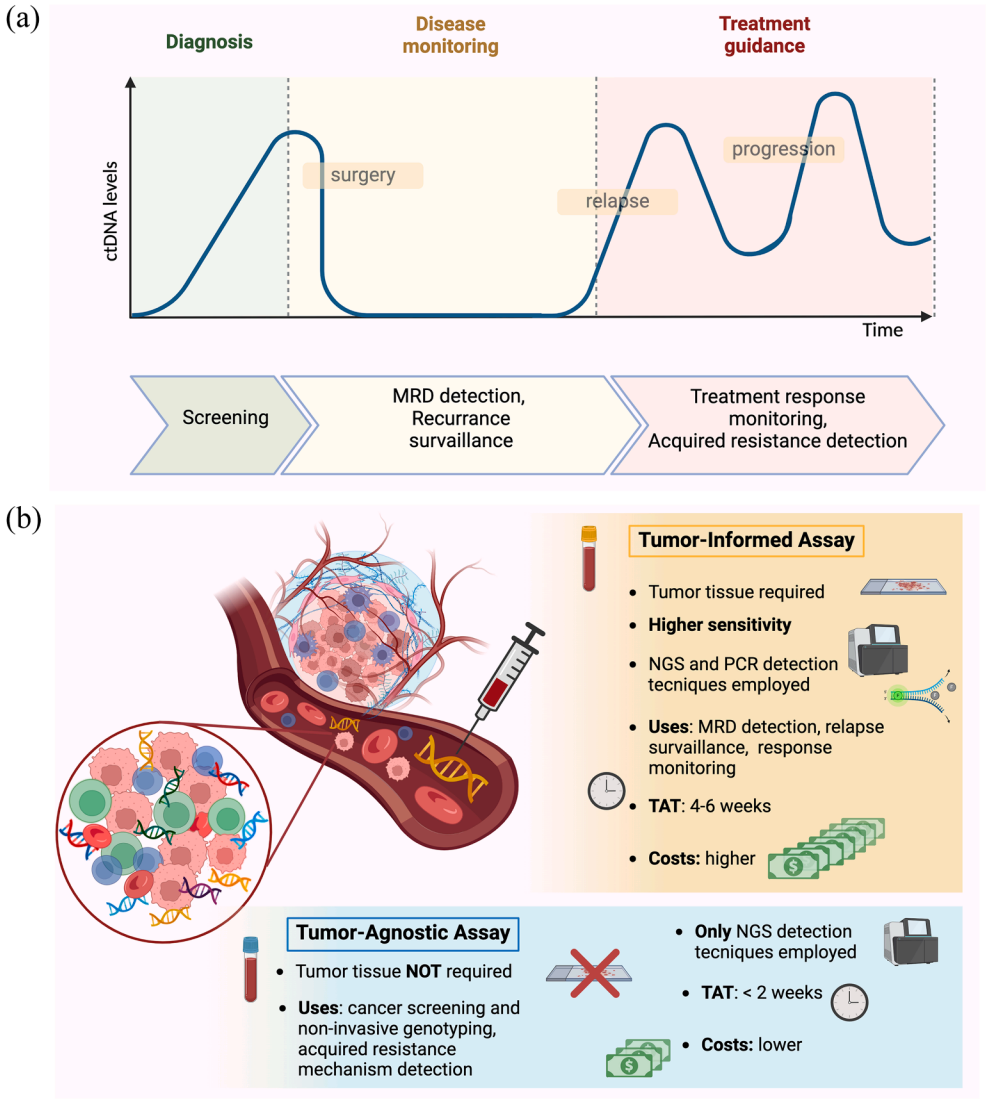

Fig. 1. A. The different clinical settings for ctDNA's applicability. LB finds a wide range of uses, starting from cancer screening to treatment monitoring in advanced stages, thus portraying dynamically the disease's evolution in response to surgical and medical interventions.Abbreviations NGS, Next-Generation Sequencing, PCR, Polymerase Chain Reaction, MRD, Minimal Residual Disease, ctDNA Circulating Tumor DNA); LB, liquid biopsy.Image created with BioRender.com, accessed on 28 August 2024. **B.** Types of ctDNA testing modalities. Tumor-informed assays track specific plasmatic mutations based on previously sequenced data from tissue samples, enhancing sensitivity at the cost of higher expenses and longer turnaround times (TAT). Conversely, tumor-agnostic methods permit a broader but less targeted plasma analysis, without prior knowledge of the tumor's characteristics.Abbreviations NGS, Next-Generation Sequencing, PCR, Polyme*RAS*e Chain Reaction, MRD, Minimal Residual Disease, ctDNA Circulating Tumor DNA); LB, liquid biopsy; TAT, turnaround time.Image created with BioRender.com, accessed on 28 August 2024.

detection of dynamic changes over time (Gerlinger et al., 2012; Findlay et al., 2016; Venesio et al., 2018; Dagogo-Jack and Shaw, 2018). Lastly, the prolonged turnaround time (TAT) for histological reports delay treatment decision-making (Lone et al., 2022).

To address these challenges, LB has emerged as a non-invasive tool for tumor analysis, enabling the detection of cancer-derived components in the bloodstream, including circulating cell-free DNA (cfDNA), circulating tumor cells (CTCs), extracellular vesicles (EVs), and regulatory RNAs (siRNAs, miRNAs), aiming to identify DNA mutations, copy number variations (CNVs) of crucial genes, epigenetic patterns, and the proteome's and metabolome's characteristics (Gazzaniga et al., 2018; Overman et al., 2013; Diaz and Bardelli, 2014; Venesio et al., 2018; Nakamura et al., 2019; Siravegna et al., 2019a; Kim et al., 2016; Mayers et al., 2014) Circulating tumor DNA (ctDNA) represents the tumor-specific fraction of cfDNA, accounting for 0.1–10 % of total cfDNA and characterized by shorter DNA fragments (~145 bp vs. ~167 bp for cfDNA). Its levels vary widely based on tumor location, histology, disease burden and setting, thus influencing the choice of the detection method (Fig. 1**. A**). In cases of low shedding rates, sensitive targeted approaches are required, such as PCR-based (i.e., droplet digital PCR (ddPCR), BEAMing-PCR) or Next Generation Sequencing (NGS)-based (Safe-SeqS, CAPP-Seq, and TAmSeq) techniques. Post-surgical minimal residual disease (MRD) detection represents a solid application of these targeted strategies, where plasma analysis is guided by the molecular profiling of the resected tumor (i.e., tumor-informed method; Fig. 1**. B**). Conversely, high ctDNA levels favor untargeted strategies, such as the NGS-based genome-wide analysis (i.e. whole exome sequencing (WES), whole-genome sequencing (WGS), or whole methylome sequencing), which capture a wide range of mutations but with lower sensitivity. This approach is applied in advanced cancer care to identify new therapeutic targets without relying on tissue specimens (i.e. tumor agnostic technique; Fig. 1**. B**) (Mauri et al., 2022; Krebs et al., 2022; Xin et al., 2023; Rolfo et al., 2020).

These technologies found several applications in the CRC field, due to its optimal shedding properties compared to other tumors (Mauri et al., 2022; Malla et al., 2022). LB-based MRD detection has proven to be a strong negative prognostic marker, predicting relapse in 95–100 % of cases and preceding radiologically proven recurrence by a median of 8.7–12 months (Diehl et al., 2008; Reinert et al., 2019; Tie et al., 2019). Thus, a ctDNA-based treatment decision making for the CRC adjuvant setting is under evaluation, obtaining promising results in recent randomized trials (Tie et al., 2022; Henriksen et al., 2021; Lonardi et al., 2020; Oki et al., 2023). In advanced stages, ctDNA displayed a high concordance with tissue sampling (84–100 %) and effectively detected drug resistance mechanisms (Nakamura et al., 2020; Loree et al., 2021; Sartore-Bianchi et al., 2019). Additionally, by assessing plasma gene expression levels, LB improved the selection of patients most likely to benefit from targeted therapies (Siravegna et al., 2019a; Oki et al., 2023; Siena et al., 2021; Meric-Bernstam et al., 2019; Sartore-Bianchi et al., 2016; Venturini et al., 2024). Finally, tumor-agnostic WGS profiling showed promising results as a diagnostic tool for early-stage CRC screening (Chung et al., 2024).

1.2. RAS heterogeneity in mCRC and NeoRAS WT concept

Treatment failure in mCRC is driven by its intrinsic heterogeneity (Chan and Buczacki, 2021). Under therapy-induced selective pressure, distinct resistant tumor subclones emerge and predominate (Siena et al., 2018a, 2018b; Morelli et al., 2015; Misale et al., 2012; Siravegna et al., 2019b). LB has demonstrated its ability to track this evolving mutational landscape, capturing the emergence of resistance mechanisms such as acquired *RAS, BRAF, MEK*, and *EGFR* mutations, as well as *ERBB2* and *MET* amplifications (Cremolini et al., 2019; Parseghian et al., 2019; Peeters et al., 2015). For instance, emerging evidence now supports anti-EGFR rechallenge after anti-EGFR-based second-line therapies, based on the absence of *RAS* MT subclones in ctDNA samples (Martinelli et al., 2021; Cremolini et al., 2019; Ciardiello et al., 2021; Kagawa et al., 2022; Sartore-Bianchi et al., 2022; Sunakawa et al., 2022). The rationale behind this approach is rooted in the pulsatile behavior of *RAS* WT subclones, whose levels exponentially decline in the absence of EGFR blockade (Morelli et al., 2015; Parseghian et al., 2019). Within this framework, a similar dynamic process has been described in primarily *RAS* MT mCRC, known as the Neo*RAS* WT phenomenon. It is hypothesized that, in response to first-line treatments, *RAS* WT subclones outgrow the initially predominant *RAS* MT ones, which decline below the detection threshold, thus creating a transient "*RAS*-WT window"(Fig. 2) (Nicolazzo et al., 2022; Klein-Scory et al., 2020; Sato et al., 2022). This pattern suggests a process of "conversion" to a different subclonal population rather than a true biological "reversion" of *RAS* MT cells. While some evidence supports the contribution of both chemotherapy and antiangiogenic therapy to *RAS* WT conversions, (Bouchahda et al., 2020; Nicolazzo et al., 2020; Henry et al., 2020) multiple LB-based studies highlight the greater impact achieved by bevacizumab-containing regimens (Raimondi et al., 2019; Sunakawa et al., 2020; Osumi et al., 2023). Notably, the same Neo*RAS* WT converting effect of VEGF-inhibition was also confirmed by Epistolio et al. on 28 tissue specimens from liver metastases (Epistolio et al., 2021). To explain this, it has been postulated that bevacizumab-induced hypoxia enhances the sensitivity of *RAS* MT cells to ROS-mediated cell death (Henry et al., 2020). In light the growing evidence, the following sections will explore the retrospective and prospective investigations into the treatment of Neo*RAS* WT mCRC as a distinct pathological entity.

2. Current evidence on Neo*RAS* WT

Research on the Neo*RAS* WT phenomenon has primarily focused on three key aspects: identifying the most effective diagnostic techniques and optimal timing for assessment, evaluating its predictive value for anti-EGFR treatment response, and determining its prognostic significance following anti-angiogenic therapy. The main findings are detailed in Table 1.

The first studies investigated whether *RAS* MT subclones clear over time or simply fall below detection thresholds. The study analyzed ctDNA samples from 12 *RAS*-MT mCRC patients at four key time points: before treatment (T0), after four months of therapy (T1), at first progression (T2), and at second progression (T3). A confirmatory DNA

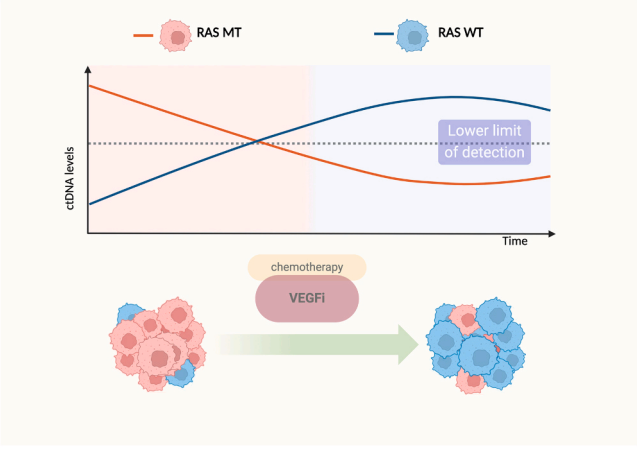

Fig. 2. The "*RAS* Dynamic Model". Rather than an independent phenomenon, the Neo*RAS* WT concept reflects a broader dynamic model, where *RAS* MT subclones undergo negative selection in response to treatment. This plasmatic clearance, known as the *RAS* WT window, could offer new therapeutic opportunities with EGFR inhibitors.Abbreviations ctDNA, Circulating Tumor DNA; *RAS* WT, *RAS* Wild Type; *RAS* MT, *RAS* Mutated; VEGFi Vascular Endothelial Growth Factor inhibitor.Image created with BioRender.com, accessed on 28 August 2024.

Table 1
Summary of trials investigating the Neo*RAS* WT phenomenon.

	Study type	N patients enrolled	N Neo*RAS* WT patients (% Neo*RAS* WT)	Methods	N patients treated with anti-EGFR moAbs	Treatment	PFS	OS	ORR	DCR
Nicolazzo et al., 2020	**Prospective**	12	7(58 %)	rtPCR Idylla™ + Methylation test	-	-	-	-	-	-
Moati et al., 2020	**Prospective**	36	2 (5.5 %)	ddPCR, NGS, methylation detection of *WIF* and *NPY* genes by ddPCR	-	-	-	-	-	-
Klein-Scory et al., 2020	**Prospective**	12	10 (91 %)	ddPCR and BEAMing PCR	-	-	-	-	-	-
Garcìa De Santiago et al., 2021	**Retrospective**	23	17 (74 %)	PCR assay: Idylla™ ct*KRAS* Mutation Assay and Idylla™ct *NRAS/BRAF* Mutation Assay	-	-	-	-	-	-
Nicolazzo et al., 2023	**Prospective**	70	42 (60 %)	rtPCR Idylla™ + Methylation test or NGS	-	-	15 mo	42 mo	-	-
Nicolazzo et al., 2021	**Prospective**	40	14 (35 %)	rtPCR Idylla™ + NGS Oncomine + Methylation test	10	anti-EGFR monotherapy	6,5 mo	-	-	-
Bouchahda et al., 2020	**Prospective**	16	9 (56 %)	Massarray Ult*RAS*eek technology	9	FOLFIRI + cetuximab	9 mo	-	55.6 %	77.8 %
Osumi et al., 2023	**Prospective**	107	23 (21.5 %)	OncoBEAM™ *RAS* CRC assay	23	anti-EGFR monotherapy	-	-	-	-
Osumi et al., 2024	**Prospective**	429	42 (9.8 %)	NGS	6	anti-EGFR monotherapy (N = 2) and chemotherapy+ anti-EGFR (N = 4)	-	-	50 %	20 %
JACCRO CC−1159	**Prospective**	29	24 (82.2 %)	TaqMan® PCR (castPCR) assays	-	FOLFOXIRI + bevacizumab	-	27mo	-	-
Nicolazzo et al., 2022	**Prospective**	72	2 (22 %) CT; 41 (82 %) CT+bevacizumab	rtPCR Idylla™ + Methylation test or NGS	-	CT (N = 22) or CT+Bevacizumab (N = 50)	5.9 mo CT; 9.3 mo CT+bevacizumab	-	-	-

Abbreviations EGFR mAb, anti-epidermal growth factor receptor monoclonal antibodies; PFS, Progression Free Survival; OS, Overall Survival; ORR, Objective Response Rate; DCR, Disease Control Rate; NGS, next-generation sequencing; mo, months; ddPCR, droplet-based digital polymerase chain reaction; WIF, WNT-Inhibitory-Factor 1; NPY, Neuropeptide Y; PCR, polymerase chain reaction; rtPCR, Real time PCR; CRC, colon rectal cancer.

methylation test was performed to assess ctDNA presence. At T1, 83 % of patients appeared *RAS* WT, but the absence of methylated ctDNA suggested that this was due to undetectable ctDNA rather than a biological clearance. By T2, the same 83 % remained *RAS* WT, but 62.5 % had methylated ctDNA, supporting the hypothesis that in many cases, the mutation had truly disappeared. At T3, 50 % of wild-type cases still had methylated ctDNA, while others, particularly those with lung or brain metastases, had none, likely due to lower ctDNA shedding from these tumor sites. This study highlights the utility of a five-gene methylation test in confirming ctDNA presence (Nicolazzo et al., 2020).

Moati et al. selected 36 patients from the PLACOL study (Nicolazzo et al., 2020) who were diagnosed with *RAS* MT mCRC through both histology and ctDNA. By performing LB at multiple time points, they analyzed the behavior of *RAS* MT subclones in response to treatment. *RAS* mutation clearance was considered valid only after ctDNA persistence confirmation, with NGS-based research on additional gene mutations or ddPCR-based detection of WNT-Inhibitory-Factor 1 (*WIF1*) and Neuropeptide Y (*NPY*) gene methylation. At progression, *RAS* mutation clearance was observed in 8/36 patients (22.2 %). However, only 2 of these cases were confirmed using the designated molecular assays (Moati et al., 2020).

A similar strategy was adopted by Nicolazzo et al., using both NGS-mutational panel and PCR-based methylation profiling to confirm the presence of ctDNA after *RAS* MT clearance detection. The authors prospectively collected data from 40 *RAS* MT mCRC patients at PD from any line of therapy. Of these, 18/40 cases (45 %) were defined as "*RAS* converters" since no *RAS*/BRAF ctDNA mutations were found. These plasma samples were then analyzed with the NGS panel, and in 11/18 cases (61 %), at least one somatic mutation other than *RAS* was identified, classifying them as the "true *RAS* converters" subgroup. This result was confirmed in 14/18 patients by the methylation assay, showing a concordance of approximately 70 % between the two techniques. Considering the whole population, the authors noted a trend towards improved PFS in patients receiving EGFR blockade as second or further treatment lines compared with those who did not (Nicolazzo et al., 2021).

To address the risk of false-negative results in non-shedding tumors, Klein-Scory et al. employed the more accurate BEAMing PCR assay, as a

confirmatory test for *RAS* MT ctDNA detected with ddPCR at screening. The authors selected 12 *RAS* MT mCRC patients who achieved stable disease (SD) or partial response (PR) during first-line therapy. A reduction in plasmatic *RAS* mutational allele frequency (MAF) was observed in all 12 cases (100 %). In 10/12 patients (91 %), ctDNA-based *RAS* mutational load dropped below the ddPCR detection threshold (<0.25 %), which was interpreted as Neo*RAS* WT conversion ($p < 0.0001$). Reversion time was not significantly associated with either the tumor's sidedness ($p = 0.955$) or treatment regimen ($p = 0.266$) (Klein-Scory et al., 2020).

Moreover, a high Neo*RAS* WT conversion rate was reported by García De Santiago et al. in a retrospective analysis of a LB series of 23 *RAS* MT mCRC patients treated with anti-VEGF therapies. Through PCR assays (i.e., Idylla™ ctK*RAS* Mutation Assay and Idylla™ct *NRAS/BRAF* Mutation Assay), the authors observed the disappearance of *RAS* MT in up to 17/23 patients (73.9 %) (De Santiago et al., 2021).

In 2023, Nicolazzo and colleagues published a prospective trial enrolling 70 patients with *RAS* MT mCRC refractory to first-line treatment. At first, the authors distinguished the 'Neo*RAS* WT' (45/70, 64 %) from the '*RAS* MT' (25/70, 36 %) population based on plasma ctDNA sampling at PD. To confirm *RAS* clearance, a sequential validation approach was used: a primary NGS analysis identified at least one additional somatic mutation in 36/45 cases (80 %), while in the remaining 9/45 (20 %) a methylation assay was performed, yielding a positive result in 6/9 cases (67 %). By combining these two methods, a 'true *RAS* conversion' was confirmed in 42/45 (93 %) samples. Finally, the authors highlighted the prognostic impact of *RAS* mutation clearance, with both median OS and post-progression survival favoring Neo*RAS* WT patients over the *RAS* MT persistent group (64 vs. 27 months, $p < 0.001$, and 42 vs. 16 months, $p < 0.001$, respectively) (Nicolazzo et al., 2023).

The subsequent research delved deeper into the clinical implications of Neo*RAS* WT patients, focusing on the predictive and prognostic value of *RAS* conversion and clarifying on the impact of anti-EGFR therapies in this context.

Bouchahda et al. conducted a study enrolling 16 pre-treated, anti-EGFR-naïve mCRC patients harboring *RAS* mutations at baseline. At disease progression, LB was performed to classify patients into two distinct groups: *RAS* MT persistent (Group 1, N = 7) and *RAS* WT converted (Group 2, N = 9). All patients in Group 2 were treated with the cetuximab + FOLFIRI regimen, while Group 1 received the investigator's choice of therapy. Anti-EGFR treatment was associated with significantly improved outcomes: mPFS was 3.5 months (95 % CI, 0.8–6.1) for Group 1 compared with 9.0 months (95 % CI, 4.7–13.3) for Group 2. Similarly, mOS was 4.7 months (95 % CI, 1.1–8.3) in Group 1, whereas it was not reached at 18.7 months in Group 2. ORR was 42.9 % and DCR 85.7 % in Group 1, while in Group 2, ORR reached 55.6 % and DCR 77.8 % (Bouchahda et al., 2020).

Osumi et al. later expanded on this topic, conducting two key studies. The first was a multi-institutional, observational study analyzing clinicopathological features associated with ctDNA-defined *RAS* conversion among 107 mCRC patients, refractory to prior chemotherapies. Using BEAMing PCR (OncoBEAM™ *RAS* CRC assay), 23/107 (21.5 %) patients were identified as Neo*RAS* WT. In the logistic regression multivariate analysis, factors significantly associated with Neo*RAS* WT conversion were the absence of liver metastases ($p = 0.019$), smaller tumor diameter ($p = 0.012$), and non K*RAS* exon 2 mutations ($p = 0.026$). Anti-EGFR monotherapy was attempted only in 3/23 cases, showing poor results: one patient had decreased tumor marker levels but discontinued due to skin toxicity, while for the other two patients mPFS was 2 months (Osumi et al., 2023). Building upon prior findings, some of the same authors investigated a subgroup of 478 tissue-diagnosed *RAS* MT mCRC patients from the GOZILA study. Plasmatic *KRAS* and *NRAS* mutations were detected in 387/478 cases via ctDNA sampling, while the remaining 91 patients (19.0 %) were classified as Neo*RAS* WT. This population was further divided into two subgroups: Group A, with no additional detectable mutations, and Group B, carrying other non-clonal somatic alterations (e.g., TP53, ATM, and GNAS). For Group A, the multivariate analysis showed a significant association of Neo*RAS* conversions with the absence of liver ($p < 0.00001$), and lymph node ($p = 0.033$) metastases. Similarly, in Group B, Neo*RAS* WT status was significantly correlated with the absence of liver metastasis ($p = 0.0005$) and non *KRAS* exon 2 mutations ($p = 0.049$). As for the clinical outcome, 6 Neo*RAS* WT patients were treated with anti-EGFR, ranging from fourth to seventh line. Among them, one achieved PR, while two maintained SD for at least six months (Osumi et al., 2024).

Further prognostic implications were explored by Sunakawa et al., drawing data from the Japanese phase II JACCRO CC-11 trial, where 64 *RAS* MT patients received modified FOLFOXIRI+bevacizumab as first-line treatment. CtDNA analysis was carried out at three time points in 36 patients: at baseline, week 8, and PD. *RAS* mutations disappeared in 24/29 (82.8 %) cases at 8 weeks and remained undetectable at PD in 18/29 (62.1 %) cases. Moreover, Neo*RAS* conversions showed significantly improved clinical outcomes, with mOS of 27 vs 16 months (HR 0.31, $p = 0.0073$) and post-progression survival of 15.1 vs 7.3 months (HR 0.21, $p = 0.0046$). Of note, the Neo*RAS* WT conversion was associated with a significantly lower tumor burden which could have influenced ctDNA shedding and *RAS* MT detection (Sunakawa et al., 2022).

The group led by Nicolazzo et al. investigated the role of bevacizumab in *RAS* conversion in a cohort of 72 patients with unresectable *RAS* MT mCRC, whose baseline plasma ctDNA mutational status was concordant with that of the primary tumor tissue. In the overall population, 43 cases (60 %) showed Neo*RAS* WT conversion, with 88 % maintaining it across all time points. Bevacizumab was identified as the only independent factor associated with *RAS* conversion (OR = 45, 95 % CI 9–230). Among patients receiving chemotherapy alone, *RAS* conversion was rare (2/22, 9 %), whereas in those treated with chemotherapy plus bevacizumab, 41/50 (82 %) converted to *RAS* WT. Moreover, patients who lost their *RAS* mutation had significantly better outcomes than those with persistent *RAS* mutations in the bevacizumab-treated group (mPFS 9.3 vs. 5.9 months, $p = 0.001$), supporting *RAS* conversion as a potential positive prognostic marker for treatment response (Nicolazzo et al., 2022).

3. Neo*RAS* WT mCRC: ongoing trials

Numerous trials are currently underway to prospectively assess the effectiveness of anti-EGFR in the context of LB-defined Neo*RAS* WT mCRC. These studies vary in terms of design, treatment regimens, and endpoints (Table 2).

The single-arm phase II CETIDYL trial (NCT04189055) is investigating the safety and efficacy of cetuximab vs cetuximab+irinotecan for 107 Neo*RAS* WT patients, as third or further treatment lines. The study focuses on a very selected population with liver-limited advanced disease.

The phase II randomized MoLiMoR trial (NCT04554836) is assessing the efficacy and safety of FOLFIRI + /- intermittent cetuximab vs FOLFIRI as first-line setting in left-sided mCRC. The study aims to enroll 144 patients, with PFS as the primary endpoint. Unlike other trials, MoLiMoR incorporates real-time molecular monitoring to guide treatment decisions. In the experimental arm, anti-EGFR treatment is either halted or resumed according to the plasma dynamics of *RAS* MT subclones, detected by ctDNA ddPCR analysis.

The Japanese C-PROWESS single-arm phase II trial (jRCTs031210565) employs BEAMing PCR technology to identify *RAS* WT mCRC patients at screening. The study is evaluating irinotecan+panitumumab as a third or further-line regimen. The primary endpoint is ORR, with the target enrollment of 30 patients.

The CONVERTIX (2017–003242–25) study was a single-arm, phase II study attempting to assess the efficacy of panitumumab+FOLFIRI as second-line treatment after receiving first-line FOLFOX/CAPOX+bevacizumab. The primary endpoint was PFS, but the trial was

Table 2
Summary of Ongoing Clinical trials assessing Neo*RAS* WT phenomenon.

Study	Phase	Method	Status	Line of treatment	Estimated N	Primary endpoint	Treatment regimen
CETIDYL trial (NCT04189055)	II	rtPCR	Recruiting	Third or further line	72	ORR	Cetuximab or Cetuximab + Irinotecan
MoLiMoR (NCT04554836)	II	ddPCR	Active, not recruiting	First line	144	PFS	FOLFIRI + intermittent Cetuximab vs FOLFIRI
KAIROS (EudraCT number 2019–001328–36)	II	rtPCR		Second line	112	ORR	Chemotherapy + Cetuximab
C-PROWESS (jRCTs031210565)	II	BEAMing PCR	Recruiting	Third or further line	30	ORR	Cetuximab + Irinotecan
CONVERTIX (2017–003242–25)	II	BEAMing PCR	Closed	Second line	40	PFS	FOLFIRI + Panitumumab
NEORAS-SYSUCC (NCT05962502)	II	NA	Recruiting	Third or further line	54	ORR	Cetuximab + Irinotecan

Abbreviations PFS, Progression Free Survival; ORR, Objective Response Rate; rtPCR, Real time PCR; ddPCR, droplet-based digital polymerase chain reaction; PCR, polymerase chain reaction.

prematurely closed due to insufficient accrual.

NEORAS-SYSUCC (NCT05962502) is a Chinese phase II single-arm trial, testing the efficacy and safety of cetuximab+irinotecan as third-line treatment for Neo*RAS* WT left-sided mCRC. The primary endpoint is ORR, with a target enrollment of 54 patients. Recruitment began in August 2023.

Finally, the KAIROS study (EudraCT number 2019–001328–36) another single-arm phase II trial, faced early termination due to poor accrual. Designed to assess the efficacy and safety of cetuximab + a chemotherapy doublet as second-line treatment for 112 *RAS* WT converted mCRC patients, its primary endpoint was ORR.

4. Discussion

Current international guidelines base the mCRC therapeutic algorithm on a dichotomous model, historically shaped by key determinants such as tumor sidedness and mutational profiling (Cervantes et al., 2023; Benson et al., 2024). However, the introduction of LB is reshaping this paradigm, better aligning with the dynamic nature of mCRC, where mutant subclones expand or decline in response to treatment pressure and microenvironmental changes. As described in this article, *RAS* MT subclones exhibit a pulsatile behavior, undergoing exponential time-dependent decay, not only in baseline *RAS* WT patients but also in those initially *RAS* MT, defining the Neo*RAS* WT phenomenon. This evolving perspective suggests that EGFR blockade could be guided by an LB-defined *RAS* WT therapeutic window rather than relying solely on baseline tissue genotyping. Supporting this concept, recent data from the FIRE-4 trial showed how LB detected *RAS* mutations in 13 % of the *RAS* WT enrolled population. In these cases, switching from anti-EGFR to anti-VEGF therapy resulted in numerically improved outcomes (PFS 10.1 vs. 6.4 m, HR 0.82 (95 % CI 0.47–1.41), p = 0.54; OS 24.9 vs. 16.3 mo, HR, 0.57 (95 % CI 0.31–1.07), p = 0.10) compared with continuous anti-EGFR therapy, further highlighting the role of LB in addressing mCRC's molecular heterogeneity (Stintzing et al., 2025). The Neo*RAS* WT phenomenon could therefore be considered part of a broader concept, the "*RAS* mutation dynamic model," where the tumor's molecular status is tightly linked to the shifting predominance of different subclonal populations.

However, several key limitations must be addressed before considering a change in clinical practice. First, the Neo*RAS* WT concept lacks a solid biological rationale, as the underlying mechanisms driving *RAS* conversions remain poorly understood. Current theories suggest that selective treatment pressure, particularly anti-angiogenic therapy, induces tumor microenvironment changes (Osumi et al., 2023). Several studies have hypothesized that bevacizumab-induced hypoxic damage induces a massive and deadly increase of intracellular ROS production, exceeding the high scavenging proprieties of *RAS* MT cells (Henry et al., 2020; Osumi et al., 2023; Lim and Leprivier, 2019; Bartolacci et al., 2021). An additional challenge lies in the lack of standardization in LB diagnostics. The different Neo*RAS* WT incidences reported across studies stem from inconsistent designs, sample sizes, and, most notably, plasma diagnostic methodologies. Despite the known differences in sensitivity and specificity, both PCR-based and NGS-based detecting techniques have been used across trials, leading to discrepancies in results (Nicolazzo et al., 2022, 2020; Raimondi et al., 2019). Furthermore, diagnostic accuracy is affected by ctDNA shedding variability, which depends on factors such as tumor burden and metastatic sites (Bouchahda et al., 2020; Osumi et al., 2021). Notably, higher ctDNA clearance has been observed in non-liver metastases (e.g., lung, brain, peritoneum), likely due to the presence of biological barriers and increased circulating DNAse activity (Uhlén et al., 2015). Similarly, Neo*RAS* WT conversion correlates with fewer metastatic sites and greater tumor shrinkage, but a reduction in tumor volume alone does not necessarily reflect true *RAS* MT clearance. False-negative results may occur when ctDNA levels drop below detection thresholds (Bando et al., 2019). To address false negative results for low shedding tumors, several studies adopted confirmatory tests, such as NGS-based screening for truncal APC/TP53 mutations or methylation profiling of WIF1/NPY genes (Klein-Scory et al., 2020; Henry et al., 2020; Sunakawa et al., 2020; Osumi et al., 2023; Nicolazzo et al., 2023). These methods help differentiate true *RAS* clearance from undetectable ctDNA, refining the actual incidence of Neo*RAS* WT mCRC. By applying these strategies, Osumi et al. reported a decrease in *RAS* conversion incidence from 19 % to 9.8 %, while a post-hoc CO.26 trial analysis registered a 50 % reduction (Osumi et al., 2021; Wu et al., 2024). The same authors have highlighted the scarce durability of *RAS* conversions, with the reacquisition of the baseline *RAS* mutations within a median time of 162.5 days (Wu et al., 2024). This raises another key debate: the optimal timing for ctDNA sampling. While LB is typically performed at disease progression, the decision to rely solely on plasma analysis or integrate tissue biopsy is challenging. Plasma testing is favored for its non-invasiveness, but in low-shedding tumors, tissue biopsy may be necessary to distinguish true *RAS* clearance from false negatives. In a heterogeneous disease like mCRC, combining LB with tissue biopsy may offer a more comprehensive approach, but it also raises significant cost-related barriers. High-sensitivity NGS-based ctDNA testing remains expensive, and many centers lack the necessary infrastructure and expertise for reliable real-time molecular monitoring. Without standardized reimbursement policies, its widespread implementation remains largely restricted to clinical trials, thereby limiting broader accessibility. As a final point, despite suggesting a better prognostic potential, the predictive value of *RAS* MT clearance remains unclear. The transient nature of this phenomenon, the small sample sizes examined, and the inconsistency in diagnostic methods have prevented

a definitive conclusion in the evaluation of the anti-EGFR treatments in this setting (Bouchahda et al., 2020; Henry et al., 2020).

5. Conclusions

While the Neo*RAS* WT concept holds promise and could redefine treatment strategies in mCRC, its clinical utility needs to be validated through prospective, randomized trials. Addressing current limitations, improving standardization of testing, and ensuring cost-effective implementation will represent crucial steps toward the integration of this approach into routine clinical practice.

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Declaration of Competing Interest

D.R. received honoraria from Amgen, MSD and Takeda. The other authors declare no competing interests

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