


# Detection of RAS mutations in circulating tumor DNA: a new weapon in an old war against colorectal cancer. A systematic review of literature and meta-analysis

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## Abstract

**Background:** Tissue evaluation for RAS (KRAS or NRAS) gene status in metastatic colorectal cancer (mCRC) patients represent the standard of care to establish the optimal therapeutic strategy. Unfortunately, tissue biopsy is hampered by several critical limitations due to its invasiveness, difficulty to access to disease site, patient's compliance and, more recently, neoplastic tissue spatial and temporal heterogeneity.

**Methods:** The authors performed a systematic literature review to identify available trials with paired matched tissue and ctDNA RAS gene status evaluation. The authors searched EMBASE, MEDLINE, Cochrane, www.ClinicalTrials.gov, and abstracts from international meetings. In total, 19 trials comparing standard tissue RAS mutational status matched paired ctDNA evaluated through polymerase chain reaction (PCR), next generation sequencing (NGS) or beads, emulsions, amplification and magnetics (BEAMing) were identified.

**Results:** The pooled sensitivity and specificity of ctDNA were 0.83 (95% CI: 0.80–0.85) and 0.91 (95% CI: 0.89–0.93) respectively. The pooled positive predictive value (PPV) and negative predictive value (NPV) of the ctDNA were 0.87 (95% CI: 0.81–0.92) and 0.87 (95% CI: 0.82–0.92), respectively. Positive likelihood ratio (PLR) was 8.20 (95% CI: 5.16–13.02) and the negative likelihood ratio (NLR) was 0.22 (95% CI: 0.16–0.30). The pooled diagnostic odds ratio (DOR) was 50.86 (95% CI: 26.15–98.76), and the area under the curve (AUC) of the summary receiver operational characteristics (sROC) curve was 0.94.

**Conclusion:** The authors' meta-analysis produced a complete and updated overview of ctDNA diagnostic accuracy to test RAS mutation in mCRC. Results provide a strong rationale to include the RAS ctDNA test into randomized clinical trials to validate it prospectively.

**Keywords:** circulating tumor DNA, diagnostic accuracy, meta-analysis, metastatic colorectal cancer, liquid biopsy, RAS

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## Introduction

Colorectal cancer (CRC) represents one-third of neoplasms in the world by incidence, with approximately 1.3 million new cases diagnosed every year.<sup>1</sup> Although many screening efforts are being made to diagnose CRC at an early stage, at least 50% of patients will have local or distant disease

recurrence, or present with advanced disease.<sup>2,3</sup> Retrospective analyses from phase III randomized controlled trials in metastatic colorectal cancer (mCRC) have shown that a tissue-based mutation in one of the RAS family of genes in a more comprehensive evaluation of RAS, commonly called expanded RAS (KRAS exons 2,3,4 and NRAS

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exons 2,3,4), can more accurately select patients for an anti-EGFR therapy rather than KRAS codons 12 and 13 testing alone.<sup>4-7</sup> Consequently, RAS testing is considered a suitable method to negatively predict the efficacy of anti-EGFR monoclonal antibodies (mAbs) in mCRC patients (representing approximately 55% of the total number).<sup>8</sup> Therefore, tumor tissue genotyping has become the routine approach in the management of these patients because of its ability to guide through the best first-line options.<sup>9,10</sup> Today, more in-depth understanding of the mechanisms linked to tumor heterogeneity enable researchers to determine that a tumor is made up of a multitude of cellular clones with their proper genomic structure (spatial heterogeneity).<sup>11-13</sup> Consequently, the monitoring of acquired resistances represents a fundamental step in establishing the best time to stop drug administration because of its ineffectiveness and the number of side effects. In addition, tissue biopsy, although it is able to provide useful genomic information, remains burdened by various limitations related to its invasiveness, difficult reproducibility over time, and poor representation of the entire tumor mass.<sup>14</sup> A number of reports have recently shown how liquid biopsy used in the oncology field could be a potential new alternative to traditional tissue biopsy, due to its abundance of genetic material as circulating tumor DNA (ctDNA), circulating-free DNA (cfDNA), plasma exosomes and circulating microRNAs.<sup>15</sup> Recently, the results from a large randomized controlled trial (AURA3) demonstrated that the T790M mutation evaluation performed by tissue or plasma through ctDNA detection in EGFR mutated non-small cell lung cancer (NSCLC) patients progressing after a first generation EGFR tyrosine kinase inhibitor achieved similar efficacy outcomes, when compared with a platinum-pemetrexed regimen.<sup>16</sup> Outcomes were considered so consistent that the major international scientific societies including the American Society of Clinical Oncology (ASCO), the European Society of Medical Oncology (ESMO), the National Comprehensive Cancer Network (NCCN) recommended the evaluation of the T790M mutation status on blood ctDNA as the first option compared with the traditional evaluation of tumor tissue biopsy.<sup>17</sup> The authors' current work systematically evaluated the performance of T790M analysis on blood ctDNA compared with the standard tissue in NSCLC demonstrating high specificity and sensitivity.<sup>18</sup> To date, however, the diagnostic accuracy of the RAS test on blood

ctDNA in mCRC patients remains controversial. A recent meta-analysis provided an estimate of the prognostic relevance of cfDNA as a source of KRAS mutations by evaluating different conventional polymerase chain reaction (PCR) techniques.<sup>19</sup> Therefore, the objective of this article was to carry out a systematic review of trials comparing matched blood and tumor tissue to provide a precise estimate of the diagnostic accuracy of the RAS gene mutation on ctDNA, in addition new generation technologies in mCRC patients will be assessed.<sup>20-38</sup>

## Materials and methods

### Search for clinical trials

The authors reviewed all published studies that reported the plasma specificity and sensitivity data of the RAS mutation (KRAS or KRAS/NRAS if available) tested by ctDNA in mCRC patients. The authors selected trials published up to December 2018 using electronic databases including Medline (PubMed), EMBASE, and Cochrane libraries. The search strategy on PubMed used the following terms, 'colorectal neoplasms', 'CRC', 'colorectal', 'liquid biopsy', 'serum', 'plasma', 'liquid biopsy', 'circulating DNA', 'circulating cell-free DNA', 'cell-free DNA', 'ctDNA', and 'circulating tumor DNA'. This search was then modified for searches using the other databases (EMBASE, Controlled Cochrane Trial Register - CCTR).

Only human studies were selected and an English language restriction was used. The authors have also explored sources of gray literature including the abstracts presented at the ASCO, ESMO, and Gastrointestinal Cancer Symposium meetings. The web-site [www.clinicaltrials.gov](http://www.clinicaltrials.gov) was also investigated as a source of unpublished data.

### Selection Criteria

To select clinical trials the following criteria were used: (1) patients with histologically confirmed diagnosis of advanced CRC, (2) studies that contain RAS mutational evaluation (KRAS or KRAS / NRAS) in tumor tissue and matched plasma, (3) studies that included the sensitivity and specificity value of the RAS mutation tested by ctDNA using traditional (PCR) or new generation techniques (ddPCR, BEAMing, NGS), and (4) studies in which basal blood sampling was carried out before drug therapy. Any ongoing trials were excluded. In the case of trials with several follow-ups, the

most recent trial was selected. The trials that did not include both the specificity and sensitivity values of the detection of blood RAS mutation by ctDNA were excluded from this review.

#### Data extraction

Data extraction and assessment were performed by two authors separately (A.G and S.T.). Disagreements were solved following discussions with a third author (A.R.). The following data were extracted from the selected studies: name of the first author, author nation, number of patients, year of publication, type of blood sample (plasma or serum) name of the journal, method of detection (PCR, BEAMing or NGS), evaluated gene (KRAS or KRAS/NRAS), true positive (TP), true negative (TN), false positive (FP), and false negative (FN) rates. The meta-analysis was drawn up according to the PRISMA guidelines for reporting of systematic reviews.<sup>39</sup>

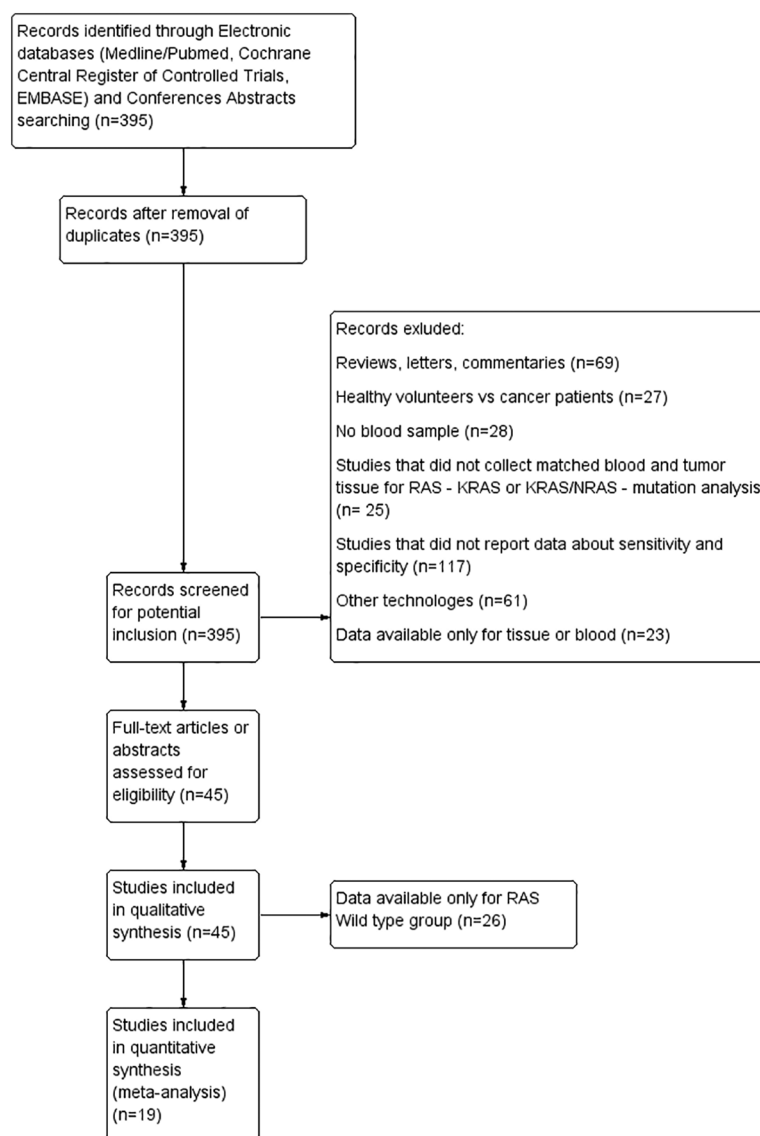
#### Quality assessment

The analysis of the overall quality of the studies included in the meta-analysis was conducted by two different investigators (A.G. and S.T) according to the quality assessment of diagnostic accuracy studies 2 (QUASAD-2) tool which designed for diagnostic accuracy analysis.<sup>40</sup> The quality assessment included four areas: patient selection, index test, reference standard, and flow and timing. For each study, the authors declared 'yes' as a low risk of bias and 'no' as a high risk of bias. The authors also declared 'unclear' if the data was not sufficient for a precise judgment. In addition, two authors (A.G. and S.T.) evaluated the risk of selective outcome reporting bias and any disagreements were resolved by consensus.

#### Statistical analysis

The data was extracted as TP, TN, FP, FN and the sample size in each study included the status of RAS mutation on tumor tissue, which is considered as a gold standard, and ctDNA considered as an experimental group (mutations in KRAS or KRAS/NRAS found in both tissue and liquid biopsy). The authors considered: TP as the number of patients with RAS mutation found in both tissue and liquid biopsy, TN as the number of patients with RAS mutation not found either in the tissue or in liquid biopsy, FP as the number of patients with RAS mutation not found in the tissue but found in liquid biopsy, and FN as the number

of patients with positive tissue biopsy and negative liquid biopsy. The following values were calculated: specificity, sensitivity, concordance, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR,) and the respective 95% confidence intervals (95% CI). The sensitivity was calculated as the ratio between TP and the sum of TP and FN  $\times 100$  ( $TP/[TP + FN] \times 100$ ). The specificity was calculated as the ratio between TN and the sum of TN and FP  $\times 100$  ( $TN/[TN + FP] \times 100$ ). The concordance was calculated as  $([TP + TN]/[TP + FN + TN + FN]) \times 100$ . PLR was calculated as  $sensitivity/(1-specificity)$  and represents the likelihood that a positive liquid biopsy for a RAS mutation can be found in a patient with a positive tissue biopsy for RAS mutation. NLR was calculated as  $(1-sensitivity)/specificity$  and represents the likelihood that a negative liquid biopsy for a RAS mutation can be found in a patient with a negative tissue biopsy for RAS mutation. The DOR was calculated as the ratio between PLR and NLR (PLR/NLR). This measure expresses the magnitude of the odds that a patient is a carrier of a tissue mutation of RAS in the case of positive liquid biopsy or negative liquid biopsy for a RAS mutation. The sample sizes of the included studies were used as weight to pool specificity, sensitivity, PPV, and NPV. PLR, NLR, and DOR were pooled using the DerSimonian Laird method (random effects model) to consider variance between studies.<sup>41</sup> Furthermore, the authors have elaborated a summary receiver operating characteristics (sROC) curve and calculated the area under the curve (AUC). A subgroup analysis was also performed with regard to the new generation detection techniques included in this meta-analysis (BEAMing, PCR, and NGS). To assess the presence of a possible bias linked to the threshold effect, Spearman's rank correlation coefficient was used between the sensitivity logit and the 1-specificity logit. The threshold effect was considered significant if the  $p$  value  $<0.05$ . The other sources of heterogeneity not dependent on the threshold effect were calculated using t Cochran's Q test (as the weighted sum of squared differences between individuals) or the index of inconsistency ( $I^2$ ) that indicates the percentage of variance between the studies that is explained by heterogeneity and not by chance. Heterogeneity was considered significant if the  $p$  value of Cochran's Q test was  $<0.05$  or high if  $I^2$  value  $>75\%$ . A meta-regression was also performed to identify other sources of heterogeneity and the publication bias test to evaluate



**Figure 1.** Flow chart of the trials included in the meta-analysis.

any other bias among the studies was included in our analysis. The related funnel plot (for visual inspection) and Egger's test were produced and considered significant if  $p$  value  $<0.05$ . All analyses were conducted using the MetaDisc statistical software (version 1.4).<sup>42</sup> The publication bias calculation was performed using the MetaEssential software.<sup>43</sup>

## Results

### *Characteristics of eligible studies*

The search of literature trials found a total of 395 articles published up to December 2018. A small

number of patients lacked information on treatments (140/1810 = 7.7%)

Of these patients, nineteen met the inclusion criteria and were therefore included in the pooled analysis with a total of 1810 patients (Figure 1). The study by Sefrioui<sup>35</sup> evaluated the RAS mutation status by both digital chip PCR and cast PCR and the data was reported in two independent studies, with a total of 20 items included in the pooled analysis. All of the studies included patients with histologically confirmed mCRC with the collection of tissue matched to blood (plasma or serum) for the ctDNA RAS mutation detection. The most frequently used technology

was NGS (8/20 studies). Six studies used the PCR technique (6/20) and six studies used BEAMing (6/20). With regard to positivity thresholds, although 2/20 studies did not report a precise threshold, 18/20 studies expressed it as mutant allele concentration (copies/ml) or mutant allele fraction (%). All of the studies which underwent pooled analysis included the values of specificity and sensitivity of ctDNA for the detection of RAS mutation (KRAS or KRAS/NRAS) compared with the tumor tissue considered as the gold standard. The authors included studies in which the basal blood sample was carried out before drug therapy. Only three studies, for which this information was not available and for a small number of patients,<sup>21,27,31</sup> were included in the results to guarantee the systematic nature of the meta-analysis. Sensitivity ranged from 50.0 to 100%, while specificity ranged from 66.7 to 100% among the different studies. The main characteristics of the included trials are summarized in Table 1.

#### *Diagnostic accuracy analysis*

The pooled sensitivity and specificity of ctDNA in the authors' analysis were 0.83 (95% CI: 0.80–0.85) and 0.91 (95% CI: 0.89–0.93) respectively (Figure 2). The pooled PPV and NPV of the ctDNA were 0.87 (95% CI: 0.81–0.92) and 0.87 (95% CI: 0.82–0.92), respectively. PLR was 8.20 (95% CI: 5.16–13.02) and NLR was 0.22 (95% CI: 0.16–0.30). The pooled DOR was 50.86 (95% CI: 26.15–98.76) and the AUC of the sROC curve was 0.94 (Figure 3). A subgroup analysis was carried out to evaluate if different methods could influence the diagnostic accuracy of the ctDNA and their results are shown in Figures 4 and 5 and summarized in Table 2.

#### *Threshold effect and heterogeneity*

To evaluate the presence of bias associated with the threshold effect the authors calculated Spearman's rank correlation coefficient and the relative *p* value. Spearman's rank correlation coefficient was  $-0.186$  ( $p$  value = 0.433) and therefore not significantly related to bias. However, a source of high heterogeneity that was not dependent on the threshold effect was found and, therefore, a meta-regression was performed. The results demonstrated how the different methods of ctDNA analysis were not associated with heterogeneity.

#### *Quality analysis and publication bias*

The presence of publication bias was investigated using the MetaEssential software. The plot did not reflect particular asymmetries and in addition, the Egger test was not significant ( $p = 0.89$ ) (Figure 6). The overall quality of the trials included in the meta-analysis was assessed as good by using the QUADAS-2 tool (Figure 7).

#### **Discussion**

The authors' meta-analysis included 20 studies with a total of 1810 mCRC patients in which the tissue matched paired with blood were available for the assessment of the mutational status of RAS (KRAS or KRAS/NRAS) due to ctDNA. The results demonstrated acceptable performance of ctDNA in terms of overall specificity (0.91) and sensitivity (0.83) for the RAS mutation if compared with tumor tissue, considered as gold standard. However, this analysis demonstrated that approximately 20% of patients defined as negative by liquid biopsy were actually positive for tissue analysis. This could potentially be due to the different technologies used although the analysis did not appear to indicate this (Figures 4 and 5, and Table 2).

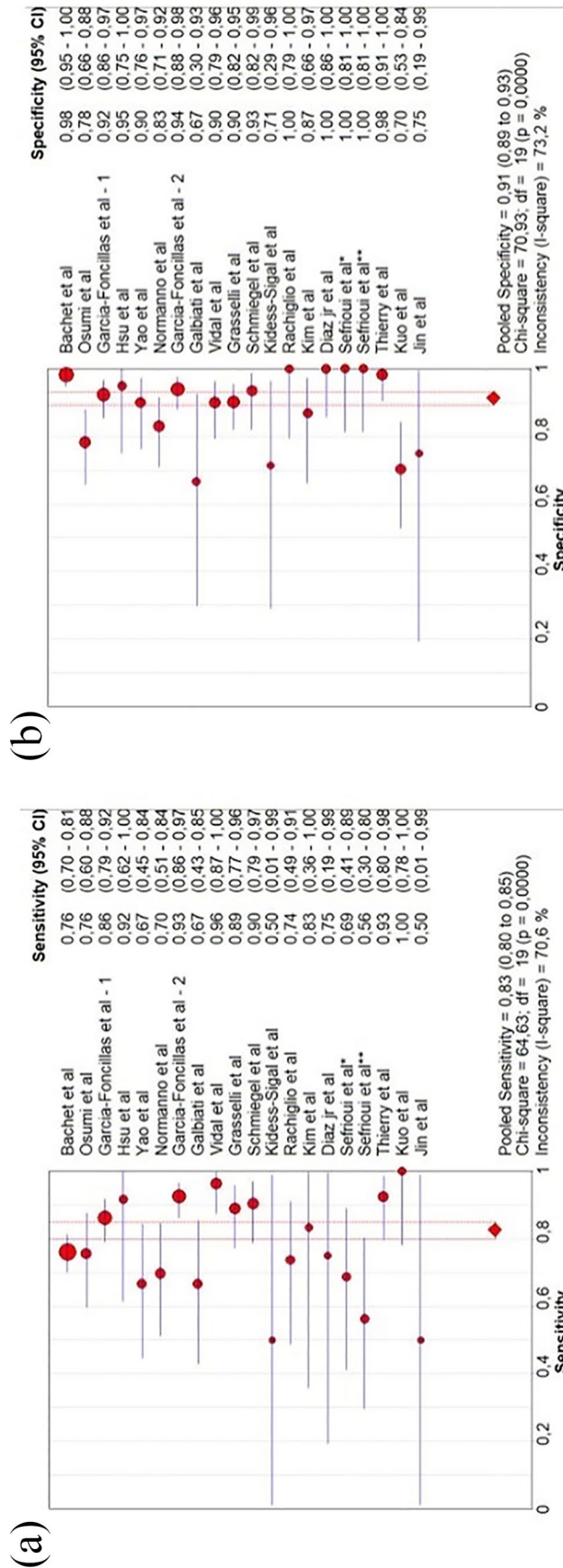
In addition, the novelty of the results resides in the overall finding that liquid biopsy influences by 30% the probability of having an RAS mutated ctDNA in patients with RAS mutated tissue test, as well as the probability of having an RAS wild type ctDNA in patients with wild type RAS tissue tests, therefore indicating a moderate impact according to the PLR and NLR values.<sup>44</sup> Finally, sROC curve (AUC 0.94) suggests a high performance of ctDNA in terms of sensitivity and specificity. Overall, according to Youden and colleagues<sup>45</sup> it appears that 0.74 should be the pooled threshold value able to minimize the FP rates. BEAMing has the most acceptable performance (Youden Index 0.83).<sup>45</sup> However, the authors' results reinforce the concept that these tools should be used at the most opportune time and at the most appropriate phase of the disease course.

The mutations in ctDNA can be detected with PCR-based or sequencing-based assays. NGS as a high throughput method allows deep sequencing of amplicons, which is useful for the detection of somatic mutations in ctDNA.<sup>46</sup> Given the multiplex nature of NGS, it is possible broadly to adopt this method for the identification of a

**Table 1.** Characteristics of the trials included in the meta-analysis.

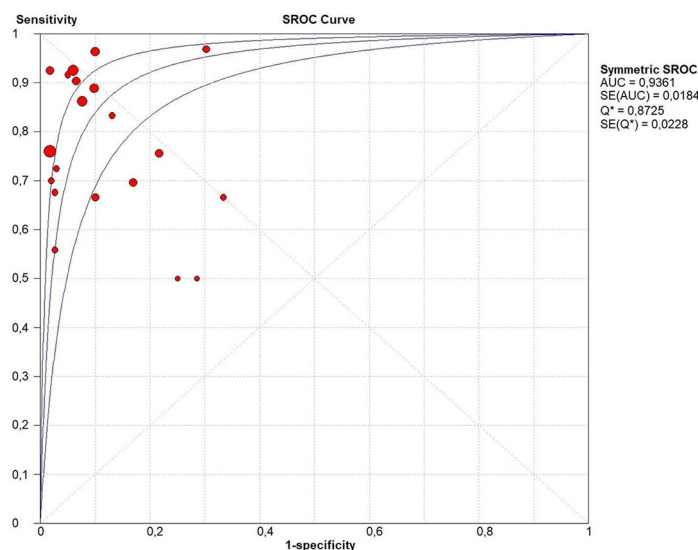
Study (reference)	Country	Number of patients n	Sample	Detection method	Gene	Sensitivity n. (%)	Specificity n. (%)	Concordance n. (%)	NPV n. (%)	PPV n. (%)
Bachet <i>et al.</i> <sup>20</sup>	France	412	Plasma	NGS	KRAS, NRAS	184/242 (76)	167/170 (98.2)	351/412 (85.2)	167/225 (74.2)	184/187 (98.4)
Osumi <i>et al.</i> <sup>21</sup>	Japan	101	Plasma	NGS	KRAS, NRAS	31/41 (75.6)	47/60 (78.3)	78/101 (77.2)	47/57 (82.4)	31/44 (70.4)
Garcia-Foncillas <i>et al.</i> <sup>22</sup>	Spain	236	Plasma	Beaming	KRAS, NRAS	113/131 (86.2)	97/105 (92.4)	210/236 (89)	97/115 (84.3)	113/121 (93.3)
Hsu <i>et al.</i> <sup>23</sup>	Taiwan	32	Plasma	NGS	KRAS	11/12 (91.6)	19/20 (95)	30/32 (93.7)	19/20 (95)	11/12 (91.6)
Yao <i>et al.</i> <sup>24</sup>	China	76	Plasma	NGS	KRAS	16/24 (66.7)	36/40 (90)	52/64 (81.2)	36/44 (81.8)	16/20 (80)
Normanno <i>et al.</i> <sup>25</sup>	Italy	92	Plasma	NGS	KRAS, NRAS	23/33 (70)	49/59 (83)	72/92 (78.3)	49/59 (83)	23/33 (70)
Garcia-Foncillas <i>et al.</i> <sup>26</sup>	Spain	238	Plasma	Beaming	KRAS, NRAS	112/121 (92.5)	110/117 (94)	222/238 (92.6)	110/119 (92.4)	112/119 (94.1)
Galbiati <i>et al.</i> <sup>27</sup>	Italy	30	Plasma	ddPCR	KRAS	14/21 (66.7)	6/9 (66.7)	20/30 (66.7)	6/13 (46.1)	14/17 (82.3)
Vidal <i>et al.</i> <sup>28</sup>	Spain	115	Plasma	Beaming	KRAS, NRAS	53/55 (96.3)	54/60 (90)	107/115 (93)	54/56 (96.4)	53/59 (89.8)
Grasselli <i>et al.</i> <sup>29</sup>	Spain	92	Plasma	Beaming	KRAS, NRAS	48/54 (88.8)	83/92 (90.2)	131/14 (89.7)	83/83 (93.2)	48/57 (84.2)
Schmiegel <i>et al.</i> <sup>30</sup>	Germany	98	Plasma	Beaming	KRAS, NRAS	47/52 (90.3)	43/46 (93.5)	90/98 (91.8)	43/48 (89.5)	47/50 (94)
Kidess-Sigal <i>et al.</i> <sup>31</sup>	USA	9	Plasma	NGS	KRAS	1/2 (50)	5/7 (71.4)	6/9 (66.7)	5/6 (83.3)	1/3 (33.3)
Rachiglio <i>et al.</i> <sup>32</sup>	Italy	35	Plasma	NGS	KRAS, NRAS	14/19 (73.6)	16/16 (100)	30/35 (85.7)	16/21 (76.2)	14/14 (100)
Kim <i>et al.</i> <sup>33</sup>	Korea	29	Plasma	NGS	KRAS	5/6 (83.3)	20/23 (87)	25/29 (86.2)	20/21 (95.2)	5/8 (62)
Diaz jr <i>et al.</i> <sup>34</sup>	USA	28	Serum	Beaming	KRAS	3/4 (75)	24/24 (100)	27/28 (96.4)	24/25 (96)	3/3 (100)
Sefrioui <i>et al.</i> <sup>35</sup>	France	34	Plasma	dPCR (chip)	KRAS	11/16 (69)	18/18 (100)	29/34 (85.2)	18/23 (78.2)	11/11 (100)
Thierry <i>et al.</i> <sup>36</sup>	France	95	Plasma	castPCR	KRAS	9/16 (56)	18/18 (100)	27/34 (79.4)	18/25 (72)	9/9 (100)
Kuo <i>et al.</i> <sup>37</sup>	Taiwan	52	Plasma	Intplex PCR	KRAS	37/40 (92.5)	56/57 (98.2)	93/97 (96)	56/59 (95)	37/38 (97.3)
Jin <i>et al.</i> <sup>38</sup>	Korea	6	Plasma	PNA-PCR	KRAS	15/15 (100)	26/37 (70.2)	41/52 (78.8)	26/26 (100)	15/26 (57.6)
			DTBP + PCR	KRAS		1/2 (50)	3/4 (75)	4/6 (66.6)	3/4 (75)	1/2 (50)

BEAMing, beads, emulsions, amplification and magnetics; CAST, competitive allele-specific TaqMan; CI, confidence intervals; dPCR, digital polymerase chain reaction; DTBP, Dimethyl 3,3'-dithiopropanimidate dihydrochloride; NGS, next generation sequencing; NPV, negative predictive value; PCR, polymerase chain reaction; PNA, peptide nucleic acid; PPV, positive predictive value.



**Figure 2.** Forest plots of sensitivity and specificity of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) mutation. Figure 2(a) pooled sensitivity, (b) pooled specificity.

Forest plots of overall sensitivity and specificity of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) mutation. Figure 2(a) pooled sensitivity, (b) pooled specificity. The different circle size indicates the different weight of the study.



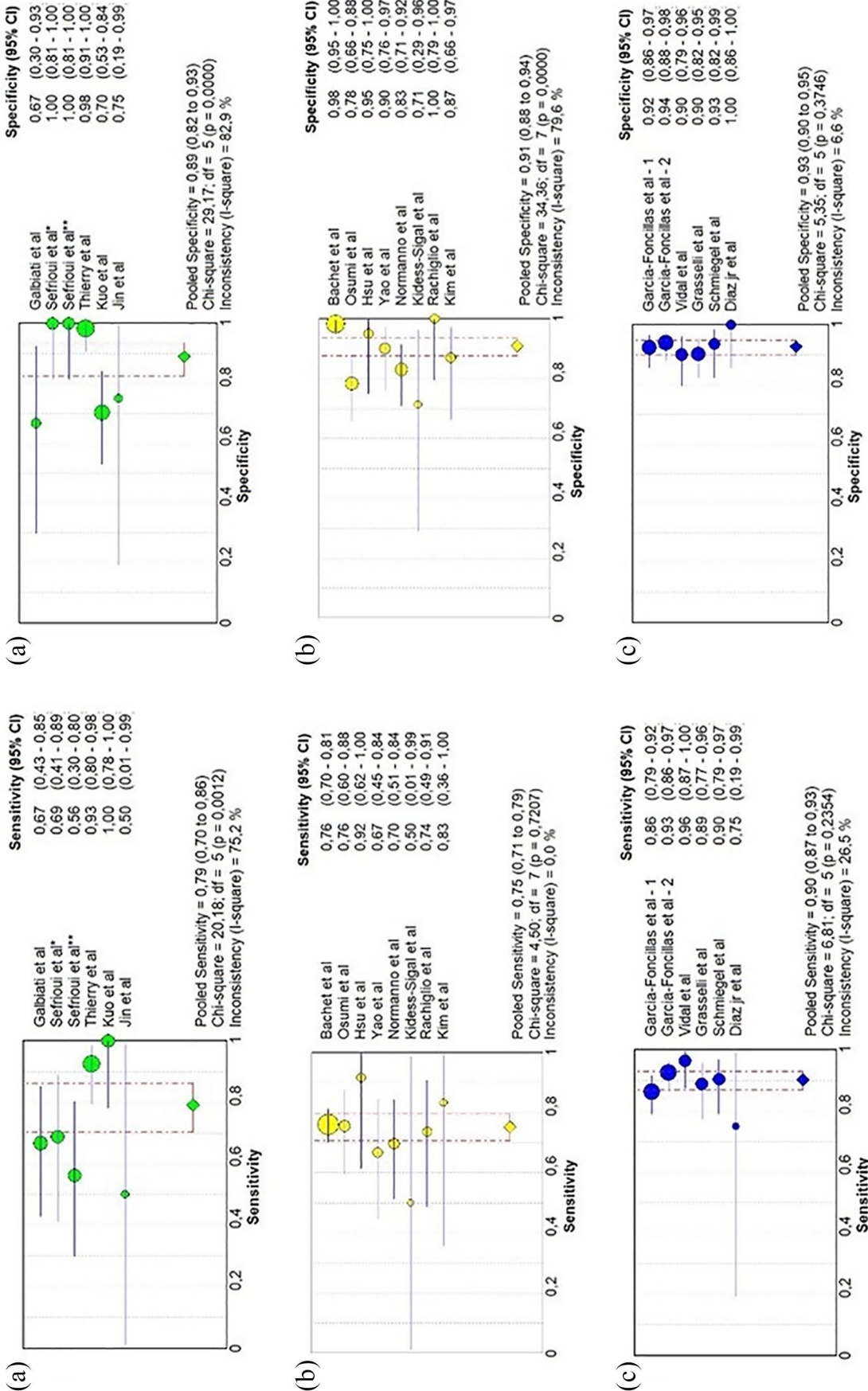
**Figure 3.** sROC curve of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) mutation. sROC curve of overall ctDNA for the detection of RAS (KRAS or KRAS/NRAS) mutation. The different circle size indicates the different weight of the study.

gene's mutational status with ctDNA as the analyte. While the PCR-based methods can detect preferentially specific hotspot mutations, NGS has the benefit of detecting novel mutations in addition to the hotspots. NGS, in addition, also the advantage of being cost effective in screening for multiple genes and hotspots in a single assay. The NGS assay is a highly sensitive technology that allows the detection of mutations in ctDNA with high accuracy when compared with other next generation technologies including digital real time PCR.

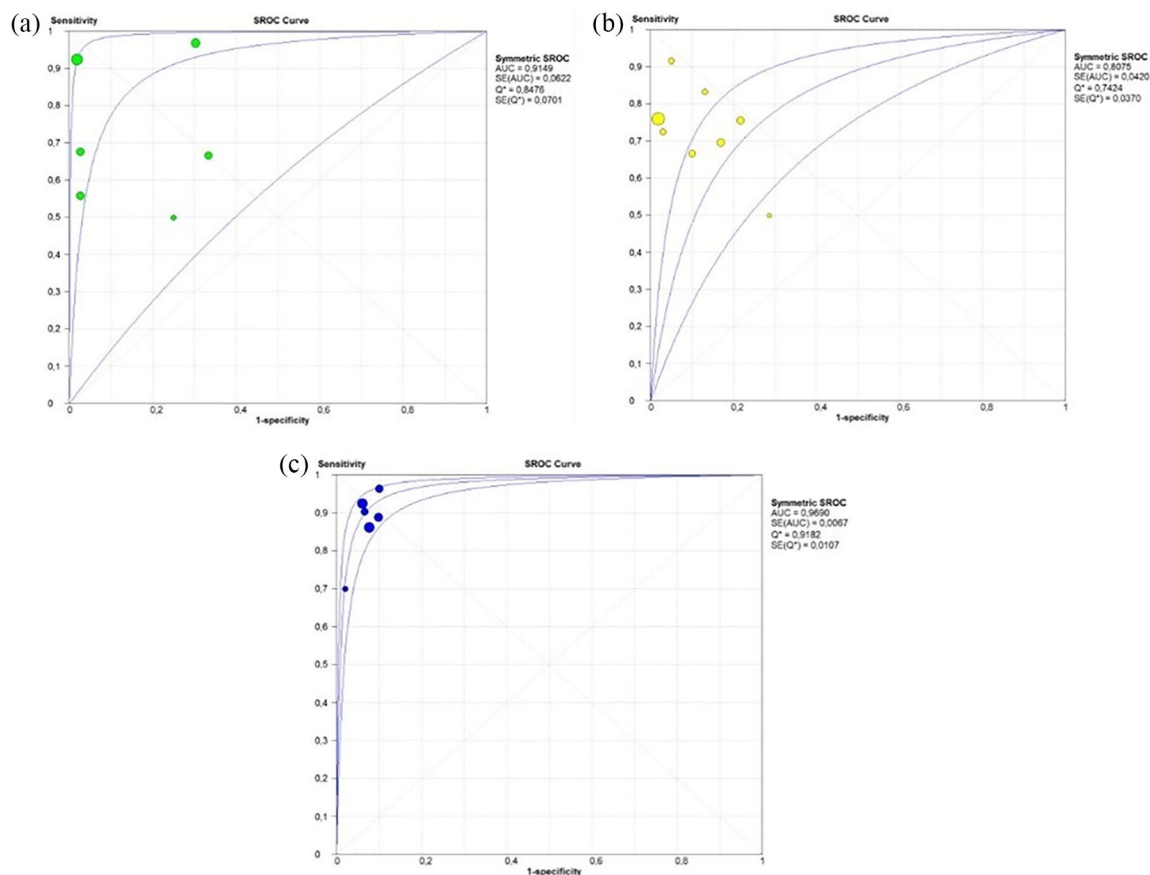
Originally, the potential of liquid biopsies was limited technically by the relatively high error rate of NGS systems, as real ctDNA mutations were masked by intrinsic errors in the DNA library preparation and sequencing. Modern NGS systems produce errors at a per-base rate of  $10^{-2}$  to  $10^{-3}$ , but clinically relevant mutations have been revealed to be at, or below, that level making many true variants undetectable.<sup>47</sup> In recent years error correction strategies have been developed and today NGS and liquid biopsy have a great potential in a gene's mutational status detection and impact positively on diagnostics and cancer management. Recently, ctDNA liquid biopsies have been adopted for several personalized medicine applications, including guiding treatment selection during monitoring, minimum residual disease detection, and even screening (ClinicalTrials.gov NCT02808884; NCT02889978).

Technologies including quantitative PCR and NGS could potentially be reserved for the first mutational assessment in the metastatic settings, reserving other technologies such as BEAMing for the monitoring of already identified genetic alterations. Despite the fact that the different technologies under investigation have shown an overall good performance, making circulating DNA analysis a promising and unavoidable area of research, the authors' data suggest that ctDNA has some limitations for its use in routine clinical practice due predominantly to the lack of a real standardization of sensitivity limit that could increase the concordance between tissue and plasma. The early knowledge of the disease's mutational profile is one of the most relevant clinical needs because it can have a considerable impact at different phases of clinical histories including diagnosis, first-line approach, and dynamic monitoring for the detection of acquired resistance.<sup>48</sup> New generation technologies allow the analysis of multigenic panels and several patient samples at the same time, with high performance standards and economic savings. It has also been hypothesized that elevated levels of ctDNA in CRC are correlated with poor outcome, as its primary post-surgery finding may suggest the presence of minimal residual disease and its potential role for the definition of actionable targets. As mentioned previously, although data is fragmented, some evidence seems to suggest a potential role in the monitoring of secondary





**Figure 4.** Forest plots of sensitivity and specificity of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) according to the different diagnostic methods: Figure 4(a) PCR, (b) NGS, (c) BEAMing. Forest plots of sensitivity and specificity of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) according to the different diagnostic methods: Figure 4(a) PCR, (b) NGS, (c) BEAMing. The different circle size indicates the different weight of study.

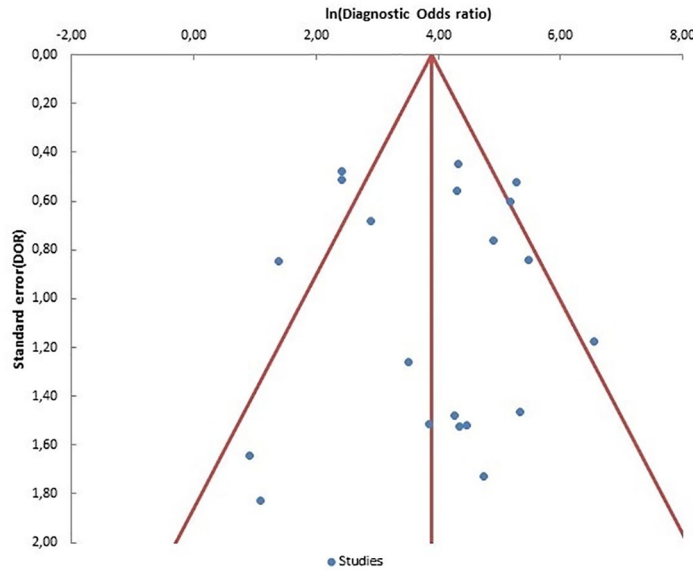


**Figure 5.** sROC curve of ctDNA for detection of RAS (KRAS or KRAS/NRAS) mutation according to the different diagnostic methods: Figure 5(a) PCR, (b) NGS, (c) BEAMing. sROC curve of ctDNA for detection of RAS (KRAS or KRAS/NRAS) mutation according to the different diagnostic methods: Figure 5(a) PCR, (b) NGS, (c) BEAMing. The different circle size indicates the different weight of the study.

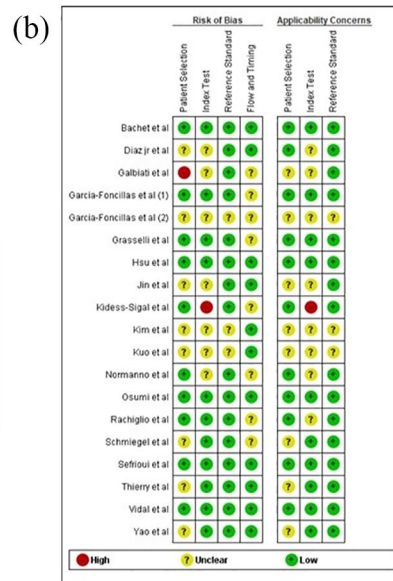
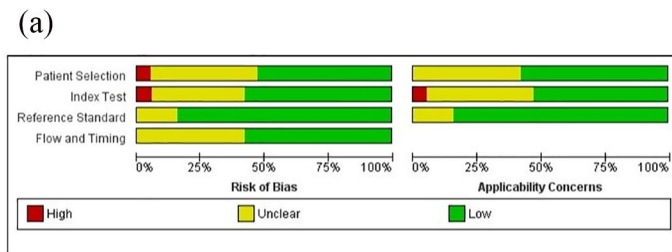
**Table 2.** Meta-analysis results.

	Number of patients <i>n</i>	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)	NLR (95% CI)	PLR (95% CI)	AUC
All Studies	1810	0.83 (0.80 – 0.85)	0.91 (0.89 – 0.93)	51.35 (26.85 – 98.18)	0.22 (0.16 – 0.30)	8.27 (5.25 – 13.03)	0.94
NGS	786	0.75 (0.71 – 0.80)	0.91 (0.88 – 0.94)	28.28 (10 – 79.94)	0.27 (0.23 – 0.32)	7.39 (3.2 – 17.13)	0.81
BEAMing	807	0.90 (0.87 – 0.93)	0.93 (0.90 – 0.95)	113.26 (68.72 – 186.66)	0.11 (0.08 – 0.17)	11.41 (8.23 – 15.82)	0.97
PCR	217	0.79 (0.70 – 0.86)	0.89 (0.82 – 0.93)	36.6 (5.6 – 239.55)	0.29 (0.14 – 0.63)	6.72 (1.84 – 24.54)	0.91

AUC, area under the curve; BEAMing, beads, emulsions, amplification, and magnetics; CI, confidence intervals; DOR, diagnostic odds ratio; NGS, next generation sequencing; NLR, negative likelihood ratio; PCR, polymerase chain reaction; PLR, positive likelihood ratio.



**Figure 6.** Funnel plot of DOR for ctDNA detection of RAS (KRAS or KRAS/NRAS) mutation. Each circle represents a study and the vertical line represents the pooled effect estimate.



**Figure 7.** Quality assessment of included trials by the QUADAS-2 tool. Figure 7(a) methodological quality graph, (b) methodological quality summary.

resistances. Misale and colleagues<sup>49</sup> demonstrated for the first time in mCRC patients treated with anti-EGFR (panitumumab or cetuximab) that the discovery of KRAS amplification through liquid biopsy was able to anticipate the radiological progression by 10 months with a sensitivity of 60%. Furthermore, Siravegna and colleagues<sup>50</sup> demonstrated that the early discontinuation of

treatment with anti-EGFR reduced the number of mutated KRAS clones allowing reacquisition of the sensitivity to anti-EGFR agents. This finding suggested novel rationales potentially useful for the management and continuum of mCRC care. Recently, Goldberg and colleagues<sup>51</sup> proposed that ctDNA evaluated by liquid biopsy can become an optimal tool for decision making

between the strategy of cetuximab continuum or re-challenge, in order to extend the number of available therapeutic options and optimize the management. Rossini and colleagues<sup>52</sup> reported the results of the CRICKET study in which 27 mCRC wild type RAS/BRAF patients with acquired resistance during the first-line anti-EGFR regimen were treated with irinotecan-cetuximab as re-challenge. In this experience, the ctDNA evaluated by ddPCR and NGS at the re-challenge baseline was able to predict a greater efficacy of irinotecan-cetuximab in wild type ctDNA RAS patients compared with RAS mutant ctDNA in terms of progression-free survival (3.9 versus 1.9 months, Hazard Ratio 0.48), identifying RAS mutations in 9/25 among patients evaluated by liquid biopsy (48%). These data show how liquid biopsy can offer a possible solution to the important challenge of inter- and intra-tumor heterogeneity and consequently to drug resistance. Although CRC is highly heterogeneous, their intra-tumor heterogeneity features have been less investigated than in other cancer types. Recent findings indicate that intra-tumor heterogeneity can be tested by single cell analysis, the single cell genome sequencing allows quantitative characterization of both single nucleotide and somatic copy number variations in individual tumor cells.<sup>53</sup> Recently, a *Nature* opinion article suggested that the main operative unit of cancer is the genetically and epigenetically modified single cell. The single cell analysis can allow clarification of the intra-tumor genetic heterogeneity and cancer genome evolution in order to develop new tools able to provide robust interpretation of the mechanisms related to diagnosis, tumor recurrence and the activity of new generation molecular targeted agents, and the development of secondary resistance modifying cancer patient management strategies.<sup>54</sup> Despite this potential, the use of liquid biopsy in routine clinical practice has historically been burdened by substantial costs, heterogeneity in methodology, and results from studies or in different threshold values that could be influenced by the noise caused by normal tissue cfDNA. For these reasons, the level of evidence to date is not yet considered as enough to recommend the use of ctDNA RAS testing in routine clinical practice. The authors' meta-analysis, therefore, provides for the first time clear and comprehensive evidence of the feasibility and potentially acceptable performance of ctDNA RAS testing in mCRC patients offering a strong rationale for prospective clinical trial validation.

## Conclusion

The results of the authors' analysis demonstrates that ctDNA is a very promising testing tool. In particular, the technology used (PCR, BEAMing, NGS) and the disease course phase (initial diagnosis, real time monitoring) can influence the diagnostic accuracy of the test. Therefore, there is a need both to standardize ctDNA analysis and that the results are validated prospectively within large randomized trials in order to improve the clinical applicability of ctDNA in mCRC.

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## Conflict of interest statement

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## Supplemental material

Supplemental material for this article is available online.

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