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Running title: ctDNA-guided anti-EGFR therapy in later-line mCRC

ctDNA retesting to guide anti-EGFR therapy in later-line metastatic colorectal cancer

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Circulating tumor DNA (ctDNA) represents a promising tool for dynamic molecular profiling in metastatic colorectal cancer (mCRC), enabling monitoring of clonal tumor evolution and the detection of mechanisms of acquired resistance during treatment. The relevance of ctDNA has increased, particularly in the context of anti-EGFR therapy, where the emergence of RAS gene mutations constitutes a key resistance mechanism. The aim of this study was to evaluate the clinical utility of reassessing mutational status using ctDNA prior to rechallenge with anti-EGFR therapy in later treatment lines in patients with mCRC. A total of 17 patients with confirmed RAS and BRAF wild-type status in tumor tissue before initiation of first-line therapy were included in the analysis. All patients received anti-EGFR monoclonal antibody-based therapy in the first line and subsequently underwent ctDNA analysis from peripheral blood prior to initiation of third-line treatment using the Idylla™ qPCR platform. RAS gene mutations were detected in ctDNA in 4 patients, while 13 patients retained a wild-type status. Patients without detectable mutations achieved longer progression-free survival in the third-line setting compared with patients harboring RAS mutations. Overall survival from the start of third-line therapy was also prolonged in the wild-type ctDNA group. These findings suggest that the absence of resistant RAS clones in ctDNA may predict greater clinical benefit from anti-EGFR rechallenge. Liquid biopsy-based retesting may therefore represent an important tool for treatment individualization in later lines of mCRC therapy. This study also illustrates the translational potential of ctDNA analysis, linking dynamic molecular characterization of tumor evolution with clinically relevant treatment decision-making and the implementation of personalized oncology approaches.

Key words: colorectal carcinoma; liquid biopsy; ctDNA; anti-EGFR

Liquid biopsy represents a minimally invasive approach enabling the detection and analysis of tumor-associated genetic and molecular biomarkers in body fluids. It allows continuous monitoring of the tumor molecular profile and the dynamics of genetic alterations throughout disease progression without the need for repeated tissue biopsies. Peripheral blood is the most commonly utilized source of biological material for liquid biopsy, from which circulating tumor cells (CTCs),

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circulating tumor DNA (ctDNA), and extracellular vesicles can be isolated [1]. Compared with conventional tissue biopsy, liquid biopsy offers several advantages, particularly its minimal invasiveness, the feasibility of repeated sampling, and the ability to capture both spatial and temporal molecular heterogeneity of the tumor, including clonal evolution during the course of the disease. Among currently available liquid biopsy approaches, ctDNA analysis has emerged as the most clinically relevant application, enabling the detection of tumor-associated genetic and epigenetic alterations [2].

ctDNA is primarily released into the bloodstream through apoptosis- and necrosis-mediated shedding of nucleic acid fragments from tumor cells. Its concentration in circulation partially reflects tumor burden as well as the biological characteristics of the malignancy. Importantly, the quantity of detectable ctDNA varies substantially among different cancer types [2]. ctDNA analysis is primarily utilized for molecular tumor genotyping, detection of minimal residual disease (MRD), monitoring of treatment response, identification of resistance mechanisms, and selection of targeted therapeutic strategies [3]. Despite its broad potential, the clinical utility of ctDNA varies across different tumor entities and is closely associated with the biological characteristics of the respective malignancy. ctDNA analysis has gained particular clinical relevance in solid tumors of the gastrointestinal tract, especially in colorectal cancer, where molecular tumor heterogeneity and the emergence of acquired resistance during treatment substantially influence therapeutic decision-making [2].

Colorectal cancer (CRC) is among the most prevalent malignancies worldwide and represents one of the leading causes of cancer-related mortality. Its development and progression are driven by the accumulation of genetic and epigenetic alterations affecting key signaling pathways involved in the regulation of cellular proliferation, differentiation, and survival [4]. International clinical guidelines emphasize the importance of molecular testing of tumor tissue, particularly the determination of KRAS, NRAS, and BRAF mutational status, as well as the assessment of defects in the mismatch repair (MMR) system responsible for microsatellite instability (MSI) status determination. In addition to RAS testing, genomic profiling in patients with CRC should also include evaluation of tumor mutational burden and alterations involving ERBB2, POLE, POLD1, RET, and NTRK1, NTRK2, and NTRK3 gene fusions https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf [5]. Determination of RAS mutational status is of critical importance for the selection of anti-EGFR monoclonal antibody-based therapy [6]. In patients with metastatic CRC (mCRC) harboring RAS wild-type tumors, anti-EGFR treatment is associated with improved treatment response, prolonged progression-free survival (PFS), and overall survival (OS), whereas the presence of RAS mutations constitutes a predictive biomarker of primary resistance to this therapeutic approach [7].

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Despite an initial therapeutic response, most patients treated with anti-EGFR monoclonal antibodies eventually develop acquired resistance, reflecting dynamic changes in the clonal architecture of the tumor. Characterization of these alterations in clinical practice is limited by the restricted availability of repeated tissue biopsies. However, advances in liquid biopsy technologies have enabled real-time monitoring of genetic alterations through ctDNA analysis. Resistant RAS clones have been shown to emerge in peripheral blood during anti-EGFR treatment, while their levels may gradually decline following treatment discontinuation, supporting the concept of temporally reversible acquired resistance and providing a rationale for subsequent re-administration of anti-EGFR therapy [8, 9]. The observation of progressive regression of resistant RAS clones after cessation of anti-EGFR therapy has established the biological basis for reusing this therapeutic strategy in selected patients. In clinical practice, the terms reintroduction, rechallenge, and retreatment are used to describe distinct clinical scenarios involving repeated administration of anti-EGFR therapy [10, 11]. The concept of rechallenge is based on the assumption that following a period without anti-EGFR selective pressure, resistant subclones undergo gradual decline ("clonal decay"), accompanied by the relative restoration of EGFR-dependent tumor cell populations [11]. In this manuscript, the term anti-EGFR rechallenge is used to describe the re-administration of anti-EGFR therapy following documented disease progression on previous anti-EGFR-based treatment and an intervening anti-EGFR-free treatment period.

One of the major limitations in selecting patients suitable for anti-EGFR rechallenge is the limited representativeness of archived tumor tissue, which may not reflect the current molecular evolution of the tumor. ctDNA analysis enables real-time assessment of the current molecular profile of the malignancy while more comprehensively capturing both intertumoral and intratumoral heterogeneity. Prospective clinical trials, including CRICKET and CHRONOS, have demonstrated that the absence of RAS mutations in ctDNA is associated with a higher likelihood of achieving clinical benefit from anti-EGFR rechallenge [12, 13]. Consequently, ctDNA analysis is shifting the paradigm of patient selection from reliance on historical molecular profiling derived from tumor tissue toward dynamic monitoring of the evolving biological profile of the tumor.

In addition to the identification of RAS gene mutations, other genetic alterations also play a significant role in mechanisms of resistance to anti-EGFR therapy, particularly BRAF mutations and mutations affecting the extracellular domain of EGFR, both of which can be detected through ctDNA analysis and may influence therapeutic response. Dynamic changes in genetic alterations detected in ctDNA reflect the clonal evolution of the tumor and support the biological model suggesting that the duration of the anti-EGFR-free interval may influence the efficacy of the rechallenge strategy [14].

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Despite its growing clinical relevance, ctDNA analysis is also associated with several limitations, including interindividual variability in ctDNA shedding, reduced detectability in certain metastatic sites, the potential influence of clonal hematopoiesis, and the absence of uniformly established cut-off thresholds for clinical decision-making [15, 16].

Within this context, molecular retesting using ctDNA represents a promising approach for identifying patients with metastatic colorectal cancer who may benefit from anti-EGFR therapy in later treatment lines. From a translational medicine perspective, ctDNA provides a direct link between the molecular evolution of the tumor and clinical treatment decision-making, enabling biological information obtained through molecular diagnostics to be translated into individualized therapeutic strategies. Based on these considerations, we analyzed a cohort of patients with metastatic colorectal cancer to evaluate the clinical significance of ctDNA-based molecular retesting prior to administration of anti-EGFR therapy in later-line treatment settings.

Patients and Methods

Patients. Patients with metastatic colorectal cancer (mCRC) with confirmed RAS and BRAF wild-type (WT) status prior to initiation of first-line treatment and no previous treatment for metastatic disease were enrolled in the study between June 2017 and December 2020. Patients were recruited from several hospitals across Slovakia and subsequently followed longitudinally at five hospitals throughout successive lines of systemic treatment until the end of February 2024. A total of 52 patients were initially enrolled in the study.

Prior to initiation of first-line treatment, RAS and BRAF mutational status was determined from primary tumor or metastatic tissue using the Idylla™ (Biocartis) molecular testing system, based on real-time PCR. The same Idylla™ platform was used for all subsequent plasma ctDNA analyses throughout the longitudinal follow-up. Peripheral blood samples were scheduled for collection before initiation of first-line treatment, at the three-month follow-up assessment, and following disease progression, i.e., before initiation of second- and third-line treatment.

First-line treatment consisted of cetuximab in combination with irinotecan- or oxaliplatin-based chemotherapy. Following progression on first-line treatment, patients received second-line anti-VEGF treatment in combination with chemotherapy at the discretion of the treating oncologist. All patients included in the final analysis subsequently received cetuximab as third-line anti-EGFR rechallenge, irrespective of the RAS mutational status determined by ctDNA analysis prior to initiation of third-line treatment. Clinical outcomes following rechallenge were subsequently compared between patients with RAS WT and RAS MT status in ctDNA.

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Of the 52 initially enrolled patients, the planned assessment of RAS mutational status prior to initiation of second-line treatment could not be performed in 12 patients. The reasons were death (n=8), continuation of first-line anti-EGFR treatment (n=2), refusal to continue participation in the study (n = 1), and prolonged hospitalization during follow-up (n=1). Thus, 40 patients remained eligible for assessment prior to second-line treatment and were further followed until initiation of third-line treatment. In 23 patients, the planned assessment of RAS mutational status prior to third-line treatment could not be performed because of death (n=12), detection of a RAS mutation prior to initiation of second-line treatment (n=7), ongoing second-line treatment at the end of follow-up (n=2), or ongoing first-line treatment (n=2). The final analysis cohort therefore comprised 17 patients with available RAS mutational status prior to initiation of third-line treatment. The patient selection process is summarized in Figure 1. Detailed demographic, clinical, and treatment characteristics of the patients included in the final analysis are presented in Table 1. Molecular ctDNA analysis was performed at the Department of Clinical Genetics, St. Elizabeth Cancer Institute. The study was approved by the Ethics Committee of St. Elizabeth Cancer Institute on 20.04.2017. The study was conducted in accordance with the Helsinki Declaration, and all patients provided written informed consent for molecular genetic testing and for the use of clinical data for scientific purposes.

Blood collection and plasma separation. Peripheral blood samples were collected into Cell-Free DNA BCT CE stabilization tubes (Streck), which ensure the stability and integrity of circulating cell-free DNA (cfDNA). Particular emphasis was placed on minimizing the interval between sample collection and processing during the pre-analytical phase. Immediately after receiving, blood samples were centrifuged at 1,500 × g for 10 min at 18-25 °C. Subsequently, approximately 1.2 ml of plasma was carefully transferred into 2 ml tubes, followed by a second centrifugation step at 15,000-20,000 × g for 1 min at the same temperature. After the second centrifugation, approximately 1.1 ml of plasma from each tube was transferred into new tubes in order to minimize contamination by cellular debris. Processed plasma samples were either analyzed immediately or stored at -80 °C until molecular analysis.

Molecular analysis of ctDNA (Real-Time PCR). ctDNA analysis was performed using Biocartis qPCR assays on the fully automated Idylla™ platform using the Idylla™ ctKRAS Mutation Assay and the Idylla™ ctNRAS-BRAF-EGFR S492R Mutation Assay according to the manufacturer's instructions. In cases where plasma samples had been stored at -80 °C, they were thawed at room temperature prior to analysis and subsequently centrifuged at 16,000 × g for 1 min at 18-25 °C. From each sample, 1 ml of plasma was loaded into a single-use test cartridge and analyzed using the Idylla™ system in accordance with the manufacturer's protocol and recommendations. According

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186 to the manufacturer, the limit of detection (LOD) is below 5% mutant allele frequency for the most
187 prevalent KRAS, NRAS and BRAF mutations. The ctDNA analysis performed in this study was
188 restricted to clinically relevant RAS and BRAF alterations detectable by the Idylla™ platform.
189 Other potential mechanisms of resistance to anti-EGFR therapy were not systematically assessed.
190 **Statistical analysis.** The Kaplan-Meier method was used to analyze survival outcomes according to
191 RAS/BRAF mutational status determined prior to initiation of third-line treatment. Three time-to-
192 event outcomes were evaluated: progression-free survival during third-line treatment (PFS3),
193 survival during third-line treatment, and survival from the initiation of third-line treatment,
194 including the duration of third- and fourth-line treatment (PFS3+PFS4). Differences between the
195 RAS/BRAF WT and MT groups were assessed using the log-rank test, and median survival times
196 were reported with 95% confidence intervals (95% CIs).
197 To quantify differences in the risk of an event between the groups, univariable Cox proportional
198 hazards regression analysis was performed for each of the evaluated time-to-event outcomes.
199 Results were expressed as hazard ratios (HRs) with 95% CIs, with the WT group used as the
200 reference category. The proportional hazards assumption of the Cox models was assessed using
201 Schoenfeld residuals. A p-value < 0.05 was considered statistically significant.
202
203 **Results**
204 **Treatment characteristics of patients included in the study.** This study was designed to test the
205 hypothesis that ctDNA-based molecular analysis focused on the detection of acquired resistant
206 clonal alterations may represent a suitable tool for selecting patients with mCRC for anti-EGFR
207 rechallenge in later lines of therapy. A total of 52 patients with mCRC, in whom continuation or
208 rechallenge of anti-EGFR therapy was considered, were initially evaluated. The final analyzed
209 cohort consisted of 17 patients who had confirmed wild-type status of the KRAS, NRAS, and
210 BRAF genes in tumor tissue prior to initiation of first-line therapy and who subsequently received
211 two prior lines of systemic anticancer treatment. All patients (100%) had previously received anti-
212 EGFR therapy (cetuximab in combination with chemotherapy) in the first-line setting. Between
213 first-line anti-EGFR treatment and subsequent anti-EGFR rechallenge, patients received second-line
214 systemic therapy consisting of chemotherapy with FOLFOX or FOLFIRI in combination with the
215 anti-VEGF agent bevacizumab. Molecular ctDNA analysis performed prior to initiation of third-line
216 treatment using the Idylla™ (qPCR) platform revealed a change in RAS mutational status in 4
217 patients, indicating dynamic clonal tumor evolution and the emergence of subclones associated with
218 acquired resistance to anti-EGFR therapy. The primary objective of the study was to compare PFS
219 and OS following anti-EGFR rechallenge in the third-line setting between patients with RAS wild-

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type (WT) ctDNA status and those with detectable RAS mutations (MT) in ctDNA prior to initiation of the rechallenge strategy.

During first-line treatment with cetuximab in combination with chemotherapy, 12 patients (70.6%) achieved a partial response, 4 patients (23.5%) had stable disease, and 1 patient (5.9%) experienced disease progression.

The median duration of second-line treatment, representing the anti-EGFR-free interval between the initial exposure to cetuximab and its subsequent rechallenge in the third-line setting, was 14 months (range, 2-40 months).

Third-line systemic treatment was initiated following disease progression during second-line treatment. More detailed data on disease burden or patient performance status immediately prior to initiation of third-line treatment were not systematically recorded in this study.

Clinical outcome after anti-EGFR rechallenge. A total of 17 patients were indicated for anti-EGFR rechallenge in the third-line setting. Of these, 13 patients (76.5%) retained a WT status of RAS/BRAF genes in ctDNA throughout all lines of treatment, whereas 4 patients (23.5%) exhibited detectable mutations immediately prior to initiation of third-line therapy. The median OS from diagnosis in patients with persistent WT status was 36 months (95% CI: 31-NA), while in the group of patients with detectable mutations prior to third-line therapy, the median OS was 56.5 months (95% CI: 37-NA).

A more detailed analysis of the preceding treatment lines showed that patients in whom a RAS mutation was detected prior to third-line treatment had a longer combined PFS during the first and second treatment lines compared with patients with persistent RAS WT status (median PFS1+PFS2: 52 vs. 26 months; p=0.03). Median PFS1 was 18.5 vs. 13 months (p=0.4), whereas a more pronounced difference was observed during second-line treatment, with median PFS2 of 31.5 vs. 14 months (p=0.01).

Key differences were observed in the analysis of the efficacy of the rechallenge strategy in the third line.

Progression-free survival in the third line (PFS3). The median PFS in patients with RAS MT was 5 months (95% CI: 3-NA) compared with 7 months in RAS WT patients (95% CI: 6-NA). This difference was statistically significant (p=0.04; Figure 2A). Univariable Cox proportional hazards regression analysis showed a higher risk of progression in patients with MT status compared with the WT group (HR=4.51; 95% CI: 1.02-19.98).

Overall survival during third-line therapy. Median survival in the third-line setting was 6 months (95% CI: 3-NA) in MT patients versus 9 months in WT patients (p=0.2; Figure 2B). Cox regression

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analysis showed a higher risk of the event in the MT group compared with the WT group; however, the difference was not statistically significant (HR=3.71; 95% CI: 0.62-22.07).

Overall survival from initiation of third-line therapy (PFS3+PFS4). The median survival from the start of rechallenge strategy was 6 months (95% CI: 3-NA) in MT patients, compared with 11 months (95% CI: 6-NA) in WT patients (p=0.1; Figure 2C). Cox regression analysis also showed a higher risk of the event in the MT group; however, the difference was not statistically significant (HR=2.94; 95% CI: 0.73-11.96).

The proportional hazards assumption was not violated in any of the three Cox models based on the analysis of Schoenfeld residuals (all $p > 0.70$). These findings suggest that the absence of resistant RAS mutations in ctDNA prior to initiation of third-line therapy predicts a more favorable clinical benefit from anti-EGFR rechallenge.

Discussion

During the course of metastatic disease, more than 30% of patients with mCRC eventually proceed to third-line or later lines of therapy [17]. In this clinical setting, a rechallenge strategy defined as the re-administration of previously administered agents is frequently employed in routine practice [12]. The present study represents a translational approach in which dynamic molecular information obtained from ctDNA is evaluated in the context of an actual therapeutic decision, namely the selection of patients for anti-EGFR rechallenge. In line with this empirical approach, anti-EGFR rechallenge is utilized based on the described decay of resistance mechanisms, its favorable toxicity profile, and its potential to induce tumor shrinkage [18]. Although widely implemented, this strategy is increasingly supported by serial molecular profiling; however, robust prospective evidence remains an area of ongoing investigation. Several studies suggest that ctDNA-based analysis focusing on resistance-associated alterations to anti-EGFR therapy may substantially improve patient selection for this therapeutic approach [12, 18, 19].

Our results suggest that ctDNA-based reassessment of mutational status prior to anti-EGFR rechallenge may provide meaningful clinical utility for patient selection in later lines of therapy. We observed that patients with persistent WT status of RAS and BRAF genes in ctDNA achieved a statistically significant longer PFS in the third-line setting compared with those in whom mutations emerged prior to treatment initiation (7 vs. 5 months; p=0.04). This finding supports the hypothesis that the absence of resistant clones detectable in peripheral blood is associated with a higher likelihood of clinical benefit from anti-EGFR rechallenge [12].

An initially unexpected finding was the longer OS from diagnosis observed in patients in whom a RAS mutation was detected prior to initiation of third-line treatment compared with patients with

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287 persistent RAS WT status (56.5 vs. 36 months). However, a more detailed analysis of the preceding
288 treatment lines suggests that this difference may primarily be related to longer disease control
289 before initiation of third-line treatment. Patients in whom a RAS mutation was detected prior to
290 third-line treatment achieved a median combined PFS during the first and second treatment lines of
291 52 months, compared with 26 months in patients with persistent RAS WT status (p=0.03).
292 The difference was less pronounced during first-line treatment, with a median PFS1 of 18.5 months
293 in patients with RAS mutations detected prior to third-line treatment and 13 months in patients with
294 persistent RAS WT status (p=0.4). A more pronounced difference was observed during second-line
295 treatment, with a median PFS2 of 31.5 months in patients in whom a RAS mutation was
296 subsequently detected prior to third-line treatment compared with 14 months in patients with
297 persistent RAS WT status (p=0.01). Thus, the longer OS from diagnosis observed in the RAS MT
298 group may largely reflect longer disease control during the preceding treatment lines before
299 detection of the RAS mutation.
300 In contrast, following the detection of a RAS mutation prior to third-line treatment, shorter PFS3
301 was observed in patients with RAS MT status compared with those with persistent RAS WT status
302 (5 vs. 7 months; p=0.04). This finding is consistent with the negative predictive significance of
303 RAS-mutated resistant clones for the efficacy of anti-EGFR rechallenge. Therefore, the longer OS
304 from diagnosis observed in patients with RAS MT status should not be interpreted as indicating a
305 favorable prognostic significance of an acquired RAS mutation. Rather, it may reflect differences in
306 disease course and longer disease control before the emergence or detection of the RAS mutation.
307 However, these findings should be interpreted with considerable caution given the very small
308 number of patients with RAS MT status prior to third-line treatment (n=4). The observed
309 differences may be substantially influenced by individual disease trajectories, patient selection, and
310 random variability. Therefore, these findings should not be considered evidence of a favorable
311 prognostic significance of acquired RAS mutations, but rather an exploratory observation requiring
312 confirmation in a larger patient cohort.
313 Our findings are consistent with available clinical studies highlighting the importance of dynamic
314 monitoring of mutational status using ctDNA in the context of anti-EGFR rechallenge. One of the
315 main objectives of our study was to monitor changes in RAS mutational status across successive
316 lines of treatment. Despite the initially confirmed RAS WT status, RAS mutations were detected
317 prior to initiation of third-line treatment in 4 of 17 patients (23.5%), indicating dynamic molecular
318 evolution of the tumor during treatment. Similar changes have also been observed in previous
319 studies. In the CHRONOS study, resistance-associated alterations were detected in ctDNA prior to
320 anti-EGFR rechallenge in 16 of 52 (31%) evaluated patients, including mutations in KRAS, NRAS,

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321 BRAF, and EGFR-ECD [13]. In the CAVE study, RAS/BRAF mutations were detected in ctDNA
322 prior to rechallenge in 19 of 67 patients (28%) [18]. Together with our observations, these findings
323 support the importance of repeated molecular profiling, as the initial WT status determined before
324 first-line treatment may not reflect the current molecular profile of the tumor at the time of later-line
325 treatment.
326 Beyond the dynamics of mutational status itself, available clinical studies also demonstrate its
327 relevance to the efficacy of subsequent anti-EGFR rechallenge. In the CRICKET study, patients
328 with RAS WT ctDNA prior to rechallenge achieved longer PFS and OS compared with patients
329 with RAS mutations [12]. Similarly, in the CAVE study, patients with RAS/BRAF WT ctDNA had
330 significantly longer PFS and OS than those with mutated ctDNA [19]. The CHRONOS study was
331 subsequently the first to prospectively use the ctDNA mutational profile for molecular selection of
332 patients for anti-EGFR rechallenge [14]. Similarly, in our cohort, shorter PFS3 was observed in
333 patients with RAS MT compared with RAS WT status prior to third-line treatment (5 vs. 7 months;
334 p=0.04). Thus, despite the small sample size, our findings support the relevance of ctDNA not only
335 for detecting changes in mutational status during treatment but also as a potential tool for
336 identifying patients more likely to benefit from anti-EGFR rechallenge [12, 13, 20].
337 The biological rationale underlying this approach is the concept of "clonal decay," according to
338 which discontinuation of anti-EGFR selective pressure is followed by a gradual decline of resistant
339 subclones harboring RAS mutations. This process may result in a relative re-expansion of tumor
340 cell populations that remain sensitive to EGFR inhibition [10].
341 The relevance of these findings is further supported by their subsequent implementation in clinical
342 practice. At the initiation of the study, repeated assessment of mutational status using ctDNA was
343 performed within the framework of a research project. Subsequently, this testing became
344 reimbursed by health insurance providers and is currently performed as part of routine molecular
345 diagnostics for patients with mCRC at our institution. This progression from research-based
346 molecular testing to reimbursed routine clinical diagnostics represents a direct example of
347 translational medicine, demonstrating how findings generated in a research setting can be
348 transferred into clinical practice and incorporated into patient management. This transition from
349 project-based testing to routine clinical use reflects the growing importance of repeated assessment
350 of the current molecular profile of the tumor throughout the course of treatment.
351 However, it is important to emphasize that the absence of detectable mutations in ctDNA does not
352 necessarily indicate their complete eradication, but may reflect a low proportion of tumor cells with
353 an altered mutational status (and thus a low concentration of mutated ctDNA in blood plasma) or
354 detection limits of the employed methodology. In our study, the qPCR-based Idylla™ platform was

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355 used, which, while providing rapid and standardized results suitable for routine clinical practice, has
356 a more limited mutation coverage and lower sensitivity compared with next-generation sequencing
357 approaches.
358 The main limitation of this study is the small number of patients in the final analysis cohort,
359 particularly the very limited number of patients with detectable RAS mutations in ctDNA prior to
360 third-line treatment (n=4). The small sample size reduces the statistical power of the analyses and
361 increases the uncertainty of the estimated treatment effect. In such small subgroups, the results may
362 be more strongly influenced by individual patient characteristics, differences in disease course, or
363 the duration of previous treatment. Furthermore, the risk of random differences between the
364 compared groups is increased, limiting the generalizability of the observed findings to the broader
365 population of patients with mCRC. Therefore, the results, including the observed difference in PFS
366 between the RAS WT and RAS MT groups, should be interpreted with caution and considered
367 primarily exploratory and hypothesis-generating. These findings should therefore be confirmed in
368 further studies involving larger patient cohorts.
369 The small number of patients in the final analysis is also related to the study design and the clinical
370 course of advanced mCRC. A total of 52 patients were initially evaluated; however, inclusion in the
371 final analysis required patients to have received prior anti-EGFR treatment followed by second-line
372 systemic treatment and to remain eligible for molecular reassessment using ctDNA and potential
373 anti-EGFR rechallenge in the third or later treatment line. Given the progressive nature of
374 metastatic disease, a substantial proportion of the initially evaluated patients did not reach this stage
375 of treatment or could not be further followed. The final cohort of 17 patients therefore represents a
376 group in whom complete longitudinal follow-up through the third-line setting and ctDNA
377 assessment at the required clinical time point were feasible. However, this selection may also
378 introduce selection bias, as patients who reach later treatment lines may represent a prognostically
379 more favorable and clinically selected population.
380 An important methodological aspect of longitudinal molecular monitoring is the consistent use of
381 the same diagnostic platform. In this study, the Idylla™ system was used for all ctDNA analyses at
382 the different follow-up time points. Therefore, the observed changes in RAS mutational status
383 between consecutive blood samples cannot be attributed to differences arising from the use of
384 different diagnostic platforms. Nevertheless, despite the consistent use of the same platform, ctDNA
385 results may have been influenced by biological and analytical factors, particularly variations in the
386 amount of ctDNA released into the circulation and low mutant allele frequencies below the
387 detection limit of the assay.

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Another limitation of the present study is that molecular analysis was restricted to RAS and BRAF alterations detectable by the applied platform. Other established mechanisms of resistance to anti-EGFR therapy, including EGFR extracellular domain mutations, HER2 amplification, MET amplification, and additional genomic alterations, were not evaluated and may have contributed to treatment outcomes. Nevertheless, our findings support the use of ctDNA as a tool for dynamic molecular tumor profiling and for the personalization of treatment in later lines of mCRC. Importantly, the study demonstrates a translational continuum from molecular characterization of tumor evolution to clinically actionable biomarker assessment and individualized therapeutic decision-making. The implementation of ctDNA analysis into clinical practice may contribute to more effective selection of patients likely to benefit from anti-EGFR rechallenge while avoiding ineffective therapy in patients with persistent resistant clones. Thus, ctDNA-based molecular monitoring represents a clinically relevant example of translational medicine, in which advances in molecular diagnostics are directly translated into improved treatment stratification and personalized cancer care.

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Use of artificial intelligence tools: ChatGPT (GPT-5.6, OpenAI) was used to assist with English language editing, translation, and improvement of readability and style in selected parts of the manuscript. AI assistance was limited to language-related modifications of author-generated content and did not involve data analysis, interpretation of results, or generation of scientific conclusions. All AI-assisted text was reviewed and verified by the authors, who remain fully responsible for the final content of the manuscript.

References

[1] ZHOU H, ZHU L, SONG J, WANG G, LI P et al. Liquid biopsy at the frontier of detection, prognosis and progression monitoring in colorectal cancer. Mol Cancer 2022; 21: 86. doi: 10.1186/s12943-022-01556-2

[2] NAKAMURA Y, TANIGUCHI H, IKEDA M, BANDO H, KATO K 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. doi: 10.1038/s41591-020-1063-5

[3] PANDEY S, YADAV P. Liquid biopsy in cancer management: Integrating diagnostics and clinical applications. Pract Lab Med 2024; 43: e00446. doi: 10.1016/j.plabm.2024.e00446

Accepted manuscript

[4] BRAY F, LAVERSANNE M, SUNG H, FERLAY J, SIEGEL RL et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2024; 74: 229-263. doi: 10.3322/caac.21834

[5] CERVANTES A, ADAM R, ROSELLÓ S, ARNOLD D, NORMANNO N et al. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol 2023; 34: 10-32. doi: 10.1016/j.annonc.2022.10.003

[6] SEPULVEDA AR, HAMILTON SR, ALLEGRA CJ, GRODY W, CUSHMAN-VOKOUN AM et al. Molecular Biomarkers for the Evaluation of Colorectal Cancer: Guideline From the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and the American Society of Clinical Oncology. J Clin Oncol 2017; 35: 1453-1486. doi: 10.1200/JCO.2016.71.9807

[7] DI NICOLANTONIO F, VITIELLO PP, MARSONI S, SIENA S, TABERNERO J et al. Precision oncology in metastatic colorectal cancer - from biology to medicine. Nat Rev Clin Oncol 2021; 18: 506-525. doi: 10.1038/s41571-021-00495-z

[8] SIENA S, SARTORE-BIANCHI A, GARCIA-CARBONERO R, KARTHAUS M, SMITH D et al. Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer. Ann Oncol 2018; 29: 119-126. doi: 10.1093/annonc/mdx504

[9] PARSEGHIAN CM, LOREE JM, MORRIS VK, LIU X, CLIFTON KK et al. Anti-EGFR-resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. Ann Oncol 2019; 30: 243-249. doi: 10.1093/annonc/mdy509

[10] CREMOLINI C, MONTAGUT C, RONGA P, VENTURINI F, YAMAGUCHI K et al. Rechallenge with anti-EGFR therapy to extend the continuum of care in patients with metastatic colorectal cancer. Front Oncol 2023; 12: 946850. doi: 10.3389/fonc.2022.946850

[11] MAURI G, PIZZUTILO EG, AMATU A, BENCARDINO K, PALMERI L et al. Retreatment with anti-EGFR monoclonal antibodies in metastatic colorectal cancer: Systematic review of different strategies. Cancer Treat Rev 2019; 73: 41-53. doi: 10.1016/j.ctrv.2018.12.006

[12] CREMOLINI C, ROSSINI D, DELL'AQUILA E, LONARDI S, CONCA E 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. doi: 10.1001/jamaoncol.2018.5080

[13] SARTORE-BIANCHI A, PIETRANTONIO F, LONARDI S, MUSSOLIN B, RUA F 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. doi: 10.1038/s41591-022-01886-0

[14] MCCOY MS, WOLF S, STRUCK V, THOMAS N, HUSMAN G et al. Anti-EGFR Reintroduction and Rechallenge in Metastatic Colorectal Cancer (mCRC): A Real-World Analysis. Cancers (Basel) 2022; 14: 1641. doi: 10.3390/cancers14071641

[15] FALLAH J, XU J, JOENG HK, WEINSTOCK C, HEISS BL et al. False-Positive Circulating Tumor DNA Results Do Not Explain Lack of Efficacy for PARP Inhibitors in Patients With Castration-Resistant Prostate Cancer Harboring ATM and CHEK2 Mutations. JCO Precis Oncol 2024; 8: e2400354. doi: 10.1200/PO.24.00354

[16] ROLFO CD, MADISON RW, PASQUINA LW, BROWN DW, HUANG Y et al. Measurement of ctDNA Tumor Fraction Identifies Informative Negative Liquid Biopsy Results and Informs Value of Tissue Confirmation. Clin Cancer Res 2024; 30: 2452-2460. doi: 10.1158/1078-0432.CCR-23-3321

[17] ARGILES G, ARNOLD D, PRAGER G, SOBRERO AF, VAN CUTSEM E. Maximising clinical benefit with adequate patient management beyond the second line in mCRC. ESMO Open 2019; 4: e000495. doi: 10.1136/esmoopen-2019-000495

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[18] MARTINELLI E, MARTINI G, FAMIGLIETTI V, TROIANI T, NAPOLITANO S 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. doi: 10.1001/jamaoncol.2021.2915

[19] SIRAVEGNA G, MUSSOLIN B, BUSCARINO M, CORTI G, CASSINGENA A et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015; 21: 795-801. doi: 10.1038/nm.3870

[20] NAKAJIMA H, KOTANI D, BANDO H, KATO T, OKI E et al. REMARRY and PURSUIT trials: liquid biopsy-guided rechallenge with anti-epidermal growth factor receptor (EGFR) therapy with panitumumab plus irinotecan for patients with plasma RAS wild-type metastatic colorectal cancer. BMC Cancer 2021; 21: 674. doi: 10.1186/s12885-021-08395-2

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488 **Figure Legends**

489

490 **Figure 1.** Flow diagram of patient selection

491

492 **Figure 2.** Survival outcomes following anti-EGFR rechallenge in the third-line setting. A)
493 Progression-free survival in the third-line setting until disease progression. B) Overall survival in
494 the third-line setting. C) Overall survival from initiation of third-line therapy.

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496 **Table 1.** Clinical and treatment characteristics of patients.

Characteristic	Study population
	n=17
Age, median (range of years)	68 (43-81)
Gender, n (%)	
Male	12 (70.6)
Female	5 (29.4)
Primary tumor sidedness, n (%)	
Left-sided	16 (94.1)
Right-sided	1 (5.9)
Primary tumor location, n (%)	
Sigmoid colon	7 (41.2)
Rectosigmoid	4 (23.5)
Rectum	4 (23.5)
Splenic flexure	1 (5.9)
Descending colon	1 (5.9)
First-line anti-EGFR therapy, n (%)	
Cetuximab+chemotherapy	17 (100)
Best objective response to first-line anti-EGFR therapy, n (%)	
Partial response	12 (70.6)
Stable disease	4 (23.5)
Progressive disease	1 (5.9)
Second-line therapy, n (%)	
Anti-VEGF+chemotherapy	17 (100)
Anti-EGFR-free interval, median (range of months)	14 (2-40)
Third-line anti-EGFR rechallenge, n (%)	
Cetuximab	17 (100)
RAS/BRAF status in ctDNA before second-line therapy, n (%)	
WT	17 (100)
RAS/BRAF status in ctDNA before third-line therapy, n (%)	
WT	13 (76.5)
MT	4 (23.5)

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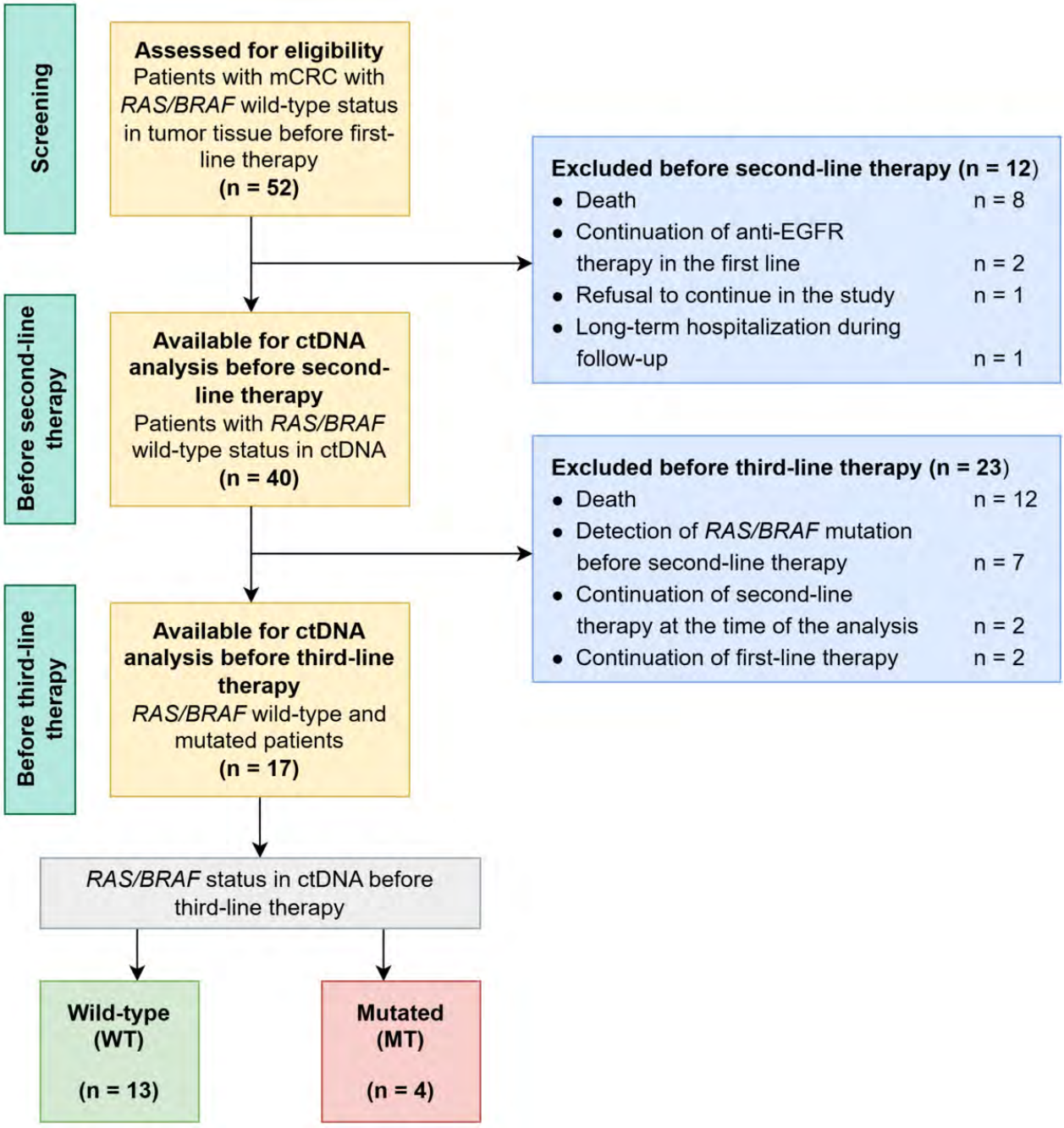
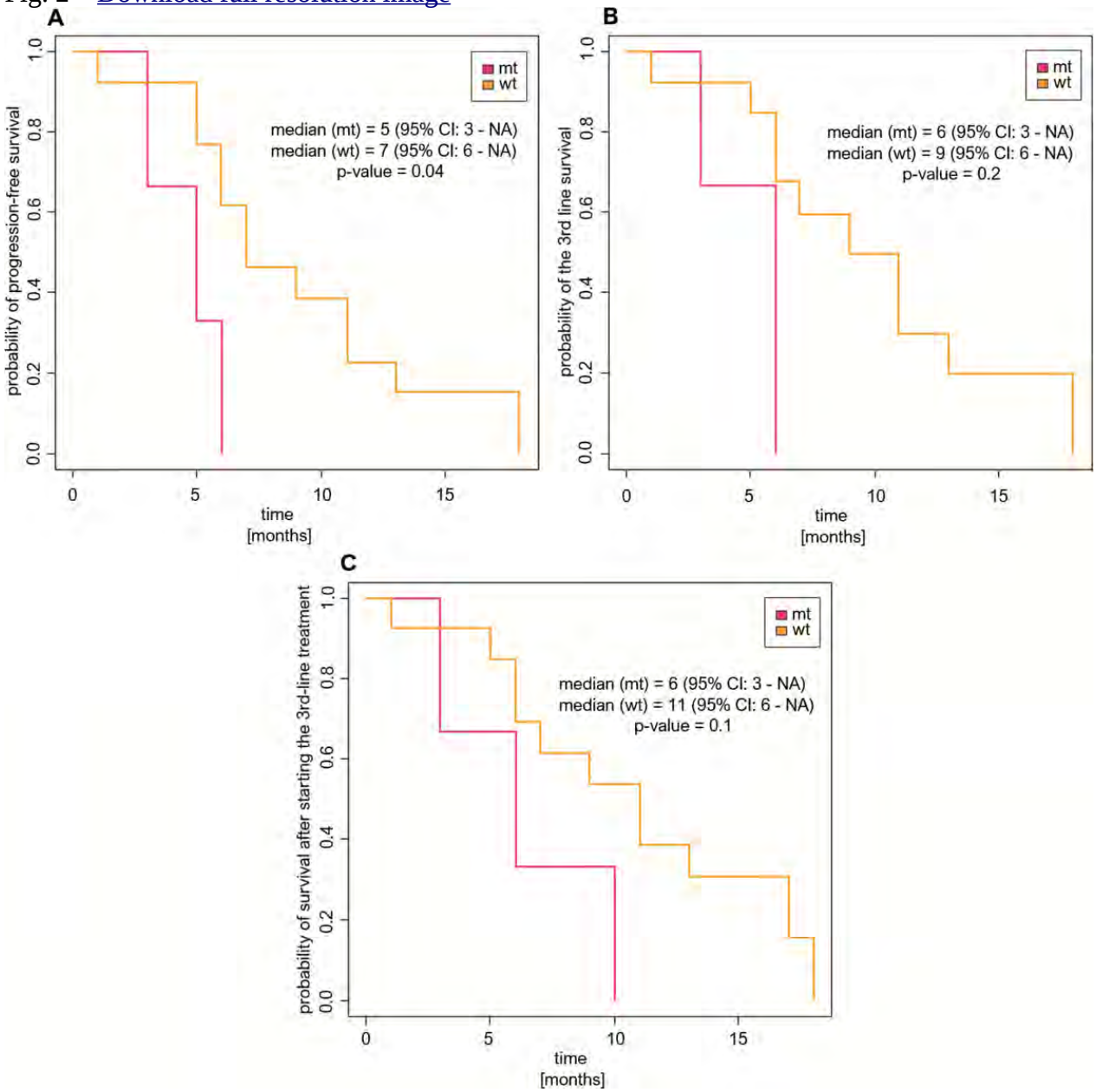
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