Title: **MicroRNA-31-3p expression and benefit from anti-EGFR inhibitors in metastatic colorectal cancer patients enrolled in the prospective phase II PROSPECT-C trial.**

Running Title: **miR-31-3p: a novel biomarker for anti-EGFR treatment.**

Authors: Gayathri Anandappa[1,2,3], Andrea Lampis[2,3], David Cunningham[1*], Khurum H Khan[1,2], Kyriakos Kouvelakis[1] ,Georgios Vlachogiannis[2,3], Somaieh Hedayat[2,3], Nina Tunariu[4], Sheela Rao[1], David Watkins[1], Naureen Starling[1], Chiara Braconi[1,5], Mahnaz Darvish-Damavandi[2,3], Hazel Lote[2,3], Janet Thomas[1], Clare Peckitt[1], Ria Kalaitzaki[1], Nasir Khan[4], Nicos Fotiadis[4], Massimo Rugge[6], Ruwaida Begum[1], Isma Rana[1], Annette Bryant[1], Jens C Hahne[2,3] , Ian Chau[1] , Matteo Fassan[6], Nicola Valeri[1,2,3,*, †]

***These authors contributed equally as senior author**

Affiliations

[1] Department of Medicine, The Royal Marsden NHS Trust, London and Sutton. United Kingdom.
[2] Division of Molecular Pathology, The Institute of Cancer Research London and Sutton, United Kingdom.
[3] Centre for Evolution and Cancer, The Institute of Cancer Research, London, UK.
[4] Department of Radiology, The Royal Marsden NHS Trust, London and Sutton. United Kingdom.
[5] Division of Cancer Therapeutics, The Institute of Cancer Research, London and Sutton, United Kingdom.
[6] Department of Medicine and Surgical Pathology, University of Padua, Padua, Italy.

† Correspondence should be addressed to:

Dr Nicola Valeri

Centre for Molecular Pathology,

The Institute of Cancer Research & The Royal Marsden Hospital, Sutton, Surrey,

15 Cotswald Road, Sutton, SM2 5PT, UK

Email: nicola.valeri@icr.ac.uk

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ABSTRACT

Purpose: Anti-Epidermal Growth Factor Receptor (EGFR) monoclonal antibodies (mABs) are effective in the treatment of metastatic colorectal cancer (mCRC) patients. *RAS* status and tumour location (sidedness) are predictive markers of patients' response to anti-EGFR mABs. Recently, low microRNA-31-3p expression levels have been correlated with clinical benefit from the anti-EGFR mAb cetuximab. Here we aimed to validate the predictive power of microRNA-31-3p in a prospective cohort of chemo-refractory mCRC patients treated with single agent anti-EGFR mABs.

Experimental Design: microRNA-31-3p was tested by *in-situ* hybridization in ninety-one pre-treatment core biopsies from metastatic deposits of forty-five mCRC patients. Sequential tissue biopsies obtained before treatment, at the time of partial response, and at disease progression were tested to monitor changes in microRNA-31-3p expression over treatment. MicroRNA-31-3p expression, sidedness, and *RAS* status in pre-treatment cell-free DNA were combined in multivariable regression models to assess the predictive value of each variable alone or in combination.

Results: Patients with low microRNA-31-3p expression in pre-treatment biopsies showed better overall response rate, as well as better progression free and overall survival, compared to those with high microRNA-31-3p expression. The prognostic effect of microRNA-31-3p was independent from age, gender and sidedness. No significant changes in the expression of microRNA-31-3p were observed when sequential tissue biopsies were tested in long-term or poor responders to anti-EGFR mABs. MicroRNA-31-3p scores were similar when pre-treatment biopsies were compared with treatment-naïve archival tissues (often primary CRC).

Conclusions: Our study validates the role of microRNA-31-3p as potential predictive biomarker of selection for anti-EGFR mABs.

STATEMENT OF TRANSLATIONAL RELEVANCE

RAS status and sidedness represent negative predictive markers of response to anti-EGFR treatment in metastatic colorectal cancer (mCRC) patients. Recently, microRNA-31-3p has emerged as a potential biomarker for the selection of candidates to first line treatment with a combination of chemotherapy and anti-EGFR treatment. Here we confirm the predictive value of microRNA-31-3p in a prospective cohort of chemo-refractory mCRC patients treated with single agent cetuximab in a phase II trial. We show that: microRNA-31-3p can be scored using *in situ* hybridization on pre-treatment biopsies; microRNA-31-3p expression is comparable between primary CRC and metastases; microRNA-31-3p expression does not change during cetuximab treatment; and that patients with low microRNA-31-3p expression had better disease control, progression free survival, and overall survival compared to patients with high microRNA-31-3p expression. We suggest that microRNA-31-3p analysis might be incorporated in the work-up of mCRC along with tumour sidedness and *RAS* testing, in order to further refine the selection of potential responders to anti-EGFR treatments.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of morbidity and mortality worldwide (1,2). Epidermal Growth Factor Receptor (EGFR) monoclonal antibodies (mABs) are effective in metastatic CRC (mCRC) and can be used alone or in combination with chemotherapy (3). Mutations in the RAS pathway are negative predictive biomarkers of response to anti-EGFR antibodies in patients with mCRC, thus *RAS* testing on tissue is routinely performed in clinical practice for patient selection (3).

We and others have recently shown that implementing *RAS* genotyping in pre-treatment circulating cell-free (cf) DNA can identify patients who are unlikely to benefit from anti-EGFR therapies (4,5). Furthermore, our mathematical modelling indicated that resistance to anti-EGFR antibodies is often polyclonal, suggesting that multiple genetic and non-genetic drivers might contribute to treatment failure (5).

MicroRNAs (miRs) are short non-coding RNAs controlling gene expression at post-transcriptional level (6). MiRs are involved in developmental and physiological processes (6), and are often dysregulated in pathological conditions such as cancer and inflammation (7). MiR dysregulation is frequently observed in CRC and multiple lines of evidence suggest that miRs affect a number of cancer hallmarks (8), and drive CRC initiation (9), progression (10) and resistance to treatment (11). Given their relative stability in tissues and other bio-fluids (12,13), miRs have been proposed as potential biomarkers for CRC early detection (14,15), diagnosis (16) and prognosis (17).

MiR up-regulation and/or single nucleotide polymorphisms in miR target genes have been postulated as potential determinants of resistance and sensitivity to anti-EGFR mABs in early and metastatic CRC (18-21). MicroRNA-31-3p (miR-31-3p) expression levels have been examined by RT-PCR in retrospective analyses of the FIRE-3, PICCOLO, NEW-EPOC and PETACC8 trials (22-26); in these studies low miR-31-3p expression was associated with improved outcome and prolonged benefit from anti-EGFR treatment. Real-Time PCR based assays for the analysis of miR-31-3p on

formalin-fixed paraffin embedded (FFPE) tissues are at an advanced stage of validation (23,27).

The PROSPECT-C trial was a phase II trial of single agent anti-EGFR antibodies in chemo-refractory mCRC. Patients underwent repeated tissue biopsies of metastatic deposits before and after treatment as well as at the time of treatment response in case of partial response (5).

In this study we aimed to: (a) validate the association between miR-31-3p expression and clinical benefit from anti-EGFR treatment in pre-treatment tissue biopsies; (b) test changes in miR-31-3p expression in serial tissue biopsies during treatment in order to assess whether miR-31-3p might be a potential biomarker of acquired resistance; (c) test whether combining miR-31-3p with *RAS* testing in cfDNA might improve patient selection.

METHODS

Trial design and patient characteristics

The Prospect-C trial (ClinicalTrials.gov identifier: NCT02994888), was a prospective, phase II, open-label, single centre, non-randomised study of biomarkers of response and resistance to anti-EGFR therapies in *KRAS*/*NRAS* wild-type (wt) chemo-refractory mCRC. Patients who were at least 18 years old and had a World Health Organisation performance status (PS) of 0-2 were considered eligible for this study if they fulfilled all the following criteria: I) chemo-refractory (at least two lines of chemotherapy) metastatic disease; II) *KRAS*/*NRAS* wt (on archival material according to clinically accredited molecular testing); III) measurable disease; and IV) metastatic sites amenable to biopsy. Patients received cetuximab/panitumumab through the Cancer Drug Fund. Written informed consent was obtained from all patients. The study was carried out in accordance with the Declaration of Helsinki and was approved by National Institutional review boards [National Research Ethics Service (NRES): 12/LO/0914]. The objectives

of the study were to validate known mechanisms and identify novel drivers of response/resistance to cetuximab. Treatment consisted of cetuximab 500mg/m^2 once every 2 weeks until progression or intolerable side effects. All but one patient received cetuximab and were anti-EGFR naïve at the time of trial entry; the aforementioned patient was switched to panitumumab due to a Common Toxicity Criteria for Adverse Events (CTCAE) 3.0 Grade II allergic reaction after the first dose of cetuximab, and had previously received 3 cycles of fluorouracil, oxaliplatin and cetuximab combination with partial response (PR) as neo-adjuvant chemotherapy for liver resection in the context of the NewEPOC trial 13 months before entering the PROSPECT-C trial.

All participants were required to have mandatory pre-treatment biopsies [baseline (BL), 6 cores], biopsies at 3 months [if PR by Response Evaluation Criteria In Solid Tumors (RECIST) v1.1 criteria (6 cores)] where clinically and technically feasible, and post-treatment at the time of progressive disease (PD) (6 cores from two suitable progressing metastatic sites). Archival material (primary cancer or original diagnostic biopsies) was assessed where available. Plasma for circulating cell free DNA (cfDNA) analysis was collected every 4 weeks until disease progression.

Analysis of miRNA 31-3p expression using In-situ hybridisation

In-situ hybridisation (ISH) assays for miR-31-3p expression in baseline tissue was performed using miRCURY® LNA® miRNA ISH Optimization Kit for FFPE (Qiagen, Hilden, Germany). Archival tumour material at diagnosis was tested if available (n=12). ISH on tissue sections was performed following the Exiqon protocol with some modifications. Initially paraffin was removed with Xylene incubation for five minutes followed by ethanol 100% incubation for another five minutes. Tissue sections were then dehydrated in ThermoBrite hybridizer (Leica Biosystems, Wetzlar, Germany) containing 20 ug/mL of Proteinase K (Roche, Basel, Swrizerland) for 15 minutes at 37 ^{o}C. The dehydration reaction was stopped by immersing the slides in PBS, and a pre-hybridization step was then performed by adding 1X ISH buffer

(Exiqon, Vedbaek, Denmark) and incubating the sections for 15 minutes at 56 °C. Following the removal of the pre-hybridization solution, previously denatured (90 °C) miRCURY® LNA microRNA detection probe (hsa-miR-31-3p, cat n. YD006116560, Exiqon, Vedbaek, Denmark) was added to the sections at a 200nM concentration; sections were incubated with the detection probe overnight at 56 °C. The following day tissue sections were sequentially immersed in 5X, 1X, and 0.2X SCC solutions at hybridization temperature for five minutes each, and finally transferred in PBS solution at room temperature. Blocking was performed at 30 °C in hybridizer followed by incubation with anti-Digoxygenin-AP fragments (Sigma-Aldrich, St. Louis, MO,) for 1h. The sections were then washed three times with PBS-Tween 0.2% for five minutes each and incubated for 2h with BCIP®/NBT Liquid Substrate System (Sigma-Aldrich) for developing the reaction. The reaction was stopped with by immersing the slides in KTBT buffer and counterstained in Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA). The sections were then dehydrated by ethanol 100% and Xylene incubations (for 5 minutes each) and covered with a coverslip.

MiR-31-3p expression was graded by two independent pathologists as follows: 0 = no staining; 1^+ = weak staining; 2^+ = intermediate staining; and 3^+ = strong staining. Patients with a 0 or 1^+ expression score were deemed as low expressors whereas those with a score of 2^+ or 3^+ were deemed as high expressors.

Analysis of miRNA 31-3p expression using Real-Time PCR

Prior to RNA extraction, samples were reviewed by the pathologist and cancer areas were marked for subsequent macro-dissection. Total RNA from FFPE slides (4 x 4um sections) was extracted using the Ambion Recover All Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. RNA quantity and quality were assessed by NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). Ten nanogram of total RNA were retrotranscribed using the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), and RT-PCR

was performed using the TaqMan® assay for miR-31-3p (assay ID 002113). RNU48 was used as housekeeping gene for normalization, and relative expression was calculated using the 2ΔCt method. MiR-31-3p expression was scored as high or low based on the median of the distribution.

Statistical Analysis

Progression free survival (PFS) was calculated from start of treatment with cetuximab to date of progression assessed radiologically, or clinically. Overall survival (OS) was calculated from start of cetuximab to date of death from any cause or last day of follow-up. Differences in PFS and OS between patients with low expression and high miR-31-3p expression pre-treatment were calculated using the Kaplan-Meier method and compared using the log-rank test. Chi-square test was used to assess the effect of miR expression on overall response to cetuximab treatment. Univariate and multivariate analysis using Cox proportional hazards method were performed to assess effects of age, gender, sidedness of tumour in all 42 patients. In the 34 patients for whom baseline ctDNA results were available, a similar approach was used and multivariate analysis performed. A p value of <0.05 was considered significant.

All the authors reviewed and approved the final manuscript. Researcher performing experiments and scoring tissues were blind to clinical outcome information. Analysis was performed by trial statisticians.

RESULTS

The PROSPECT-C trial recruited forty-five eligible patients between November 2012 and December 2016 (**Figure 1**). Forty-five percent of patients achieved disease control [partial response (PR) or stable disease by RECIST 1.1. criteria]; median PFS and OS were 2.6 months (95% confidential interval (CI): 1.9 – 4.2) and 8.2 months (95% CI: 4.2 - 12.0), respectively. These data have been previously reported by our group (5) and are

in keeping with available literature for single agent anti-EGFR treatments in chemo-refractory mCRC (28).

In order to test the association between miR-31-3p expression and benefit from anti-EGFR treatment we initially scored miR-31-3p by ISH in ninety-one baseline tissue core biopsies from forty-five patients. Forty-two of those patients (88 cores) could be tested further in this study, as, following extensive previous analyses (5,29), no cancer was left in 3 cases.

MiR-31-3p was marked as low if scored negative or 1^+, and as high if scored 2^+ or 3^+ in cancer (**Figure 2**), while stromal miR-31-3p staining due to inflammatory or immune infiltrate (**Supplementary Figure 1**) was not taken into account. Positive cells in the stroma were represented by plasma cells, macrophages and endothelial cells, and most of them showed a faint miR-31-3p expression, with only a limited number of cells characterized by a moderate expression. No significant difference in the proportion of positive stromal cells was observed between anti-EGFR responder and resistant tumours.

At least 2 different slides for each core were tested and concordance in miR-31-3p scoring among different sections from the same core was 100%. In thirty-two patients, two different cores from the same metastasis were tested, although, in four cases one of the two cores showed only necrosis and/or inflammation and thus comparison with its parental core was not possible. The concordance in miR-31-3p scoring among different cores in the remaining twenty-eight patients was 89% (3 cases scored in different categories and were attributed to the high miR-31-3p category as the average score was above 1^+). Overall, twenty-four patients were scored as miR-31-3p low and eighteen as miR-31-3p high; patients' demographics based on miR-31-3p expression are presented in **Table 1**.

In order to validate the results obtained by ISH, we performed miR-31-3p expression analysis using RT-PCR on 46 cores from 18 patients for whom material was available for RNA extraction. Two different core biopsies were available in 19 cases and the average expression (based on RT-PCR) between the two cores was used to determine the miR-31-3p scoring; for the remaining 8 cases only one core biopsy was tested. The 46 cores included 4 primary tumours, 29 pre-treatment (baseline), 4 on-treatment (at the time of partial response) and 9 post-treatment (progression) biopsies. A statistically significant correlation (chi-squared exact test p: 0.003) with 77% concordance between the two miR-31-3p expression tests was observed (**Supplementary Table 1**).

Next we tested the association between miR-31-3p score based on ISH and clinical benefit from anti-EGFR mABs. Low miR-31-3p expression was associated with better overall response rate (ORR) defined as partial response or stable disease, with 58.3% (14/24) patients showing response in the miR-31-3p low group versus 22.2% (4/18) in the miR-31-3p high group (**Supplementary Table S2**; chi-squared exact test p: 0.029). A significant depth and duration of response was observed in patients with low miR-31-3p expression (**Figure 3A** and **3B**). Median PFS was 4.21 months (CI: 1.91-5.56) and 2.27 months (CI: 1.55-2.53) in patients with low and high miR-31-3p respectively [HR for miR-31-3p high: 2.03 (CI: 1.06-3.91); p: 0.03] (**Figure 3C**). Similarly, median OS was 8.88 months (CI: 5.53-18.36) and 4.14 months (CI: 2.96-8.68) in patients with low and high miR-31-3p respectively [HR for miR-31-3p high: 2.20 (CI: 1.09-4.43); p: 0.03] (**Figure 3D**). Multivariable Cox regression analysis including miR-31-3p expression, age at diagnosis, gender, and sidedness (30) in the trial cohort (n=42) confirmed that miR-31-3p was an independent predictor of PFS (**Supplementary Table S3**) and OS (**Supplementary Table S4**).

Changes in miR-31-3p expression during single-agent cetuximab treatment have never been investigated before. Here we took advantage of repeated serial tissue sampling in our trial and we tested whether miR-31-3p scoring is altered during or after EGFR

inhibition. Analysis of nine patients with long-term response (PFS≥6 months) and patients with primary progression (PFS≤3 months) revealed no changes in miR-31-3p scoring in either of the two groups (**Supplementary Table S5** and **Figure 4A**). Indeed, analysis of liver, nodal, abdominal wall and pelvic metastases showed consistent miR-31-3p expression even when different metastases were tested (**Figure 4A** and **4B**).

Comparison of miR-31-3p score between archival, treatment naïve tissue (primary CRC in most of the cases) and pre-cetuximab tissue biopsies (**Supplementary Table S6**) was concordant in 11/12 cases (Fisher exact test p: 0.01) (**Supplementary Table S7**).

We and others have recently demonstrated the predictive value of *RAS* testing in pre-treatment cfDNA as a valuable and more specific alternative to tissue analysis in the selection of patients eligible for anti-EGFR treatments.(4,5) When we included miR-31-3p in a multivariable Cox regression analysis including age at diagnosis, gender, sidedness and *RAS* genotyping in pre-treatment cfDNA in patients for whom all the information were available (n=34), miR-31-3p showed no independent value in predicting PFS (**Supplementary Table S8**) or OS (**Supplementary Table S9**). In keeping with these data, when we generated a statistical model combining miR-31-3p status in tissues and *RAS* genotyping in pre-treatment cfDNA (presence/absence of mutations) (**Supplementary Figure 2A-C** and **Supplementary Tables S10** and **S11**), the interaction tests for ORR, PFS and OS were non-significant (p: 0.213; p: 0.178 and p: 0.067 respectively). Among patients who tested as *RAS* wt in cfDNA from baseline bloods, ORR was 78%, median PFS was 5.10 months (CI: 1.91-16.88) and median OS was 15.23 months (CI: 1.91-34.08) in patients with low miR-31-3p expression (n=9). On the contrary, ORR was 25%, median PFS was 2.27 months (CI: 1.91-16.88) and median OS was 4.67 months (CI: 1.51-12.04) in patients with high miR-31-3p expression (n=8).

DISCUSSION

MiR-31-3p expression has been tested in a number of retrospective series and retrospective analyses of prospective trials (18,22-24,31). Low miR-31-3p expression has been associated with sustained PFS and OS as well as improved ORR in response to EGFR inhibitors. Although these findings have been validated in several studies, the interpretation of these data remains challenging due to the fact that in most of these series anti-EGFR mAbs (cetuximab or panitumumab) were used in combination with different chemotherapy backbones and in different lines of treatment. In the PROSPECT-C trial (5), cetuximab was used as a single agent in a prospective and homogeneous cohort of chemo-refractory mCRC patients; furthermore miR-31-3p was tested in ad hoc pre- and post-treatment tissues biopsies. Thus, despite a relatively small sample size, the trial provided an excellent opportunity to validate the predictive role of miR-31-3p in a prospective cohort and allowed to test dynamic changes in miR-31-3p expression over-treatment. The results presented here are largely consistent with available literature (22-24,27) and suggest that low miR-31-3p might be an indicator of response and better prognosis in patients treated with anti-EGFR mAbs.

Even though our data align well with available literature, several questions remain open. Firstly, the biology underpinning a potential role for miR-31-3p in driving resistance to anti-EGFR agents is not clear. Pre-clinical *in vitro* and *in vivo* data in colon and lung cancer respectively suggest that miR-31-3p targets a number of negative regulators of the RAS-MAPK cascade (32,33). However, despite the link between miR-31-3p over-expression and RAS signalling pathway activation appears solid, no experimental evidence has, as yet, confirmed whether these mechanisms are responsible for resistance to cetuximab.

A second question relates to the source of material and the technology to be used for miR-31-3p testing. MiR-31-3p is over-expressed in early stages of sporadic and

inflammation-related CRC carcinogenesis but expression does not appear to change in more advanced or metastatic stages of disease (34-36). In keeping with these data, no significant changes in miR-31-3p scoring were observed in our trial when comparing primary cancers and metastatic sites. Similarly, in our series, no changes in miR-31-3p scoring were observed in sequential tissues biopsies collected before, during, and after cetuximab treatment. On the contrary, in the NEW-EPOC trial (22), a non-significant correlation for miR-31-3p expression was observed between paired primary CRC specimens and liver metastases in patients receiving pre-operative cetuximab, while a positive and significant correlation was observed in patients treated with chemotherapy alone, suggesting that cetuximab treatment might induce changes in miR-31-3p expression. One of the potential explanations for the discrepancy between the NEW-EPOC (22) and the PROSPECT-C trial (5) is that, in the former trial, miR-31-3p levels were tested by ISH while in the in the latter, the analysis was performed by RT-PCR. Cetuximab is known to trigger intra-tumour inflammatory infiltration in liver metastases, with an enrichment of CD3-, CD8-, and CD56-positive cells (37). Given MiR-31-3p is also involved in immune and inflammatory cells homeostasis (38-41), its over-expression might sometimes be due to intra-tumour infiltration from lymphocites or inflammatory cells. Under these circumastances, despite tissue micro-dissection, contamination by inflammatory cells might potentially lead to a bias in miR-31-3p scoring when using high sensitivity RT-PCR-based assays. In line with this hypothesis, our ISH did detect areas of miR-31-3p over-expression in the stromal compartment of tumours otherwise scored as miR-31-3p low. Furthermore, even though the comparison between ISH-based and RT-PCR-based miR-31-3p scoring in our cohort showed a good concordance, several cases were classified in different miR-31-3p expression categories by the two assays, thus highlighting some hurdles in selecting the best approach for evaluating miR-31-3p expression as a biomarker for anti-EGFR mABs. Given RT-PCR based assays have been recently validated for miR-31-3p clinical testing (23,27),

caution in the analysis and interpretation of data should be exerted in cases with intense inflammatory and immune infiltrate as these might affect miR-31-3p classification.

Selection of mCRC patients' candidate to anti-EGFR treatment relies on primary tumour location (sidedness) (30) and *RAS* testing (3). As we and others have suggested (4,5), moving *RAS* testing to plasma cfDNA might represent a more sensitive and cost/effective option than tissue analysis. In our study we combined *RAS* genotyping in cfDNA with miR-31-3p expression in order to test whether this would result in a more accurate prediction of response to cetuximab. The test for interaction between the two categorical variables was not significant possibly due to the very small sample size, however, in patients with no cfDNA *RAS* abnormalities, ORR, PFS and OS appeared better for patients with miR-31-3p low tumours. While larger studies will need to confirm these findings, a key question remains open: do we need another test to select mCRC patients for cetuximab treatment, or are we at risk of ultra-selecting patients? Our data, in line with the analyses of FIRE-3 (26) trial, suggest that miR-31-3p expression may be an indicator of depth of response to anti-EGFR inhibition; this, in our opinion, might represent the ideal scenario where a more accurate identification of patients likely to achieve resectability and/or symptom control may justify a more thorough selection of patients (**Figure 5**).

In conclusion, our results confirm the potential predictive role of miR-31-3p for the selection of patients undergoing anti-EGFR treatment. Further studies are needed to test if miR-31-3p might be combined with *RAS* testing in cfDNA to further identify best responders in specific clinical niches.

FIGURE LEGENDS

Figure 1. Schematic overview of the PROSPECT-C trial. Chemo-refractory, metastatic colorectal cancer (mCRC) patients meeting all the inclusion criteria underwent serial tissue biopsies from metastatic deposits prior to anti-EGFR treatment, after 3 months of treatment in case of partial response (PR), and at time of progression (PD). EGFR=epidermal growth factor receptor.

Figure 2. miR-31-3p *in-situ* hybridization in pre-treatment biopsies. Examples of miR-31-3p expression tested by *in situ* hybridization in tissue biopsies obtained from metastatic deposits prior to anti-EGFR treatment in the PROSPECT-C trial. The left panels show examples of cases scored as miR-31-3p "Low" based on a score of 0 (top) or 1^+ (bottom). The right panels show examples of cases scored as miR-31-3p "High" based on a score of 2^+ (top) or 3^+ (bottom). Original magnifications = 20x. In each case, a higher magnification of miR-31-3p expression in representative neoplastic cells is shown as right bottom inlet. EGFR=epidermal growth factor receptor.

Figure 3. miR-31-3p and clinical benefit from anti-EGFR therapy in the PROSPECT-C trial. Waterfall (**A**) and spider (**B**) plots show depth (based on RECIST 1.1 criteria) and duration of response based on miR-31-3p expression. Kaplan-Meier curves for progression free (**C**) and overall (**D**) survival according to miR-31-3p expression. EGFR=epidermal growth factor receptor.

Figure 4. Analysis of miR-31-3p expression in sequential tissue biopsies in the PROSPECT-C trial. (**A**) MiR-31-3p expression was tested by *in-situ* hybridization in pre- and post-treatment tissue biopsies as well as after 3 months of treatment in case of partial response. MiR-31-3p scoring did not change over treatment in liver (1024 and 1041) or in nodal (1026) cancer deposits. (**B**) Axial enhanced CT images performed at

baseline and, 20 months later at end of treatment due to RECIST 1.1 progression in non-target disease in patient 1005. The images show maintained benefit in the target pelvic lesion (yellow arrow) and a mixed response in the non-target abdominal wall disease with stable appearances of the right para-median lesion (white asterisk) and progression of the left para-median lesion (arrowhead). At baseline the biopsy was obtained from the target pelvic lesion (yellow arrow), at progression the biopsy was of the left para-median lesion (the biopsy tract can be seen in the subcutaneous tissue – white oval). No changes in miR-31-3p staining were observed in sequential biopsies from the different regions. Original magnifications = 20x.

Figure 5. Proposed workflow for the analysis of miR-31-3p expression in metastatic CRC patients. miR-31-3p might be recommended for left-sided, RAS wild-type patients eligible for resection and/or metastasectomy or for disease/symptoms control.

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TABLE

Table 1. Demographics of the PROSPECT-C Trial based on miR-31-3p expression (n=42)

	miR-31-3p low	**miR-31-3p high**
Age at registration: Median (IQR)	69.6 (62.5-75.9)	67.9 (59.3-73.3)
Gender		
Females	8 (33.3%)	8 (44.4%)
Males	16 (66.7%)	10 (55.6%)
RAS pathway aberration in pre-treatment cfDNA		
Absent	9 (50%)	8 (50%)
Present	9 (50%)	8 (50%)
Side		
Left	19 (79.2%)	12 (66.7%)
Right	5 (20.8%)	6 (33.3%)
Previous treatment lines: Median (IQR)	1 (1-2)	2 (1-2)

IQR= interquartile range; cfDNA= circulating cell-free DNA

Figure 1

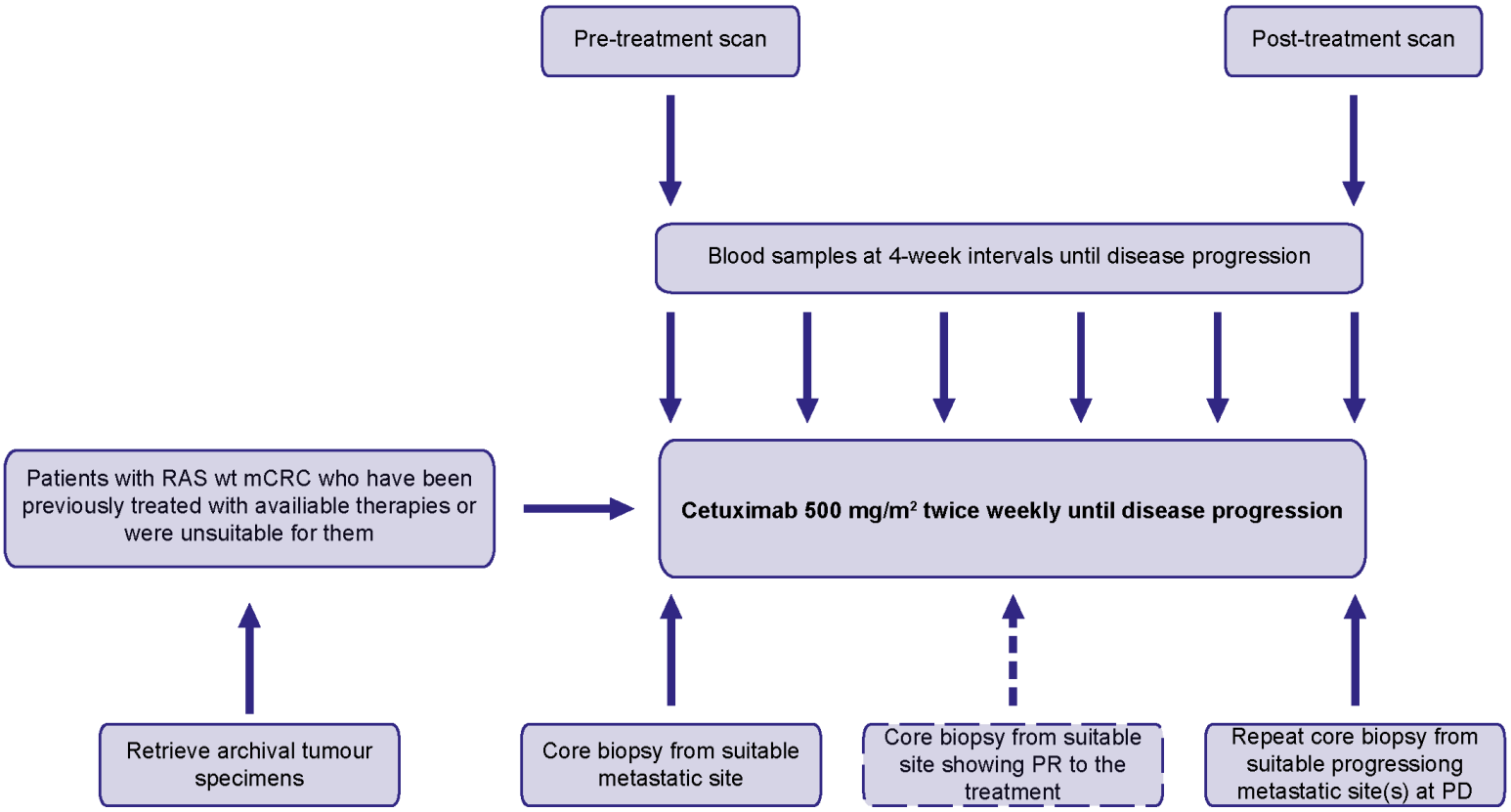


Figure 2

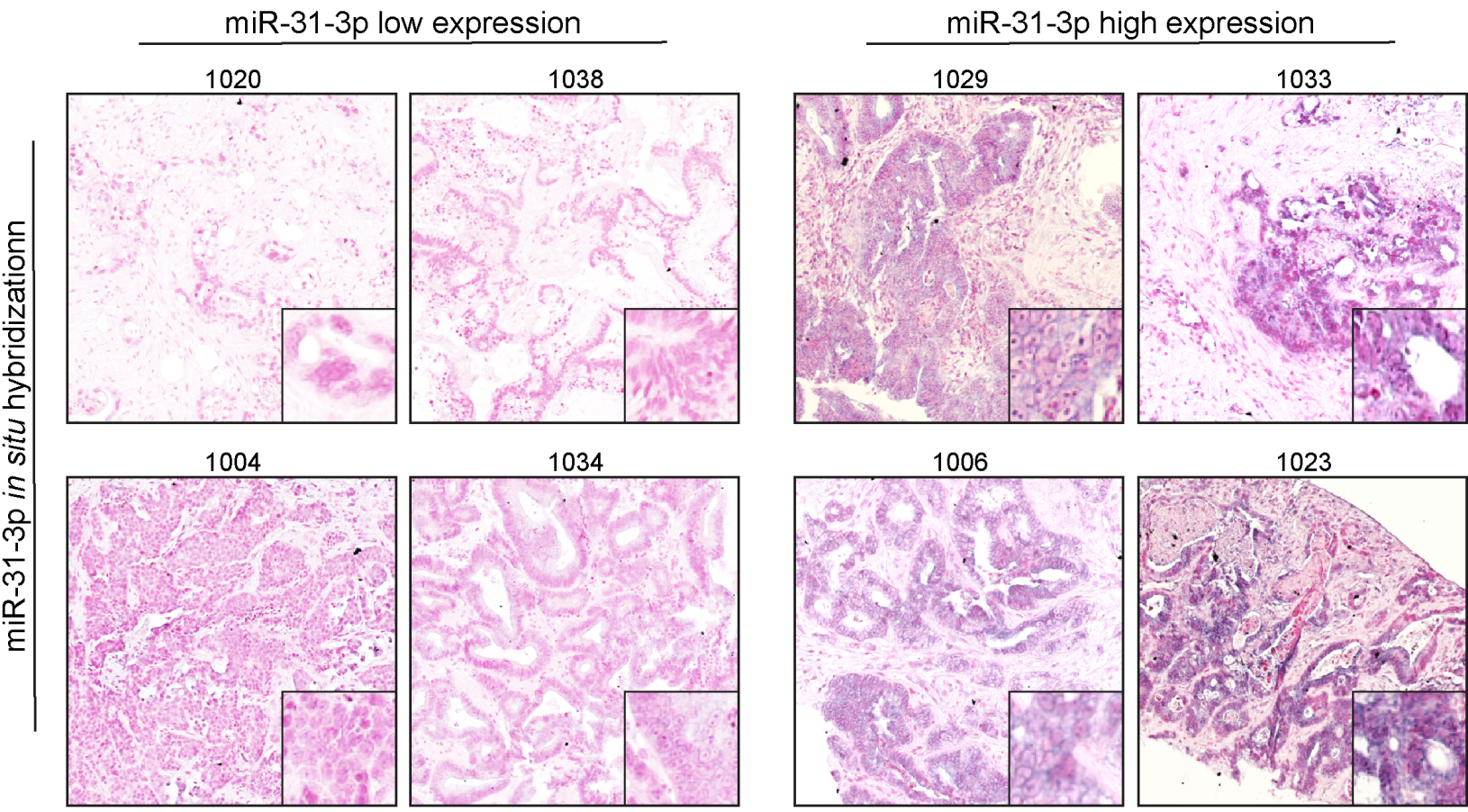
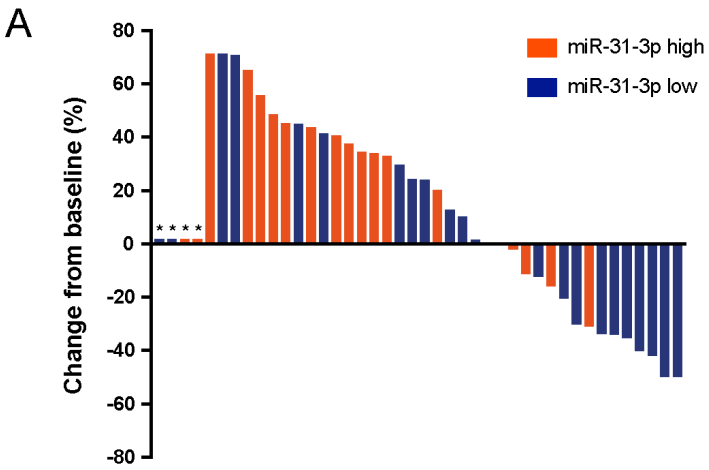
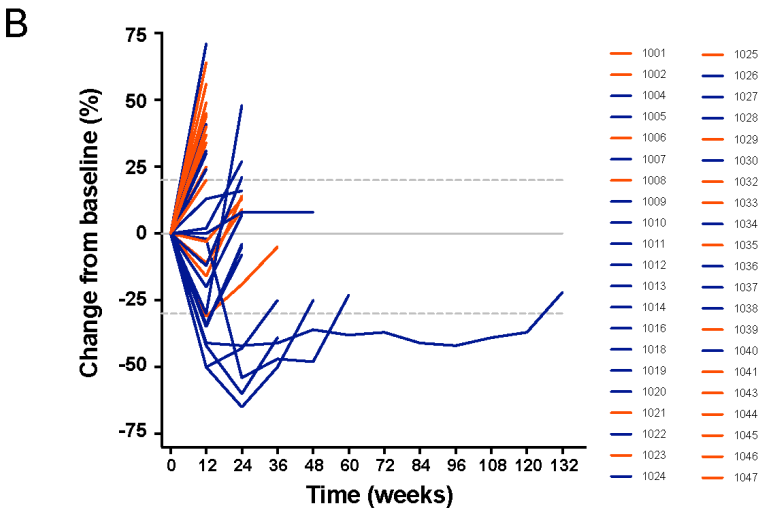
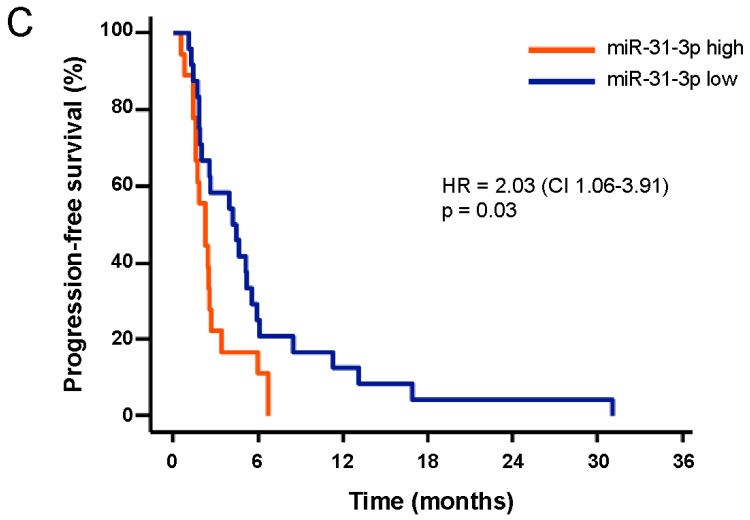
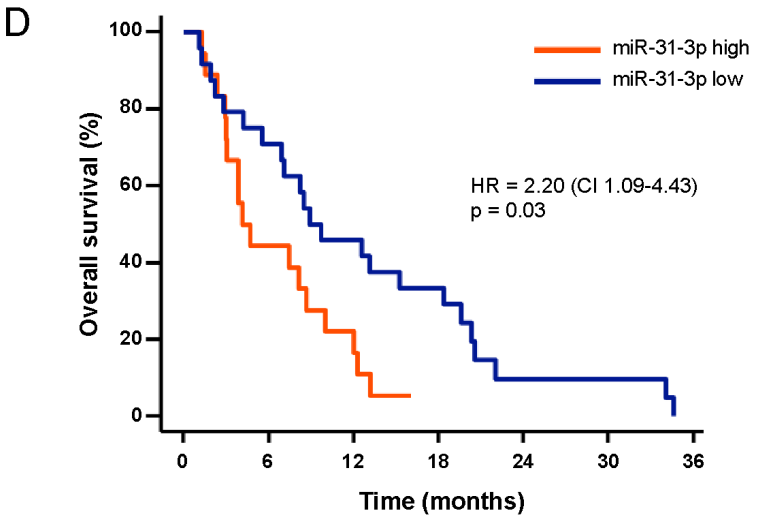


Figure 3





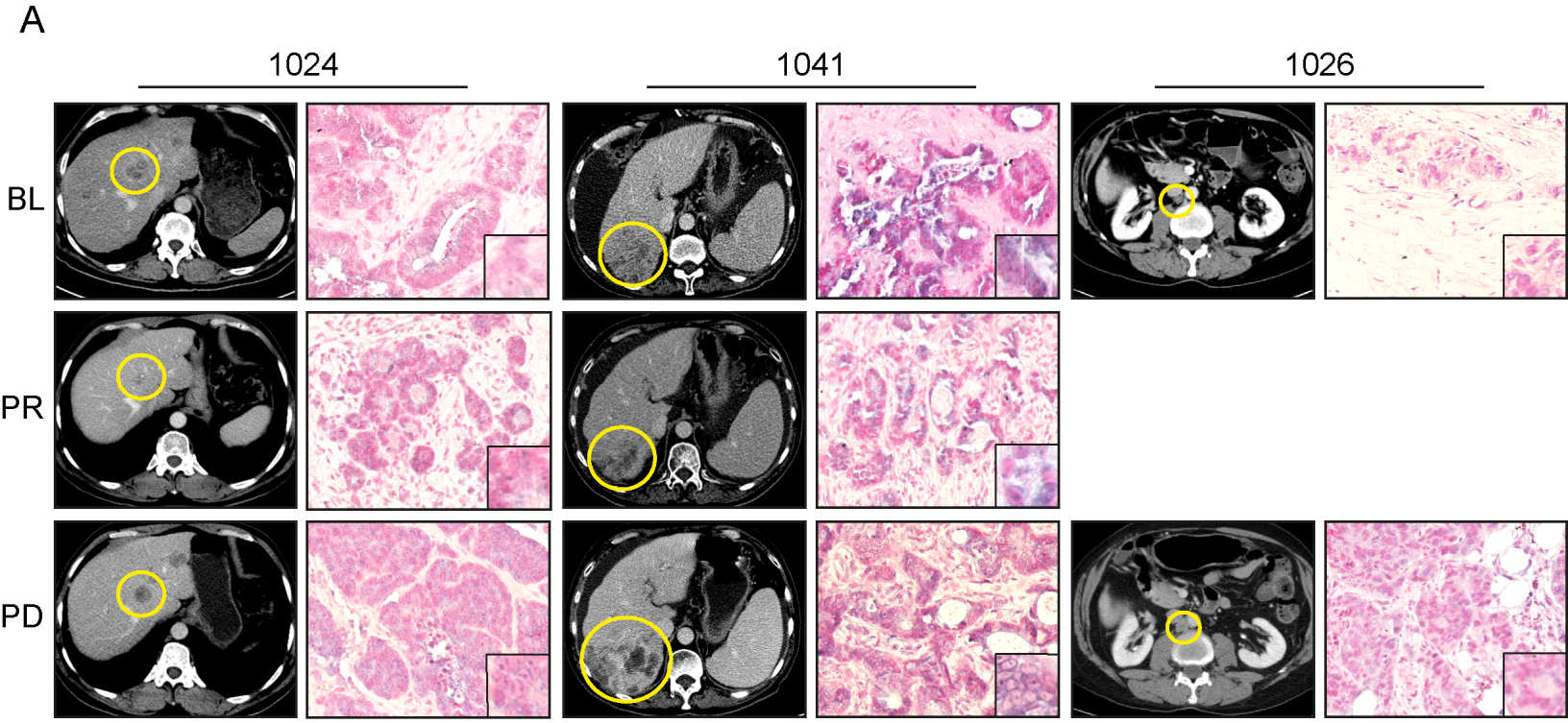




Number at risk								
	High:	18	2	0	0	0	0	0
	Low:	24	6	3	1	1	1	0

Number at risk								
	High:	18	8	4	0	0	0	0
	Low:	24	17	11	8	2	2	0

Figure 4



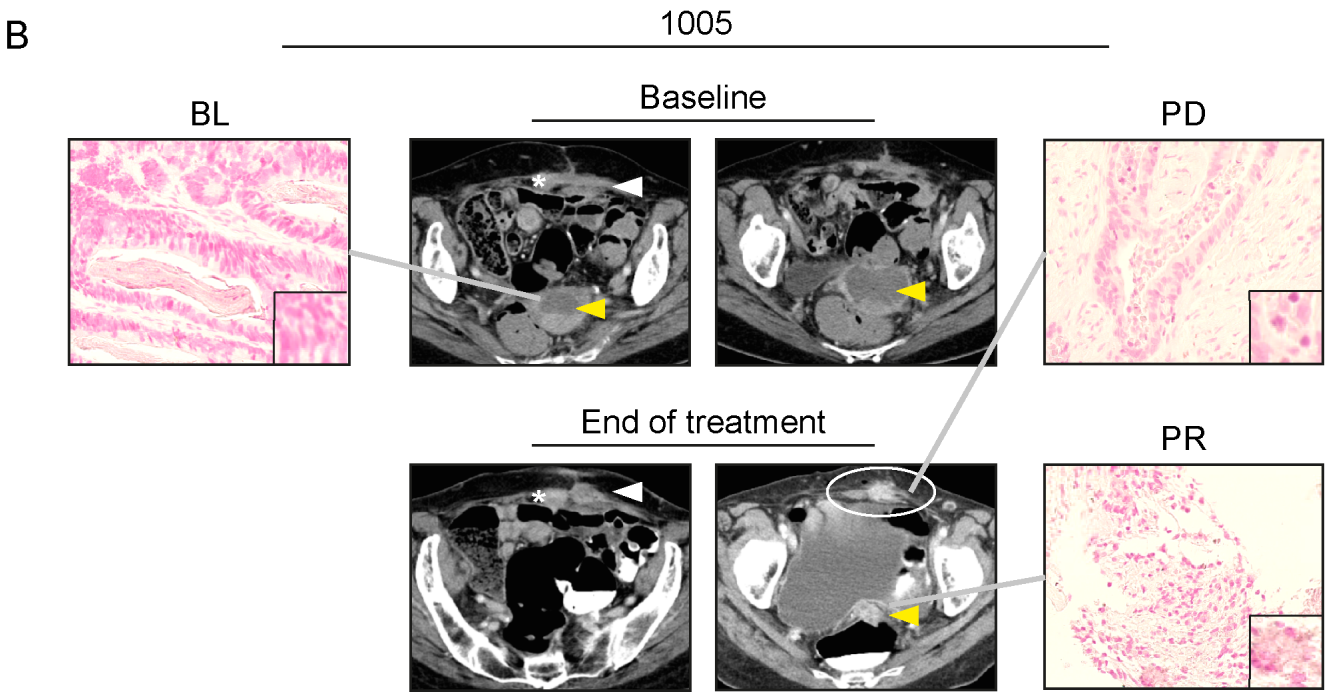
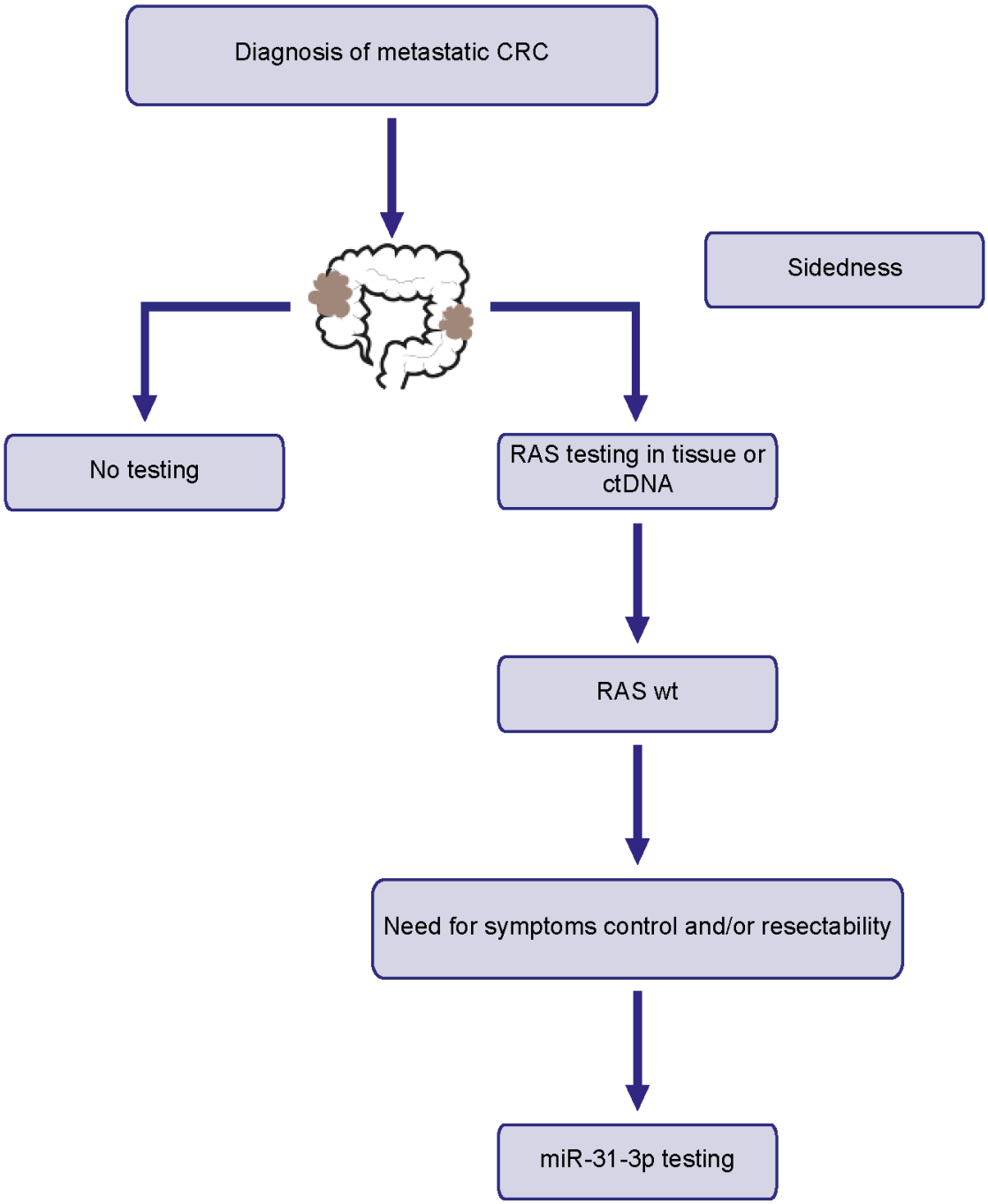


Figure 5



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