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PROGNOSTIC AND PREDICTIVE ROLE OF LIQUID BIOPSIES IN ADVANCED COLORECTAL CARCINOMA: CIRCULATING TUMOUR MUTATIONS AND MICRORNAS

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SUMMARY

Background: Genotyping tumour tissue for evaluation of genetic somatic alteration is a routine practice in daily oncology. However, evaluation of cancer tissue has some limitations, such as invasiveness and scarce reproducibility. Liquid biopsies consist on a fast, not invasive and easy collection and examination body fluid, most frequently blood and urine. Liquid biopsies are informative in regard to tumour heterogeneity and allow the contemporary evaluation of genetically different and co-occurring cell clones during the disease without being restricted to one single tumour site.

In colorectal cancer (CRC), circulating-free DNA (cfDNA) and microRNAs (miRNAs) can be relevant for early diagnosis, identification of minimal residual disease and estimation of recurrence risk. In advanced disease, liquid biopsies allow the monitoring of the response to treatment and identification new molecular alterations resulting in drug resistance and disease progression (PD).

Due to the fact that the clinical markers in use (carcinoembryonic antigen [CEA] and carbohydrate antigen 19.9 [CA19.9]) are not robust and not specific for CRC, new markers for detection and monitoring this disease are needed. In this PhD work, the prognostic and predictive role of liquid biopsies in advanced CRC has been evaluated.

Aim of the study: The primary endpoint was to monitor cfDNA mutations developing during treatment with targeted agents (bevacizumab, cetuximab and panitumumab) in metastatic CRC. As secondary endpoint, we considered the correlation between circulating molecular alterations and clinical development of resistance to these targeted agents. Moreover, early molecular alterations and changes in miRNA expression levels anticipating and predicting clinical PD were evaluated.

Materials and methods: This was a prospective, observational, multicentre study. Patients with histological diagnosis of CRC and advanced disease treated in first, second or third line with chemotherapy in combination with biological agents (bevacizumab as anti-VEGF treatment,

cetuximab or panitumumab as anti-EGFR treatment) were included. Blood and urine samples were collected before the beginning of treatment, every four weeks during treatment, within ten days of radiological evaluation of disease at radiological progression of and between one and two months after progression. Centres involved were Hospital of Cremona, ASST of Cremona and Hospital of Oglio Po-Casalmaggiore. Molecular analyses were performed in the Gastrointestinal Cancer Biology and Genomic Group at the Centre for Molecular Pathology of The Institute of Cancer Research (Sutton, UK). CfDNA was extracted from plasma, while miRNAs were extracted both from plasma and urine. Extracellular vesicles (exoxomes) were isolated from plasma and urine with further isolation of RNA. MiRNAs expression and cfDNA analysis was performed by using BioRad® droplet digital polymerase chain reaction (ddPCR) technology, while next generation sequencing (NGS) analysis using AVENIO® ctDNA Expanded Kit investigated the presence of driver mutation linked to radiological disease progression during treatment for advanced CRC.

Results: From February 2016 to October 2018, 63 patients were included, 26 patients treated with cetuximab or panitumumab (cohort 1) and 37 patients with bevacizumab (cohort 2). Median overall survival of cohort one was 18.9 months versus 15 months for cohort 2, (p=0.0362). In a previous preclinical study, let-7g-5p had higher levels in cetuximab sensible cells compared to resistant ones. Resistant cells secret a higher amount of let-7g-5p in exosomes compared to sensible cells, suggesting a potential mechanism of acquired resistance based on the intra-cellular transport of this miRNAs via exosomes. MiRNA let-7g-5p was evaluated in plasma and in urine samples of cohort 1. The ddPCR technique was used to determine let-7g-5p expression level in plasma and urine samples of 21 cases. Results were compared to CEA and CA 19.9 and disease status according to Response Evaluation Criteria in Solid Tumours (RECIST 1.1) criteria. In five cases, let-7g-5p expression followed and sometimes anticipated radiological PD, increasing its expression before or concurrently with PD, while in other cases its expression trend was not adherent to clinical outcome. Trend in urine was specular to plasma in some cases and parallel in other situations.

Secondly, we assessed *RAS* and *BRAF* mutational profile for ten patients of cohort 2. Known mutations from tissues were monitored with ddPCR on plasma samples. Mutation load followed clinical history in four cases only. Moreover, NGS analysis was performed on samples of three patients treated with bevacizumab who had long and uncommon response period to treatment. *TP53* was the acquired mutation promoting progression in two cases, while *APC* was detected together with *KRAS* mutation in a third case. Furthermore, NGS was performed on pre-surgical samples of three patients undergoing radical surgery for advanced disease after a variable period of treatment and relapsing after surgery. All three cases were found to have one or more mutations expressed before surgery. One patient showed a mutation pattern shift before and after surgery, with acquisition of *APC* and *TP53* mutations at high frequencies associated with fast disease progression.

Conclusions and future perspectives: Liquid biopsies may have a prognostic and predictive role in the management of patients with advanced CRC receiving active treatment. Tracking of known starting cfDNA mutations, detection of new mutation as well as evaluation of miRNA expression levels can become of high importance in predicting clinical trend of the disease and may be used in anticipating crucial therapeutic decisions as well as suitable candidates for radical surgery. However, liquid biopsies have some limitations, as far as the level of tumour cfDNA depends on many factors (such as cancer stage and tumour burden), levels of cfDNA among cancer patients vary and tumour-derived DNA accounts for only 0.1 to 10% of total DNA.

Future studies will include a bigger number of patients in *RAS* wild-type cohort in order to validate the potential role on miRNA let-7g-5p for resistance to anti-EGFR agents. Secondly, NGS analysis will be performed on all resected patients collected in the frame of this study, both relapsed and disease–free after surgery, in order to investigate possible differences in mutation pattern between relapsed and non-relapsed patients.

INTRODUCTION

Advanced colorectal cancer: epidemiology and clinical features

Colorectal cancer (CRC) is the third most common tumour worldwide with high prevalence in developed countries, coming after lung and breast cancer [1]. In 2018, colorectal cancer had a higher incidence in men than in female (9 versus [*vs*] 7 % of all new diagnosis of tumour) with an equal 8% rate of cancer related deaths in both sexes in the United States [2]. Therefore, CRC represents the third leading cause of cancer death regardless of the sex after lung and prostate cancer for men and lung and breast for women according to the last USA cancer statistics [2].

The majority of CRC are diagnosed on the left-hand side of the bowel, with around 60% of tumours diagnosed in the descending colon, sigmoid, recto-sigmoid junction, rectum and anus [3]. CRC incidence and mortality have declined through the years: from 2005 to 2014, rates declined annually by 2 to 3%. Recent rapid declines in the incidence and mortality have been mainly caused by the introduction and rapid diffusion of colonoscopy as predominant screening test. Alone in the United States, the endoscopic test tripled its use among adults aged 50 and older from 21% in 2000 to 60% in 2015 [2].

Unfortunately, more than half of the CRC patients develop distance metastases and the majority of them die because of the advanced disease [4]. Approximately 25% of CRC patients have metastatic disease at diagnosis (synchronous metastases); another quarter of patients develop metastases during follow-up (metachronous metastases) [4]. Location and number of metastases are known to have a prognostic role: patients with metastasis confined to one organ (liver or lung) had the longest survival in a recent series of 814 patients with stage IV CRC (2-year survival: 51.6%) [5]. On the other hand, patients with peritoneal or non-regional lymph node metastases had a lower and intermediate survival, as well as cases of liver plus lung localization (2-year survival: 39.4%). The worse survival was found for patients with metastatic disease to sites different from liver and lung,

or if metastasis are present in more than one organ, except liver plus lung [5].

Advanced colorectal cancer: molecular features

Right (RCC) and left colon cancer (LCC) have different tumorigenic pathways [6]. These differences are present both in resectable and metastatic disease [7-9].

RCC are lower in prevalence than LCC, more frequent in female, often larger in dimensions and associated with Lynch Syndrome [10-12]. Major activated pathways in RCC are the mitogen-activated protein kinase (MAPK) and the phospoinositide-3 kinase (PI3K) signalling. RCC is known to have an increased mutational burden [13-15]. Moreover, RCC shows a higher prevalence of microsatellite instability (MSI-H) resulting in higher mutation rates [7,16]. Therefore, it is not surprisingly that mutations of *KRAS* and *BRAF* genes are more common in RCC [15,17]. This high mutational burden and MSI-H make RCC an ideal candidate for immunotherapeutic options [18] .

On the other hand, LCC is more frequent in men, smaller in dimensions than RCC, associated with familial adenomatous polyposis (APC) syndrome and mainly dependent on receptor tyrosine kinase activity (RTK), such as the epidermal growth factor receptor (EGFR) signalling [10-12]. Mutations of the genes *APC*, *p53* and *NRAS* are frequent, while the mutational burden is lower than in RCC [13-15].

Rectal cancer (RC) has a significant lower prevalence of mutations in *PIK3CA* and *PTEN* genes, whose deregulation has been associated to resistance to anti-EGFR therapy [14,19]. Moreover, RC harbours a higher rate of amplification of the *Her2/neu* gene [7,20].

These biological differences in laterality and primary site in CRC are both of prognostic and predictive relevance. Indeed, RC has a higher relapse rate (21%) compared to RCC (14%) and LCC (16%) [21]. The relapse pattern is different, with RC prefer relapsing to the lung as compared to RCC and LCC [21]. Globally, LCC was shown to have a lower risk of death than RCC (hazard ratio [HR] 0.82, $p<0.001$), with a better outcome independently of stage, race and type of adjuvant chemotherapy [22]. Both *BRAF* and *RAS* mutations (*e.g*. *KRAS* in exon 2,3 and 4; *NRAS*) have a

prognostic role. *BRAF* mutation is associated with poor prognosis in CRC, with a prevalence of 7-10% [23]. The single nucleotide mutation *BRAF V600E* is the most frequent mutation in CRC patients [23]. Diversely, among all CRC metastatic patients, *RAS* mutations have a prevalence of about 50%, with *KRAS* exon 2 being the most frequent one (43% of cases) [24]. *PI3KCA* mutations have a prevalence of 10% and are significantly associated with a concurrent *RAS* mutation [25]. *RAS* mutations harbour a negative prognostic role, being associated with a reduced survival and an increased incidence of bone, lung and brain metastases [26].

Advanced colorectal cancer: treatment

The first-line treatment in advanced CRC is made by a chemotherapy backbone of fluoropirimidine (either intravenous 5-fluorouracil [5-FU] and leucovorin or oral capecitabine) used in various combinations with oxaliplatin and/or irinotecan in doublet or triplet schedules for example FOLFOX (5-fluororuracil and oxaliplatin), FOLFIRI (5-fluorouracil and irinotecan) or FOLFOXIRI (5-fluorouracil, oxaliplatin and irinotecan) [27]. It was demonstrated that combination of fluoropirimidine and leucovorin plus oxaliplatin or irinotecan increase the response rate (RR), progression free (PFS) and overall survival (OS) [28,29]. The oral fluoropyrimidine capecitabine can be used as a single agent instead of combining 5-FU and leucovorin; furthermore capecitabine can be administered in combination with oxaliplatin. Combination of capecitabine with irinotecan is not frequently used due to higher gastrointestinal toxicity than FOLFIRI - especially the risk and grade of diarrhoea is increased under treatment with capecitabine and irinotecan [27]. Thus, the chemotherapeutic options for patients with advanced CRC are cytotoxic doublets such as FOLFOX, CAPOX (capecitabine and oxaliplatin), FOLFIRI or, especially in fit and young patients, a triplet chemotherapy with FOLFOXIRI. Monotherapy with 5-FU or capecitabine is often chosen for unfit or old patients who are not good candidates for combination chemotherapy [27].

The monoclonal antibodies bevacizumab, cetuximab and panitumumab improve the clinical outcome of a subgroup of CRC patients when combined to chemotherapy in the first-line setting [27].

Anti-VEGF therapy

Bevacizumab is a monoclonal antibody that binds to circulating vascular endothelial growth factor (VEGF) exerting an antitumor effect by preventing the binding of VEGF to the VEGF receptor type 1 and 2 and by this angiogenesis is inhibited [30]. Currently, there is no validated predictive marker available for bevacizumab. Bevacizumab was shown to increase the activity of chemotherapy (in regard to RR, PFS and OS) when administered together with capecitabine or as triple-therapy in combination with 5-FU and leucovorin in first-line setting [31,32]. Combination of bevacizumab with fluoropyrimidine and oxaliplatin has been resulted in an increase of PFS but not RR and OS in a large phase III trial [33]. Similarly, the addition of bevacizumab to FOLFOX or FOLFIRI did not improve outcomes in two randomized smaller trials [34,35]. In contrast, FOLFOXIRI in combination with bevacizumab has been shown to improve RR and PFS compared to FOLFIRI + bevacizumab, resulting in one of the longest median OS recorded in the treatment of CRC patients [36].

Thus, in first-line setting, bevacizumab should be used in combination with oxaliplatin or irinotecan doublet or with triplet FOLFOXIRI, especially in fit patients for whom tumour shrinkage (cytoreduction) is the final target. Combination of FOLFOXIRI and bevacizumab may be a good choice of treatment even in fit *BRAF* mutated patients carrying a dismal prognosis and requiring a more aggressive therapeutic approach. On the other hand, a combination of bevacizumab and fluoropyrimidines may be the best treatment option for unfit and/or old patients. Bevacizumab can be also administered after progression as a second-line treatment. If not administered before, it should be considered in combination with chemotherapy in second-line [27]. Indeed, combination of bevacizumab and FOLFOX improved OS as compared to chemotherapy alone [37]. After a first-line use and progression, bevacizumab can still be used in second-line treatment as it showed

effectiveness in association with a different chemotherapeutic backbone [38]. In this study, bevacizumab was combined with oxaliplatin or irinotecan-based first-line chemotherapy and after progression with second-line chemotherapy chosen depending on the first-line regimen [38].

Anti-EGFR therapy

Cetuximab and panitumumab are monoclonal antibodies targeting the EGFR and are used for the treatment of advanced CRC alone or in combination with chemotherapy [39-41]. These agents share a similar mechanism of action and identical spectrum of activity but have a different chemical structure [40]. Panitumumab is a fully human monoclonal antibody whereas cetuximab is a mouse/human chimeric antibody [42,43]. Moreover, they have a different administration schedule (panitumumab biweekly, cetuximab weekly) and a different toxicity profile, with fewer cases of skin toxicities, fissures and pruritus for cetuximab and a higher incidence of acne like rash [40]. Common side effects of both compounds are electrolyte disorders like hypomagnesemia and hypokalemia [40]. Cetuximab and panitumumab are both equally effective as single agents, while in irinotecan refractory patients the combination of cetuximab and irinotecan is superior to cetuximab alone [27].

Mutations in *KRAS* (exons 2, 3, 4), *NRAS* (exons 2, 3, 4), *BRAF* and amplifications of *HER2* and *MET* are leading mechanisms of primary (*de-novo*) resistance to anti-EGFR treatment [44,45]. Moreover, the onset of alterations in these genes was detected in patients relapsing after a first response to anti-EGFR agents [46]. On the whole, *RAS* mutational status is predictive: the presence of the above mentioned mutations predicts lack of benefit from these biological agents [27]. Cetuximab improved RR, median PFS and OS in first-line setting in combination with FOLFIRI when compared to FOLFIRI alone [47-49]. Both cetuximab and panitumumab increased the activity of FOLFOX and FOLFIRI chemotherapy in *RAS* wild-type tumours. In contrast, no benefit was seen

when anti-EGFR agents were added to an oxaliplatin-based regimen without intravenous fluoropyrimidines [27].

In the first-line setting, there is no evidence for the superiority of one class of biological agents over another (bevacizumab *vs* anti-EGFR therapy). The combination of FOLFOX or FOLFIRI with an anti-EGFR antibody led to an increased RR both in the phase III FIRE-3 and CALGB study [50,51]. Nevertheless, only the FIRE-3 study reported an increase in OS in case of combining FOLFIRI with anti-EGFR administration, while CALGB trial didn't [50,51]. The PFS was identical for regimens containing bevacizumab and cetuximab in both studies [50,51]. Anti-EGFR treatment can also be administered in second-line or third-line (preferable option) in combination with FOLFIRI or irinotecan [27]. The addition of anti-EGFR antibodies to chemotherapy doublet in first-line treatment was associated with a significant OS benefit for patients with left-sided CRC (HR 0.69, 95% confidence interval [CI] 0.58-0.83) [52]. On the contrary, the benefit is essentially absent in RCC (HR 0.96, 95% CI 0.68-1.35, p=0.10) [52]. Consistent results were obtained when the use of anti-EGFR antibodies was compared to the addition of bevacizumab to standard chemotherapy. Indeed, an OS benefit was evident for left-sided tumours receiving chemotherapy + anti-EGFR (HR 0.71, 95% CI 0.58-0.85), while chemotherapy plus bevacizumab performed better in right-sided CRC (HR 1.30, 05% CI 0.97-1.74, p<0.001) [52,53]. In Table 1, the preferred options for first-line treatment of CRC depending on sidedness and molecular features of the disease are summarized.

Table 1. Differences of CRC due to sidedness. CRC prevalence differs between the site and also first-line treatment of advanced CRC is depending on sidedness and molecular profile of the disease. Anti-EGFR treatment is the preferred choice in *RAS* wild-type left hand side cancers and rectal cancer. For all the other cases, combination of chemotherapy (FOLFOXIRI: 5-fluorouracil, oxaliplatin and irinotecan ; FOLFOX: 5-fluorouracil and oxaliplatin; FOLFIRI: 5-fluorouracil and irinotecan) and bevacizumab is preferred. RCC: right colorectal cancer; LCC: left colorectal cancer; RC: rectal cancer; EGFR: epidermal growth factor receptor; *APC*: adenomatous polyposis coli; MSI-H: high microsatellite instability.

	RCC	**LCC**	**RC**
Prevalence in %	31	42	27
Sex prevalence	Female	Male	Male
Dimensions	Larger	Smaller	-
Associated syndromes	Lynch Syndrome	APC	-
Molecular features	*KRAS and BRAF* mutations MSI-H	*APC, p53, NRAS* mutations	*PIK3CA* and *PTEN* mutations *Her2/neu* amplification
Treatment for *RAS* wild-type	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab	FOLFOX/FOLFIRI + anti-EGFR	FOLFOX/FOLFIRI + anti-EGFR
Treatment for *RAS* mutated	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab
Treatment for *BRAF* mutated	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab	FOLFOXIRI/ FOLFOX/FOLFIRI + bevacizumab

Liquid biopsies

Liquid biopsies evaluate circulating tumour cells (CTCs) or cell-free fragments of nucleic acids shed into the bloodstream by cells undergoing necrosis or apoptosis [54]. The analysis of circulating cells or cell-free DNA and RNA, respectively, provide information about the genetic landscape of cancerous lesions and their evolution [54]. Furthermore, liquid biopsies seem to have the capacity to monitor therapy success and predict treatment failure at an earlier time point than imagine procedures do [55].

Genotyping tumour tissue for evaluation of genetic somatic alteration is a routine practice in daily oncology. Although evaluation of cancer tissue is simple and highly informative, this procedure brings some major limitations. For example, tissue biopsies are not always easy to obtain, especially in tumours with low cellularity. Moreover, the small piece of tumour tissue obtained by a biopsy is not representative in regard to tumour heterogeneity as far as it is a single snapshot in time during the history of the disease and a small portion either of the primary tumour mass or its metastases [54]. Table 2 compares liquid biopsies with solid biopsies.

Table 2: Major differences between solid and liquid biopsies. Liquid biopsies are less invasive, more reproducible and can be used to monitor the therapy. Courtesy of Dr. Nicola Valeri (unpublished).

	Solid Biopsy	Liquid Biopsy
Invasiveness	High	Low
Compliance	Low	High
Cost/Effectiveness	Low	High
Time	3 weeks	3 days
Repeated access	Very limited	Possible
Multiple Sampling (Ability to capture heterogeneity)	Not in a clinical setting	Possible
Amount of material	Variable	Variable
Sensitivity	High	Under investigation
Reproducibility	-	Good
Clinical Significance	Single biopsy not representative	Lack of threshold to separate false positive, real positive and clinically relevant positive

Liquid biopsies consist on a simple collection of body fluid. Up to today mostly limited to blood draw with subsequent isolation of plasma, serum and buffy coat. So, it is a fast, not invasive and easy examination method to be performed. It can be repeated easily and less time is required for analysis compared to solid biopsy (table 2). The sensitivity of liquid biopsies for patients with stage IV disease is around 100%, while lower stages with reduced tumour burden have lower numbers of cell-free nucleic acids, leading to a lower sensitivity [54]. Nowadays, methodologies used to analyse liquid biopsies aim to reach a sensitivity of 1% or lower [54], such as next-generation sequencing (NGS), amplification refractory mutation system (ARMS) or digital-droplet polymerase chain reaction (ddPCR) (Table 3). The limitation of the most sensitive analysis method is caused by the error rate of DNA polymerase (ddPCR, Table 3) [54]. Therefore, a sample is considered negative if a fraction of free nucleic acid in a sample is at or below 0.01%.

Table 3. Methodologies for detecting circulating cell-free nucleic acids (modified from Diaz L, JCO 2014).
Nowadays, preferred technologies reach a sensitivity of less than 1%, as it is the case for NGS, ARMS and ddPRC.
ARMS: amplification refractory mutation system; ctDNA: circulating tumour DNA; ddPCR: digital-droplet PCR; NGS: next-generation sequencing; PCR: polymerase chain reaction, TT: tumour tissue.

Technique	Sensitivity	Optimal Application
Sanger sequencing	> 10%	TT
Pyrosequencing	10%	TT
Quantitative PCR	1%	TT, ctDNA
NGS	< 1%	TT, ctDNA
ARMS	0.10%	TT, ctDNA
ddPCR	0.01% or lower	ctDNA, rare variants in TT

As already shortly mentioned above, liquid biopsies are useful for monitoring tumour burden in order to identify response to therapy and define ambiguous scenarios coming from clinics such as mixed response or stable disease [54,56]. Moreover, liquid biopsies allow a constant monitoring of the molecular make-up of the tumour, including developing resistance to a therapy by acquisition of new genetic alterations and emergence of multiple resistance clones during treatment (heterogeneity) both from primary tumour and all metastatic sites [54,56]. Another potential application is the detection of minimal residual disease, especially after a radical surgery or curative treatment, in order to identify a subclinical and early relapse [54,56]. Liquid biopsies can also detect epigenetic changes within the tumour, such as methylation changes [54,56]. Recently it was proofed that methylated circulating DNA fragments represent the degree of methylation in the tumour itself and may also correspond to tumour burden [54,56].

CTCs, cell-free DNA (cfDNA) and extracellular vesicles can be isolated from liquid biopsies [57]. During the first step of the metastatic process tumour cells penetrate the vessel wall and get into the circulatory system [57]. These penetrated tumour cells can be detected in the peripheral blood,

however their number is low (less than ten cells in 7.5 ml of peripheral blood) [57]. CTCs can also release DNA fragments into circulation [57]. In case of normal apoptotic activity, cfDNA is almost totally cleared but in patients with tumours and excessive cellular turnover, cell debris accumulates and they are not cleared properly [57]. This fact provides a rational explanation for the positive relationship between tumour burden and cfDNA amount. CfDNA evaluation, differently from CTCs, is based on measuring single tumour-specific aberrations [57]. Among extracellular vesicles, exosomes are of special interest. Exosomes are small (around 100 nm) membrane-bound particles that originate from multivesicular bodies from the endosomal compartment [58].

In Table 4, advantage and limitations of CTCs, cfDNA and exosomes are summarized. A major problem is the lack of standardized and convenient techniques to analyse CTCs, cfDNA and exosomes [57]. In addition, nearly all the clinical studies testing the utility of liquid biopsies have been retrospective, while larger and prospective studies are warranted.

Table 4: Comparison of three liquid biopsy methods (CTCs, cfDNA and exosomes).
CTCs: circulating tumour cells; cfDNA: circulating free DNA; BEAMing: beads, emulsion, amplification, magnetics; NGS: next generation sequencing. Reproduced from Zhang W et al. [57] with permission from S. Karger AG (Basel, Switzerland) © 2017. All rights reserved.

	Source	Enrich or analytic technique	Strength	Limitation
CTCs	Peripheral blood	Label dependent (e.g. CELLSEARCH)	Noninvasive	Impact of heterogeneity on various CTC enrichment methods
			High specificity of tumor-derived	Controversy of guiding therapeutic decision making
			Ex vivo functional studies available	Instability of monitoring early stage cancer
		Label independent (e.g. ISET system, ScreenCell)	Approved by FDA in clinical practice (CELLSEARCH)	
			Non-coding RNA, DNA and protein all could be evaluated	
cfDNA	Serum or plasma	PCR(e.g. BEAMing, digital PCR, Amplicon sequencing)	Noninvasive	Difficult to determine cancer-specific aberration
			High sensitivity	Superabundant data from NGS cause it's difficult to distinguish background noise from true tumour associated aberrations
		NGS	Specific gene aberration direct guide therapeutic decision making (approved by large clinical trials)	No RNA or protein could be evaluated
Exosomes	Plasma and other body fluids (ex. urine, saliva, ascites)	Ultracentrifugation	Noninvasive (available in multiple body fluids)	tumor derived exosomes lack specific marker
		Immunoaffinity methods	Ex vivo functional studies available	Impact of heterogeneity on various exosomes enrichment methods
		Chromatography	Non-coding RNA, DNA and protein all could be evalutaed	Lack of large clinical trials to validate its reliability in clinical practice
		ExoQuick polymer		

Extracellular vesicles and microRNAs

Among molecules that can be analysed through liquid biopsies, extracellular vesicles and microRNAs (miRNAs) may be a promising tool for cancer diagnosis, assessment of prognosis and early detection of disease recurrence [59]. MiRNAs are short, single-stranded non-coding RNA transcripts of 21-23 nucleotides that are acting as post-transcriptional regulators [60]. MiRNAs are often associated with tumour development, progression and response to treatment [60]. The abnormal expression of miRNAs in malignant cells is often attributed to alterations in copy numbers and gene locations, with frequent mechanisms of amplification, deletion or translocation [61]. Other possible reasons for an abnormal miRNA expression pattern are the dysregulation of key transcription

factors such as c-Myc and p-53 as well as alterations in epigenetic changes (hypomethylation, hypermethylation or histone modifications) [61]. Moreover, miRNA biogenesis machinery can be defective, with aberrant expression of enzymes and proteins that take part in the process of miRNA maturation from miRNA precursors [61]. Depending on their target genes, miRNAs can act either as oncogenes (onco-miRs) or as tumour suppressors [61]. They can be responsible for evading growth suppressors, sustaining proliferative signalling, resisting cell death, activating invasion and metastasis, inducing angiogenesis and regulating tumour microenvironment [61]. They also have a role in drug metabolism, DNA repair mechanisms, epithelial-mesenchymal transformation and cancer cell stemness [62,63].

Extracellular vesicle -based liquid biopsies reflect the physiological or pathological state of the secreting cells [59]. Extracellular vesicles can be found in nearly all body fluids, e.g. plasma, urine, saliva, ascites and bronchoalveolar lavage fluid. They are released into the extracellular environment after a fusion between these bodies and plasma membrane [57]. RNA in vesicles is protected from RNase degradation and keeps its stability regardless of different temperatures and pH conditions [64]. As far as extracellular vesicles are secreted from cancer tissue they contain proteins and miRNAs typical for the cancer cells of origin [59].

Extracellular vesicles collected from heterogeneous cancers reflect the dynamic changes during disease progression if collected at different time points during the disease [59]. Furthermore, extracellular vesicles have been identified as part of the intracellular communication system that deliver cancer pathogenic components not only among cells in the primary tumour microenvironment but also between primary tumour and metastatic sites [59]. Tumour specific proteins and miRNAs included into extracellular vesicles act in cells that are able to take up the vesicles [59]. Therefore tumour derived extracellular vesicles can prime new metastatic sites [65] and they are known to be absolute necessary for triggering the breakdown of blood-brain barrier during the process of brain metastasis [66].

Liquid biopsies and colorectal cancer

Liquid biopsies could have a relevant role in the management of CRC at different stages of the disease. Indeed, analysis of cfDNA, CTCs and miRNAs can be relevant for early diagnosis of CRC, identification of minimal residual disease and estimation of recurrence risk [67]. Moreover, in advanced disease, liquid biopsies allow the monitoring of the response to treatment and evaluation of clonal evolution of the disease, with the possibility to identify new molecular alterations resulting in drug resistance and disease progression [67]. In the early stage of disease, the presence of background signals limits the possibility to use identification of somatic mutations in cfDNA as a unique approach for diagnosis [67]. For this reason, combination of new and different biomarkers is warranted in this setting of disease [67]. The evaluation of both CTCs and cfDNA has shown promising results for detection of minimal residual disease after radical surgery and/or adjuvant chemotherapy, with analysis of somatic mutations in cfDNA and search of driver mutations by NGS analysis of the primary tumour tissue as two feasible approaches [67]. As far as advanced disease is concerned, cfDNA might provide information about the molecular evolution of disease, while CTCs might have the advantage to describe tumour heterogeneity on a single-cell basis [67]. However, despite the high number of studies and all these potential applications, the use of liquid biopsies in CRC has not entered yet into clinical practice [67].

miRNAs and colorectal cancer

As outlined before, miRNAs may act as oncomiRs or tumour suppressors in CRC [1]. In the context of CRC, miRNAs are mainly involved in 4 pathogenic pathways: Wnt/ β catenin activation, EGFR pathway, transforming growth factor β (TGF-β) and *TP53* deletion [1]. Wnt/β-catenin pathway is constitutively activated in sporadic CRC, and may lead to tumour progression [1]. Activation of EGFR pathway with *KRAS* mutations (30-60% of cases) and *BRAF* mutations (15% of cases) has a

role in cell proliferation, adhesion, cytoskeletal integrity and apoptosis [1]. *PI3KCA* mutations occur in 15-20% of cases and lead to increased cell proliferation and tumour development [1]. One negative regulator of PIK3/AKT signalling is *PTEN*, often mutated or deleted in CRC [1]. TGF-β binds to its type II receptor TGFBR2, with subsequent phosphorylation and activation of type I receptor [1]. In 30% of cases, mutations in TGFBR2 lead to activation of the pathway [1]. Deletion and mutation of the onco-suppressor transcription factor p53 (45% of cases) is another pathogenic finding in CRC [1]. The most studied miRNA in CRC is miR-21, which is often over-expressed in CRC [60]. It acts *via* lowering the expression of suppressor genes, such as *PTEN* in the PI3K/AKT signalling pathway, altering proliferation, adhesion, angiogenesis, migration, metabolism and apoptosis functions [60]. The miR-29 family, including miR-29a, miR-29b and miR-29c, is also often deregulated in CRC [1] and this miRNA family influence cell proliferation, senescence and tumour metastasis [1]. MiR-29b, for example, acts as a negative regulator of the Wnt/β-catenin signalling, directly targeting and repressing GSK3B expression [1]. The MiR-34 family (miR-34a, miR-34b and miR-34c) is also involved in CRC differentiation, drug resistance and metastatic spread [60]. These miRNAs have suppressive properties and are regulated both by p53 protein and epigenetic modifications such as hyper-methylation [60]. MiR-124 acts by down-regulating rho-associated protein kinase 1 (ROCK1), acting as an oncogene, but also inhibits the activity of cyclin-dependent kinase 4 (CDK4), which is responsible for cell cycle progression in the G1/S cellular phase [60]. In colon cells miR-130b is targeted by PPARϒ receptor, which inhibition regulates the expression of cadherin E, VEGF and *PTEN* [60]. Mir-130b is over-expressed in CRC and miR-130b-PPARϒ interaction plays a significant role in increasing tumour malignancy [60].

In figure 1, major relations between miRNAs and these pathways are shown [1].

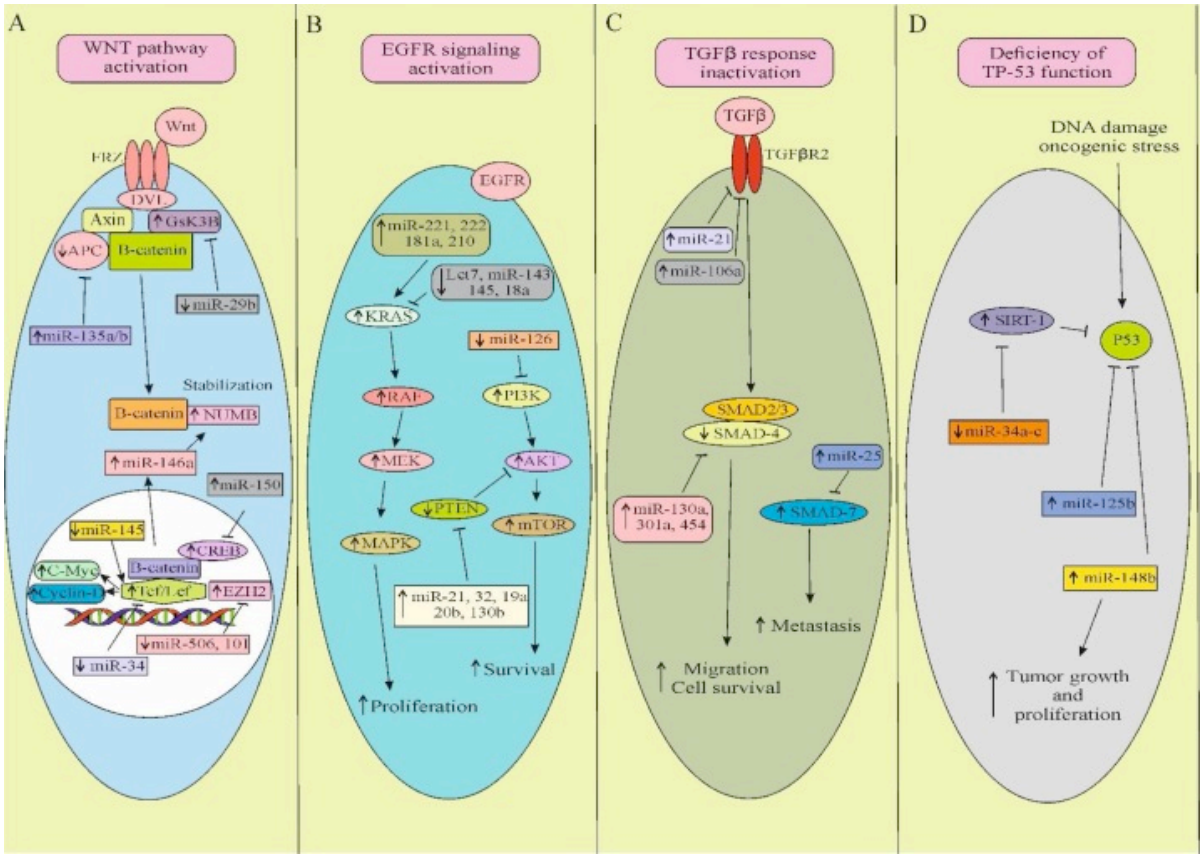


Figure 1. Overview of major signalling pathways affected by miRNAs in CRC. Wnt/ β-catenin, EGFR and TGF βR2 pathways are activated, while *p53* function is deactivated.
APC: Adenomatous polyposis coli; GSK3B: Glycogen synthase kinase 3 beta; NUMB: A protein that controls ß-catenin stability; CREB: c-AMP response element-binding protein; EZH2: Enhancer of zeste homolog 2; *EGFR*: Epidermal growth factor receptor; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *PI3K*: Phosphoinositide 3-kinase; *AKT*: Serine/threonine-specific protein kinase; *PTEN*: Phosphatase and tensin homolog; TGFβR2: Transforming growth factor beta receptor II; SIRT-1: Sirtuin 1. Down-regulation: ↓; Up-regulation: ↑). Reproduced from Shirafkan N et al. [1] with permission from Elsevier Masson SAS (Issy-les-Molineaux, France) © 2018. All rights reserved.

Due to the fact that the clinical markers in use (CEA and CA19.9) are not robust and furthermore not specific for colorectal cancer [59,68,69], there is an urgent need to establish other more reliable marker for detection and monitoring this disease. In the here presented work, the prognostic and predictive role of liquid biopsies in advanced CRC has been evaluated.

MATERIALS AND METHODS

Design of the study

This was a prospective, observational, multicentre study. Enrolment of patients started on February 2016 and clinical evaluation of patients stopped in October 2018. Two Hospitals were involved: Hospital of Cremona, ASST of Cremona and Hospital of Oglio Po-Casalmaggiore, ASST of Cremona (both located in Cremona, Italy). The time frame of this work is outlined in Figure 2.

Action	First year	Second year	Third year
Enrolment of patients			
Clinical data acquisition			
Molecular analyses			
Statistical elaboration			
Results reporting			

Figure 2. Gannt chart. Study timepoints.
Study started after approval by Ethical Committee in February 2016.

Aims of the study

The primary endpoint was to monitor cfDNA mutations that are developing during the course of anti-tumoural treatment with targeted agents (bevacizumab, cetuximab and panitumumab) in CRC patients.

As secondary endpoints, we considered the correlation between circulating molecular alterations and clinical development of resistance to the targeted agents. Moreover, another secondary

endpoint was the evaluation of early molecular alterations anticipating and predicting clinical development of resistance to targeted agents. Furthermore, changes in miRNA expression levels associated with response or resistance to targeted agents (bevacizumab, cetuximab and panitumumab) were evaluated.

Study cohorts

Patients affected by advanced CRC receiving a treatment with anti-EGFR agents (cetuximab/panitumumab, cohort 1) or bevacizumab (cohort 2) in association with chemotherapy or as single agents were included into the study. Both patients with first diagnosis of metastatic disease and cases relapsing after curative surgery were considered. First, second and third line treatment with biological agents targeting EGFR or VEGF were admitted.

Ethical aspects

The study protocol was approved by the Ethical Committee for the territory Cremona, Mantova and Lodi areas on the session of 11 December 2015. Resolution was signed by the General Director of ASST of Cremona (Cremona, Italy) on 4 February 2016 (number 38, protocol number 30769/15).
All patients included into the protocol signed an informed consent accepting to donate blood, urine, and available tissue samples (collected for clinical purposes before the protocol start). The protocol did not allow obtaining tissue samples for research purposes only (if not available from pathology archives).
During the study, all principles of good clinical practice and statements included into the Declaration of Helsinki have been taken into account.

Samples collection

Blood and urine samples were collected from the two hospitals in Cremona, Italy (Hospital of Cremona, ASST Of Cremona and Hospital of Oglio Po-Casalmaggiore, ASST of Cremona). Molecular analyses were performed in the Gastrointestinal Cancer Biology and Genomic Group at the Centre for Molecular Pathology of The Institute of Cancer Research (Sutton,UK).

Patients included were required to provide a blood draw of 30 ml for serum, plasma and buffy coat isolation. Moreover, a sample of urine (10 ml) was required. Samples were collected before the beginning of treatment, every four weeks during treatment, within ten days of radiological evaluation of disease (assessed with computed tomography [CT] scan), at radiological progression of disease (PD, assessed with CT scan) and between one and two months after progression (Figure 3).

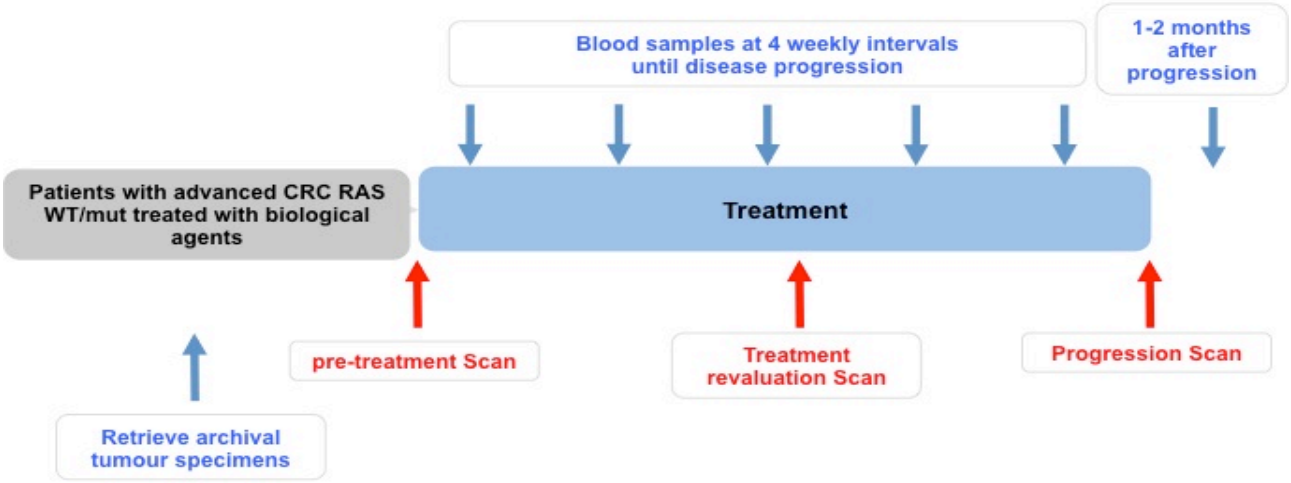


Figure 3. Time-points for samples collection
Samples were collected every four weeks from the beginning of treatment until one-two months after progression of disease. CRC: colorectal cancer WT: wild-type; mut: mutated; Scan: computed tomography scan.

Inclusion criteria

Patients have been eligible for this study if they have been an age equal or superior to 18, signed written informed consent and had histological diagnosis of colorectal adenocarcinoma and clinical diagnosis of advanced disease. They received chemotherapy in combination with biological agents (bevacizumab as anti-VEGF treatment, cetuximab or panitumumab as anti-EGFR treatment) in first, second and third line treatment.

Exclusion criteria

Patients were excluded in case of diagnosis of previous infiltrating tumour within two years from the diagnosis of CRC (patients were admitted only in case of previous cervical pre-malignancies and basal cell carcinomas). Moreover, patients included in other trials involving administration of new drugs were not admitted. The minimum permitted washout period for patients included in previous studies was 14 days.

Blood and urine samples

At baseline, every four weeks during chemotherapy, at radiological progression and between one and two months after progression, the following samples were collected: 1 x 5 ml mL blue top vacutainer tube (BD Vacutainer SST® II Advance, Becton Dickinson, Franklin Lakes, New Jersey, US) for serum; 4 x 6 mL EDTA tubes pink top vacutainer (BD Vacutainer KZE®, Becton Dickinson, Franklin Lakes, New Jersey, US) for plasma and germline DNA extraction. In addition, 10 mL urine was collected at the same timepoints.

Sample processing

After blood collection, serum was isolated from the blue top tube (BD Vacutainer SST® II Advance, Becton Dickinson, Franklin Lakes, New Jersey, US). Following incubation at room temperature for 20 minutes, the tube was inserted into the centrifuge (Eppendorf 5702 R®, Hamburg, Germany) and spinned at 4000 RPM at room temperature for 15 minutes. The obtained supernatant was transferred into labelled 1.8 mL cryovials (Cryopure 1.8 mL white, Sarsted, Numbrecht, Germany) using a plastic pipette (Eppendorf Research Plus®, Hamburg, Germany). If not processed at once samples were stored immediately in a -80 °C freezer.

For plasma was isolated from the EDTA pink top tube (BD Vacutainer KZE®, Becton Dickinson, Franklin Lakes, New Jersey, US). First the tube was inverted 8-10 times to ensure a correct mixing with the EDTA solution. Following that, samples were centrifuged at once with 3000 RPM at 4 degrees for 10 minutes. Then, plasma was transferred into labelled 1.8 mL cryovials (Cryopure 1.8 mL white, Sarsted, Numbrecht, Germany) using a plastic pipette (Eppendorf Research Plus®, Hamburg, Germany) without aspirating the buffy coat layer lying beneath the plasma. Cryovials and the EDTA tubes containing the cell pellets were immediately placed in a -80 °C freezer.

Urine was collected and then transferred into labelled 1.8 mL cryovials (Cryopure 1.8 mL white, Sarsted, Numbrecht, Germany) using a plastic pipette (Eppendorf Research Plus®, Hamburg, Germany). Samples were immediately transferred in a -80 °C freezer.

Extraction of cell-free DNA (cfDNA) from plasma

Extraction of cfDNA from plasma was performed using the QIAamp Circulating Nucleic Acid Kit® (Qiagen, Hilden, Germany). The protocol is composed of four steps (lyse, bind, wash, elute) and is similar for plasma and urine. During the first step, samples are added into centrifuge tubes together with proteinase K (100 µL for 1 mL of plasma). The solution is mixed and incubated in a water bath

(Thermo Fisher Scientific, Waltham, MA, USA) at 60 °C for 30 minutes. The obtained lysate is mixed with 1.8 mL of buffer ACB (Qiagen, Hilden, Germany). This mixture is incubated for five minutes on ice and then transferred onto columns that are inserted in QIAvac 24 Plus vacuum manifold (Qiagen, Hilden, Germany) for the binding phase. A vacuum pump (QIAvac vacuum pump, Qiagen, Hilden, Germany) is used to drawn the lysates through the columns. Then, two different buffers (600 µL of buffer ACW1 and 750 µL of ACW2; both Qiagen, Hilden, Germany) and finally 750 µL 96-100% ethanol (Merck, Darmstadt, Germany) are used for the washing phase. Once again, liquids are drawn through the columns using the QIAvac vacuum pump (Qiagen, Hilden, Germany). Finally, cfDNAs are eluted using 20-150 µL of AVE buffer (Qiagen, Hilden, Germany) by centrifugation at 14000 rpm for one minute in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA). If the cfDNAs are not used at once in down-stream analysis samples were stored immediately in a -20 °C freezer.

Extraction of miRNAs from plasma

Extraction of miRNAs from plasma was performed using the miRNeasy Mini Kit® (Qiagen, Hilden, Germany). A sample fraction of 100 µL of plasma is mixed with 500 µL QIAzol Lysis Reagent (Qiagen, Hilden, Germany) in order to lysate the plasma cells. After that, an incubation of five minutes at room temperature is required. A quantity of 3.5 µL of MiRNeasy Plasma Spike-in control (Qiagen, Hilden, Germany) and 100 µL of chloroform (Merck, Darmstadt, Germany) are then added to the lysate. After an incubation period of two-three minutes at room temperature, the lysate is centrifuged in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for 15 minutes at 14000 rpm at 4 °C. Centrifugation separates the lysate into aqueous and organic phases. The RNA is located in the upper aqueous phase. This upper phase is extracted (300 µL) and mixed with 450 µL of 100% ethanol (Merck, Darmstadt, Germany), allowing appropriate binding conditions for all RNA molecules, including small RNAs. A sample fraction of 700 µL is then applied to the

RNeasy MinElute spin column (Qiagen, Hilden, Germany), where the total RNA binds to the membrane during a centrifugation step in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) at 10000 rpm for 15 seconds at room temperature. The flow-through is discarded. The same process is repeated twice – first after addition of 700 µL of RWT buffer (Qiagen, Hilden, Germany) and then after addition of 500 µL of RPE buffer (Qiagen, Hilden, Germany). Then, a centrifugation step in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) (10000 rpm for 2 minutes) is performed after addition of 500 µL of 80% ethanol (Merck, Darmstadt, Germany). The RNeasy MinElute spin column (Qiagen, Hilden, Germany) is placed into a new collection tube, with a centrifugation at full speed for 5 minutes to dry the membrane. The purified RNA is then eluted in 30 µL RNAse-free water (Qiagen, Hilden, Germany) during a final centrifugation step in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for one minute at maximum speed.

Exosomes isolation from plasma/serum and urine

In order to obtain exosomes from plasma samples, the miRCURY® Exosomes Isolation Kit- Serum and Plasma (Exiqon, Vedbaek, Denmark) for serum and plasma was used. Samples (0.5 µL) are mixed with 6 µL thrombin (Exiqon, Vedbaek, Denmark) and then incubated for five minutes at room temperature. Following that, a centrifugation step in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for five minutes at 14000 rpm is performed in order to remove cell debris. The supernatant (0.5 mL) is mixed with 200 µL buffer A (Exiqon, Vedbaek, Denmark) and incubated for 60 minutes at 4 °C. After that, centrifugation at room temperature for 30 minutes is performed at 3660 rpm in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA). Supernatant is then discarded and the obtained pellet is resuspended in 300 µL resuspension buffer provided in the isolation kit. .

For exosomes isolation from urine, the miRCURY® Exosomes Isolation Kit for Cells, Urine and CSF was used (Exiqon, Vedbaek, Denmark). At the beginning, the 10 mL sample is centrifuged

(Allegra X-15R®, Beckman Coulter, Brea, California, US). for five minutes at 5400 rpm in a 15 mL conical tube (Corning, NY, USA) in order to remove debris. Then the supernatant is transferred into a new conical tube and 4 mL of precipitation buffer B (Exiqon, Vedbaek, Denmark) is added. The solution is incubated for 60 minutes at 4 °C and subsequent centrifuged at 5600 rpm for 30 minutes at 20 °C (Allegra X-15R®, Beckman Coulter, Brea, California, US). Supernatant is then discarded and the pellet is resuspended in 300 µL resuspension buffer provided in the isolation kit. After resuspension of the isolated exosomes RNA extraction was performed at once.

RNA isolation from exosomes

For the extraction of RNA from exosomes obtained from plasma, miRCURY® RNA isolation kit-Biofluids (BF) was used (Exiqon, Vedbaek, Denmark). A quantity of 300 µL of the resuspended exosome solution was transferred to a new tube (Eppendorf, Hamburg, Germany). After adding 60 µL of lysis solution BF (Exiqon, Vedbaek, Denmark) the solution was incubated for three minutes at room temperature. For the following protein precipitation step, 20 µL of protein precipitation solution BF (Exiqon, Vedbaek, Denmark) was added to the sample and, after an incubation of one minute at room temperature, centrifugation for three minutes at 10000 rpm is performed in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA). The supernatant is then transferred to a new 2 mL collection tube (Eppendorf, Hamburg, Germany) with subsequent addition of isopropanol (Merck, Darmstadt, Germany), Then, a microRNA Mini Spin Column BF (Exiqon, Vedbaek, Denmark) is put in a collection tube and the processed sample is load onto the column. Following incubation for two minutes at room temperature the sample was centrifuged in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for 30 seconds at 10000 rpm. The flow-through was discarded and the column was placed into another collection tube. Three steps of wash and dry followed: firstly, 100 µL of wash solution 1 BF was added to the microRNA spin column BF (Exiqon, Vedbaek, Denmark) with a centrifugation in a table centrifuge (MicroStar 17, VWR,

Radnor, PA, USA) for 30 seconds at 10000 rpm, secondly 700 µL of wash solution 2 BF (Exiqon, Vedbaek, Denmark) was added to the spin column with a centrifugation in the same centrifuge for 30 seconds at 10000 rpm, thirdly after addition of 250 µL wash solution 2 BF (Exiqon, Vedbaek, Denmark) to the spin column a final centrifugation in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) at 10000 rpm for two minutes was performed to dry completely the membrane. Finally, the spin column was placed in a new collection tube and 50 µL of RNase free water was put directly onto the membrane of the column. After incubation for one minute at room temperature, a centrifugation in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for one minute at 10000 rpm was required for extracting the purified RNA from the column.

For the extraction of RNA from exosomes derived from urine, the miRCURY® RNA Isolation Kit – Cell and Plant was used (Exiqon, Vedbaek, Denmark). First of all, 350 µL of lysis solution (Exiqon, Vedbaek, Denmark) was added to 300 µL of the resuspended exosome solution. After mixing, 200 µL of 100% ethanol (Merck, Darmstadt, Germany) was added to the solution. After assembling a column with a collection tube, up to 600 µL of the lysate with ethanol was applied onto a column. Centrifugation in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for one minute at 5600 rpm was required. After all the lysate has passed through the column, the wash step was started. A quantity 400 µL wash solution (Exiqon, Vedbaek, Denmark) was applied to the column followed by centrifugation in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for one minute at 14000 rpm. The washing step was repeated once again and then the column was centrifuged in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) for two minutes at 14000 rpm to dry the sample. In order to elute the RNA, the column was placed into a elution tube, 50 µL of elution buffer (Exiqon, Vedbaek, Denmark) was added to the column and centrifugation in a table centrifuge (MicroStar 17, VWR, Radnor, PA, USA) was performed, (two minutes at 500 rpm followed by one minute at 14000 rpm).

Digital droplet polymerase chain reaction (ddPCR)

MiRNAs expression and cfDNA analysis was performed by using BioRad® ddPCR technology (QX200 system; BioRad, Hercules, CA, USA). It is a third generation PCR with higher sensitivity compared to classic quantitative PCR (sensitivity 0.01% compared to 1%, respectively, as illustrated in Table 3) [54]. DdPCR technique allows obtaining an absolute quantification of target DNA copies per input sample without the need of housekeeping genes. Moreover, it can be used to quantify expression levels of miRNAs, detect rare sequences and genomic alterations such as copy number variations.

In ddPCR, a single PCR sample is partitioned in 20.000 discrete droplets. Before droplet generation, ddPCR reactions are prepared in the same manner as real-time PCR (Hindson CM, Nat Methods 2013). In the frame of this work, TaqMan probes labelled with reporter fluorophore FAM were used for ddPCR. With the help of a droplet generator (BioRad, Hercules, CA, USA) each sample is partitioned into 20.000 distinct nanoliter-sized droplets, uniform in size and volume. Inside each droplet, input nucleic acid, reagents for the PCR reaction and target specific probes are present, so that in each droplet a separate PCR reaction can be performed. Droplets are then transferred into a 96-well plate (twin.tec plates; Eppendorf, Hamburg. Germany) and after sealing the plate with an alufoil PCR amplification of the nucleic acid target using a thermal cycler (C-1000 Touch; BioRad, Hercules, CA, USA) is performed (1 cycle of 95^0C for 10 min, 40 cycles of 94^0C for 30 sec and 60^0C for 1min and 1 cycle of 98^0C for 10 min). Then, droplets are ready to be read with the droplet reader (BioRad, Hercules, CA, USA), which analyses each droplet individually using a two-colour detection system (Figure 4).

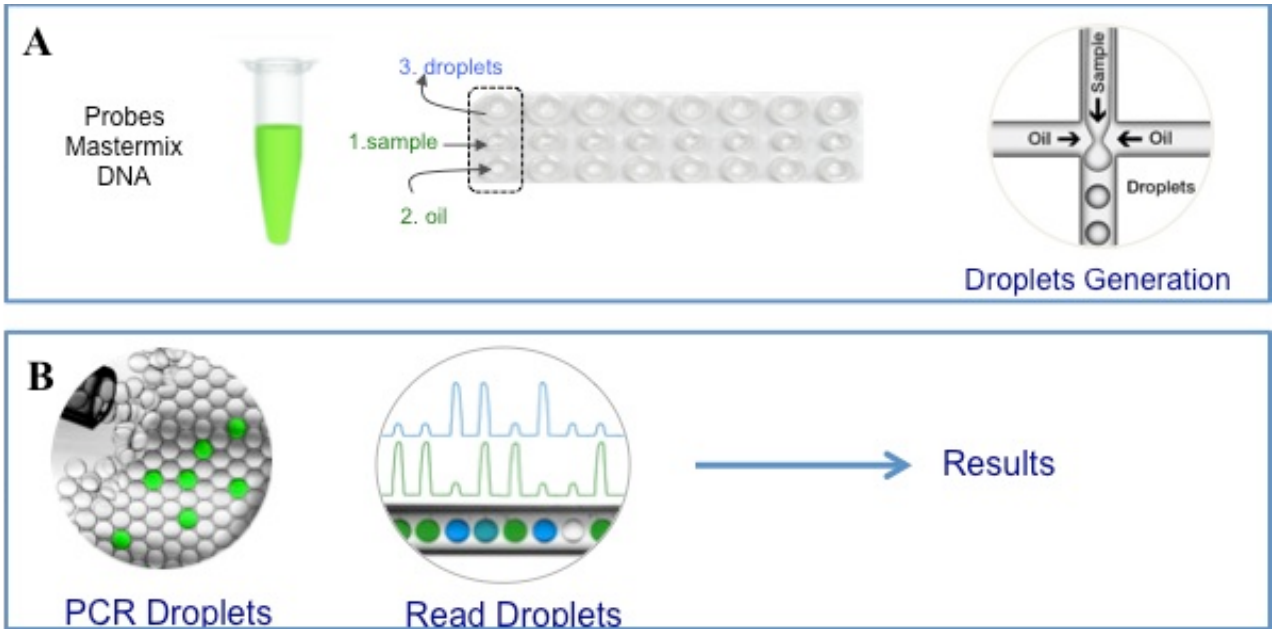


Figure 4. Preparation of droplets and analysis. Courtesy of Dr. Nicola Valeri (unpublished).
A: droplets are generated into a droplet generator using oil in order to partition the sample (made of probes, Mastermix and DNA) into 20.000 distinct nanoliter-sized droplets; B: droplets are read with a droplet reader to obtain results.

In the frame of this work, ddPCR was used for single-target evaluation (either a single miRNA expression analysis in patients receiving anti-EGFR treatment or tracking of a specific cfDNA mutation in patients treated with bevacizumab). Results were summarized into 1-D plot, with report of fluorescence intensity *vs* droplets number. All positive droplets were shown to be above a pre-arranged threshold (determined by relevant negative controls), while negative droplets were below the threshold.

Next generation sequencing (NGS)

NGS is a profiling assay that allows evaluation of multiple genomic aberrations at the same time using DNA isolated from tissue or liquid biopsy sample. For this project, AVENIO® (Roche, Basel, Switzerland) ctDNA expanded kit was used. The used panel included 77 genes (AVENIO® ctDNA Expanded Kit)

NGS analysis was used to test for four mutation classes: single nucleotide variants (SNV),

insertions and deletions (indel), fusions and copy number variations (CNV). An amount from 10 to 60 ng of cfDNA was used to obtain data. Sensitivity of the technique is above 99% and the same is for positive predictive value; only in case of CNV, sensitivity is slightly lower (but superior to 96%).

After separation of plasma from blood, with subsequent extraction and isolation of cfDNA (day 1), the sequencing library is prepared. CfDNA is linked to sample adapters and a cleaning up phase post ligation is required (days 1 and 2). Modified cfDNA is then ready for PCR amplification. Finally, target enrichment is performed with enhancement oligos placed on target DNA (days 2 and 3). Then, biotinylated probes are hybridized to the target enriched DNA. These biotinylated probes bind to streptavidin-coated magnetic beads. In this way, targets are captured with a magnet. After a washing and elution phase, the amplification phase starts. Amplified targets are then ready to be sequenced (day four) and evaluated with a bioinformatic analysis.

For this project, NGS analysis was performed on some samples of interest in order to evaluate the presence of “driver” mutation linked to radiological disease progression during treatment for advanced CRC.

RESULTS

From February 2016 to October 2018, 63 patients affected by advanced CRC were included in this study. The patients were divided into two cohorts: 26 patients were treated with anti-EGFR therapy (cetuximab of panitumumab) (cohort 1), while 37 patients received anti-angiogenetic therapy based on bevacizumab (cohort 2). Patients treated for less than a month were excluded from the analysis.

Clinical variables considered for the analysis were: age at diagnosis of metastatic disease, sex, comorbidities, previous treatments (surgery, chemotherapy, radiotherapy for non-metastatic disease), start and finish date of the treatment during which liquid biopsies were collected, including induction and maintenance phase. In addition, reasons for temporary suspensions of the treatment, reintroduction of treatment and progression (date and localization of metastases) were considered. Date of progression, date of death or last follow-up and potential treatment administered after progression were also considered. Values of tumour markers CEA and CA 19.9 were determined before start and during treatment until progression. All CT scans performed during treatment were assessed for target tumour lesions (longest diameters for each lesion). Each patient was classified according to Response evaluation criteria in solid tumours (RECIST criteria 1.1) as partial responder (PR) (reduction $\geq$ 30% in the sum of the longest diameters of target lesions), progressor (increase $\geq$ 20% in the sum of longest diameters, progressive disease [PD]), complete responder (CR) (disappearance of all target lesions) or with stable disease (SD) (neither partial response nor progressive disease) [70].

Survival variables of interest were median progression free survival (mPFS), considered as the treatment duration from start to PD, and median overall survival (mOS), namely the time between disease diagnosis and death or last follow-up. In case of no progression or death observed, mPFS and mOS were censored at the time of last follow-up, corresponding to last clinical assessment.

Among patients treated with cetuximab or panitumumab, 23 out of 28 had anti-EGFR as first-line

treatment, while the remaining received it in third line treatment (no patients were treated in second line treatment with anti-EGFR). All patients had *RAS* wild-type genotype, none of them had *BRAF* mutation.

In the second cohort, all patients received bevacizumab as first-line treatment. Three of them harboured *BRAF* mutation, while all the others presented a *RAS* mutation except for one patient with *RAS* wild-type profile.

Sixteen patients from the whole cohort had surgery for resectable metastatic disease during the course of treatment; ten of them had been under treatment with bevacizumab and six patients who received anti-EGFR treatment.

Table 5 summarises the main features of the two cohorts. Patients treated with cetuximab/panitumumab were included into cohort 1, while patients treated with bevacizumab formed cohort 2.

Table 5. Median age, survival outcomes and response rates of cohort 1 and 2. Patients treated with cetuximab/panitumumab were included into cohort 1, while patients treated with bevacizumab formed cohort 2.
mOS: median overall survival, mPFS: median progression-free survival, PR: partial response, CR: complete response, SD: stable disease, PD: progressive disease.

	Cohort 1	**Cohort 2**
Median age (years)	66	69
Age range (years)	48-81	50-82
Sex (%male / % female)	57/43	52/48
mOS(months)	18.9	15
mPFS(months)	39.7	22.9
PR (%)	23.1	33.4
CR (%)	15.4	10.9
SD (%)	42.3	42.2
PD (%)	19.2	13.5

A statistically significant difference was found in mOS between the two cohorts (18.9 months for cohort 1 *vs* 15 months for cohort 2, p=0.0362, figure 5).

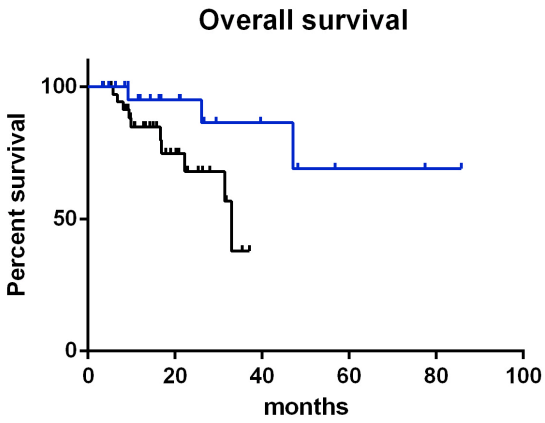


Figure 5: Median overall survival (mOS) comparison. mOS of cohort 1 was significantly longer than mOS of cohort 2.
Blue line: cohort 1 Black line: cohort 2

On the contrary, no statistical significant difference between the cohorts was noticed in regard to mPFS (39.7 *vs* 22.9 months, p=0.2, figure 6).

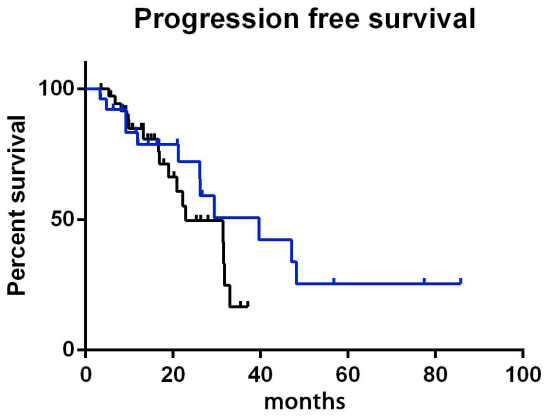


Figure 6: Median progression free survival (mPFS) comparison. No difference in mPFS was found between the two groups
Blue line: cohort 1 Black line: cohort 2

Liquid biopsies of patients treated with cetuximab/panitumumab (cohort 1)

Preclinical phase

This experimental preclinical phase was performed before the beginning of this PhD project and is reported because it provided the rational for the following clinical phase. All the content reported in this section refers to unpublished results (J.C. Hahne and N. Valeri, personal communication).

In order to detect miRNA possibly associated with development of resistance to anti-EGFR treatment, Ncounter® miRNA expression assay was performed on exosomes derived from cells harbouring *APC* and *PTEN* deletion (sensitive to anti-EGFR) after exposition to cetuximab (J.C. Hahne, N. Valeri, unpublished, personal communication). Ninety-five miRNAs were found to be significantly deregulated: 63 up-regulated and 32 down-regulated. Among them, 18 were involved in apoptosis and autophagy, 22 in proliferation and three in chemoresistance (J.C. Hahne, N. Valeri, unpublished, personal communication, Table 6 and 7).

Table 6. Up-regulated miRNAs. Adapted from Dr. J.C. Hahne and Dr. N. Valeri (personal communication, unpublished). Let-7g-5p was the highest up-regulated miRNAs. Adj.P.Val: $1.1849 * 10^{-9}$.

microRNA_ID	log2_FoldChange	adj.P.Val
hsa-let-7g-5p	**7.09**	**1.1849E-09**
hsa-miR-26a-5p	6.02	6.40161E-07
hsa-miR-378i	2.59	6.40161E-07
hsa-miR-483-3p	2.23	7.29487E-07
hsa-miR-205-5p	4.79	8.59425E-07
hsa-let-7d-5p	5.95	9.43937E-07
hsa-miR-107	3.82	1.6376E-06
hsa-miR-320e	2.83	1.9008E-06
hsa-miR-148b-3p	3.66	1.94944E-06
hsa-miR-92a-3p	3.52	1.94944E-06
hsa-miR-98-5p	4.47	2.12223E-06
hsa-miR-301a-3p	3.18	6.15195E-06
hsa-miR-30e-5p	4.43	1.22956E-05
hsa-miR-374b-5p	2.83	1.22956E-05
hsa-miR-30e-5p	4.43	1.22956E-05
hsa-miR-374b-5p	2.83	1.22956E-05
hsa-miR-425-5p	2.43	2.88985E-05
hsa-miR-30c-5p	4.28	3.86442E-05
hsa-miR-132-3p	1.66	5.72173E-05
hsa-miR-361-5p	2.08	6.77419E-05
hsa-let-7f-5p	6.57	2.60683E-09
hsa-miR-183-5p	5.18	5.5067E-09
hsa-miR-194-5p	5.43	5.5067E-09
hsa-miR-23b-3p	5.27	5.5067E-09
hsa-miR-26b-5p	5.93	5.5067E-09
hsa-miR-27b-3p	6.28	5.5067E-09
hsa-miR-296-5p	5.00	5.5067E-09
hsa-miR-30d-5p	5.06	5.5067E-09
hsa-miR-34a-5p	5.71	5.5067E-09
hsa-miR-99a-5p	4.70	5.5067E-09
hsa-miR-99b-5p	4.52	5.5067E-09
hsa-miR-15a-5p	5.23	8.09006E-09
hsa-miR-203a-3p	4.32	1.84729E-08

hsa-miR-29c-3p	4.30	1.84729E-08
hsa-miR-125a-5p	5.12	2.06124E-08
hsa-miR-342-3p	4.14	2.06124E-08
hsa-miR-19b-3p	5.45	2.23989E-08
hsa-miR-24-3p	4.28	2.58243E-08
hsa-let-7e-5p	5.04	2.79128E-08
hsa-miR-31-5p	3.79	2.79128E-08
hsa-miR-106b-5p	5.33	3.08518E-08
hsa-miR-20a-5p+hsa-miR-20b-5p	4.97	4.0512E-08
hsa-miR-30a-5p	3.75	7.38437E-08
hsa-miR-32-5p	3.50	1.25942E-07
hsa-miR-18a-5p	2.88	3.57639E-07
hsa-miR-1206	3.34	4.27118E-07

Table 7. Down-regulated miRNAs. Adapted from Dr. J.C. Hahne and Dr. N. Valeri (personal communication, unpublished). MiR-6724-5p was the major down-regulated miRNAs. Adj.P.Val: 1.20184 * 10^{-6}.

microRNA_ID	log2_FoldChange	adj.P.Val
hsa-miR-6724-5p	**-5.26**	**1.20184E-06**
hsa-miR-379-5p	-4.22	3.29798E-06
hsa-miR-2682-5p	-3.91	9.80373E-06
hsa-miR-640	-3.52	1.20442E-05
hsa-miR-561-5p	-3.66	2.87466E-05
hsa-miR-4443	-4.37	3.22559E-05
hsa-miR-520d-3p	-3.86	3.32979E-05
hsa-miR-1246	-4.34	3.58719E-05
hsa-miR-4454+hsa-miR-7975	-2.23	4.62661E-05
hsa-miR-564	-4.18	6.5884E-05
hsa-miR-575	-4.18	6.77419E-05
hsa-miR-2682-5p	-3.91	9.80373E-06
hsa-miR-574-5p	-3.90	1.34435E-07

The expression level of the highest over-expressed miRNA - let-7g-5p (Table 6) was verified by ddPCR in cells and exosomes after exposure to increasing concentration of cetuximab.

When exposed to cetuximab, let-7g-5p expression was higher in cells sensible to anti-EGFR (Figure 7A) compared to resistant cells (Figure 7B) (J.C. Hahne, N. Valeri, unpublished, personal communication).

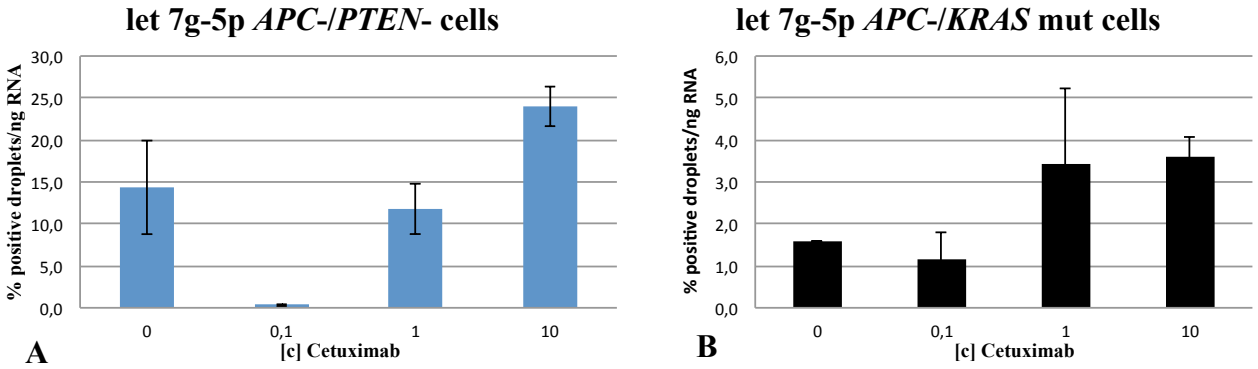


Figure 7. Percentage of positive droplets of let-7g-5p in *APC-/PTEN-* cells (sensitive to cetuximab) (A) and APC-/KRAS mut cells (resistant to cetuximab) (B) exposed to increasing concentrations of anti-EGFR. Courtesy of Dr. J.C. Hahne and Dr. N. Valeri (personal communication, unpublished). Let-7g-5p expression was higher in sensitive cells and reduced in cells resistant to cetuximab.

In parallel, increasing concentration of cetuximab brought to lower levels of let-7g-5p expression in *APC-/PTEN-* exosomes (Figure 8A) compared to *APC-/KRAS* mut exosomes (Figure 8B). Cetuximab resistant cells secrete higher amount of let-7g-5p in exosomes compared to sensitive cells. Therefore, let-7g-5p acts as a resistance vector being included into exosomes. (J.C. Hahne, N. Valeri, unpublished, personal communication).

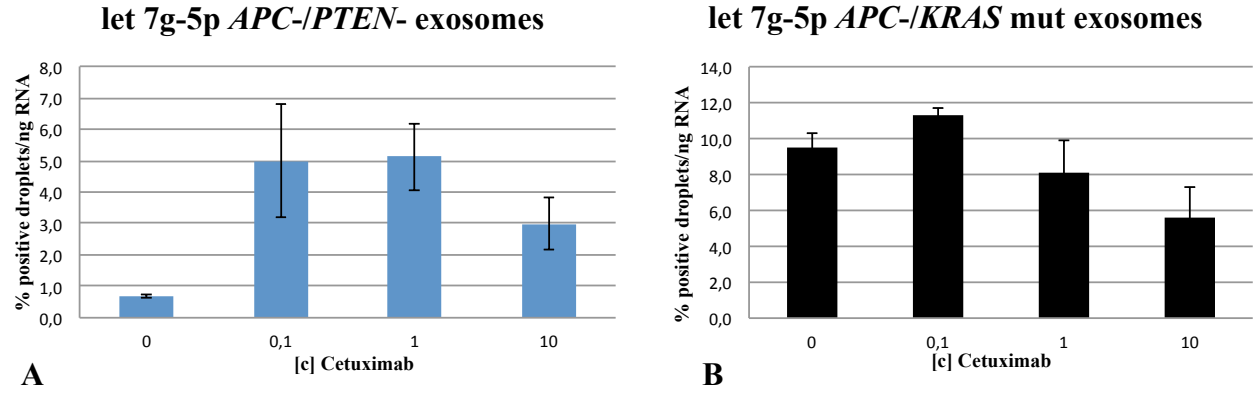


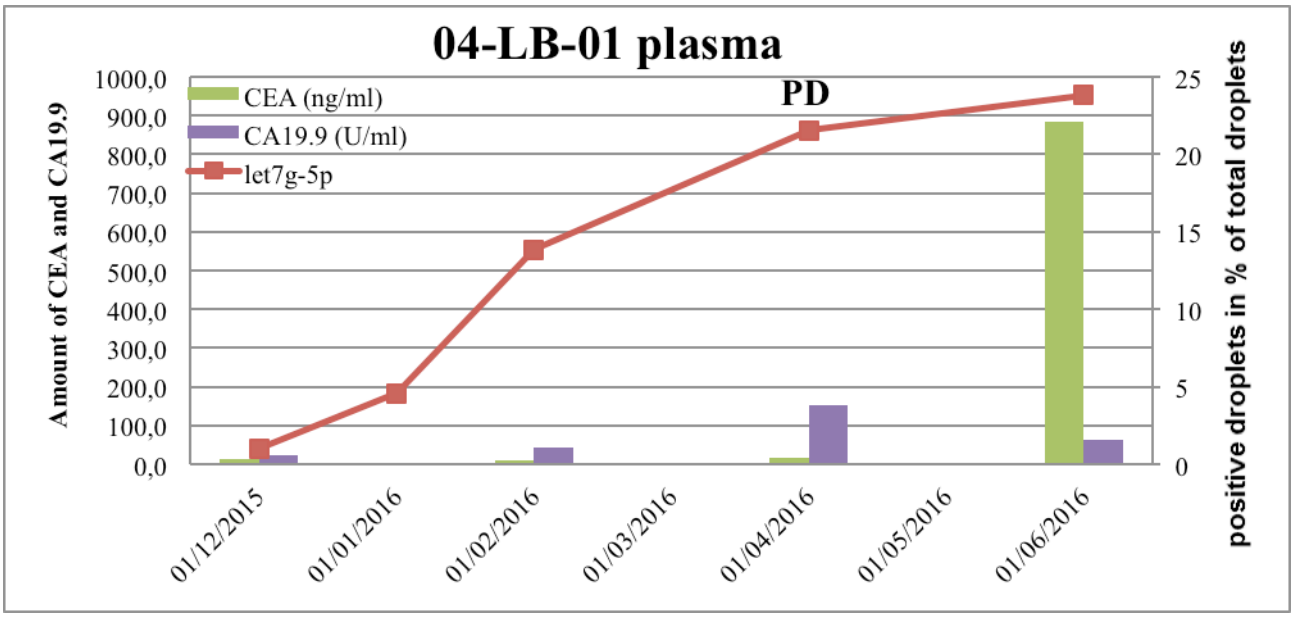
Figure 8. Percentage of positive droplets of let-7g-5p in *APC-/PTEN-* exosomes (A) and resistant *APC-/KRAS* mut exosomes (B) exposed to increasing concentrations of cetuximab. Courtesy of Dr. J.C. Hahne and Dr. N. Valeri (personal communication, unpublished). Let-7g-5p expression was higher in resistant exosomes with compared to sensitive ones.

Clinical phase

Let-7g-5p was chosen as candidate miRNA to be evaluated in plasma and in urine samples of patients treated with anti-EGFR antibody containing therapies. The ddPCR technique was used to determine the let-7g-5p expression level in plasma and urine samples taken during the whole therapy from baseline until progression. Obtained results were compared to CEA and CA 19.9 values and correlated with disease status according to Response Evaluation Criteria in Solid Tumours (RECIST 1.1) criteria. In the frame of this work, 21 cases have been analysed. Results are shown for the patients that have already progressed under anti-EGFR therapy. Collection and analysis of liquid biopsies based on blood and urine is continued in the case of the already recruited patients that have not progressed until now.

Patient 04-LB-01 received a first line treatment with FOLFOX + panitumumab for metastatic RCC with synchronous liver metastases. The patient experienced a progression in April 2016, while levels of let-7g-5p increasing from January 2016 and so anticipating radiological PD of three months roughly (Figure 9A). Differently, let-7g-5p in urine had a decreasing trend, reaching the nadir at the moment of radiological PD and then increasing again (Figure 9B).

A



B

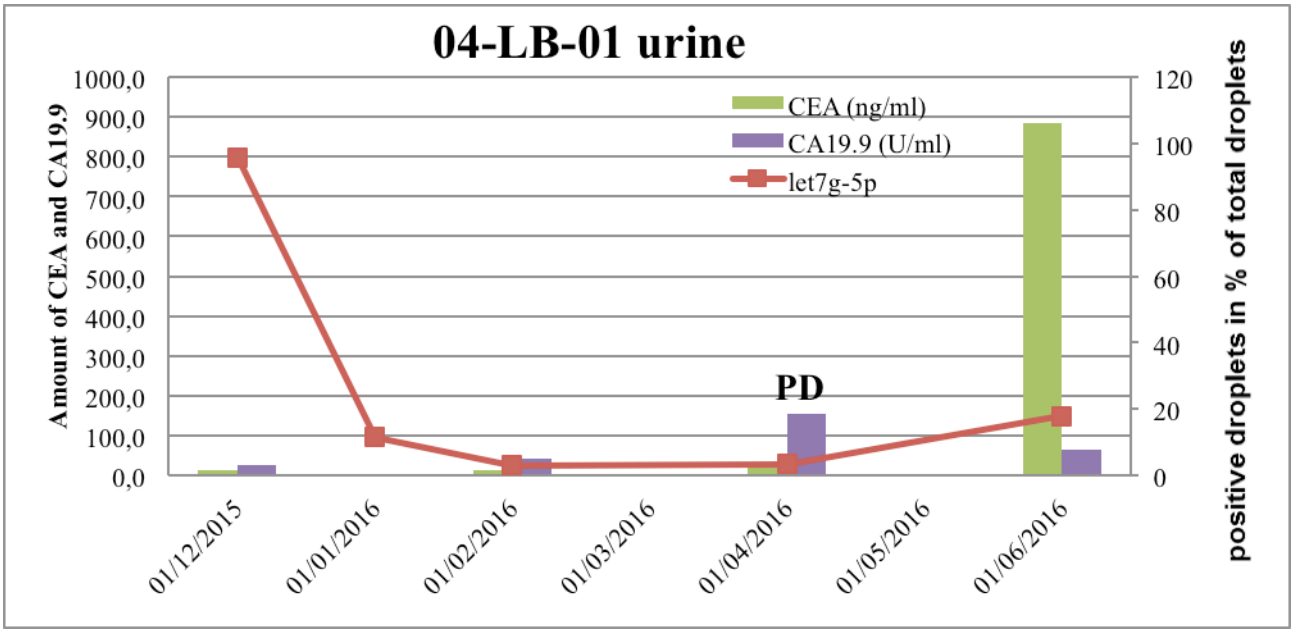
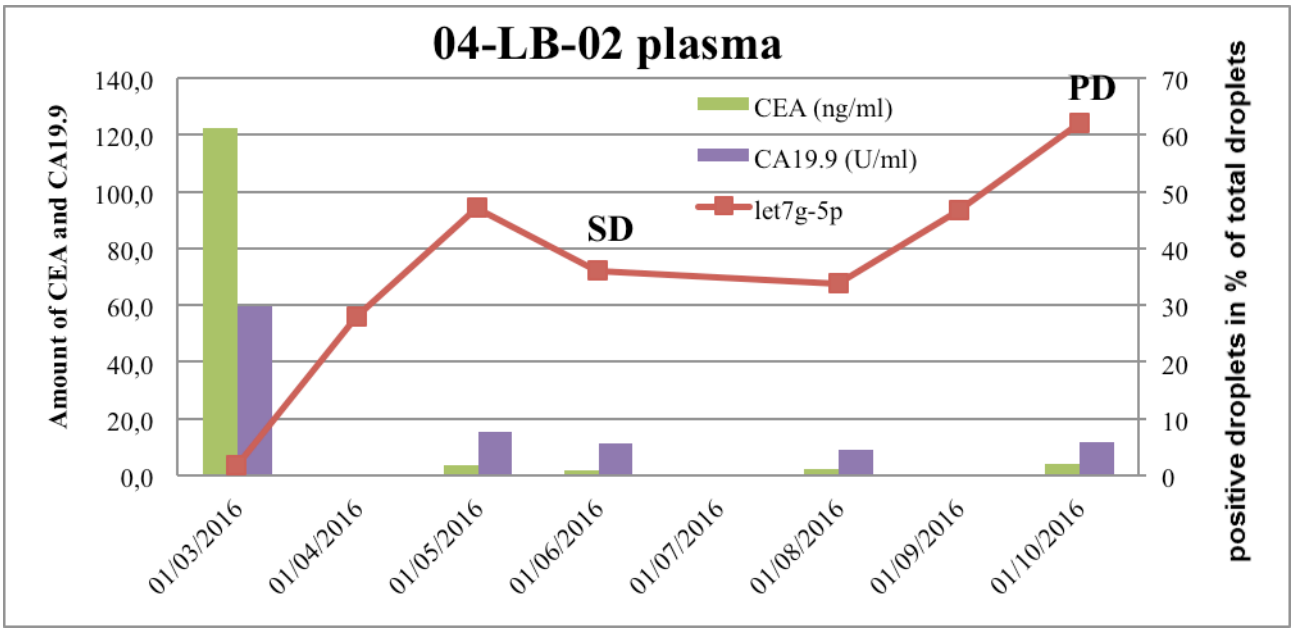


Figure 9. Let-7g-5p expression in plasma (A) and urine (B) samples of patient 04-LB-01.
Radiological PD confirmed in April 2016 with nodal and liver progression. MiRNA expression increased in plasma in parallel with biochemical (increase of marker CEA) and radiological PD (according to RECIST 1.1). On the contrary, miRNA trend in urine was opposite.
PD: disease progression.

Patient 04-LB-02 had first line chemotherapy with FOLFIRI + cetuximab after a nodal relapse of LCC with liver metastases resected in October 2014. Treatment started in March 2016, radiological PD to first line induction chemotherapy and maintenance with single agent cetuximab happened in October 2016. Let-7g-5p expression started to increase (from August 2016) two months before radiological PD of the disease (Figure 10A). Specular trend was obtained when urine samples were analysed (Figure 10B).

A



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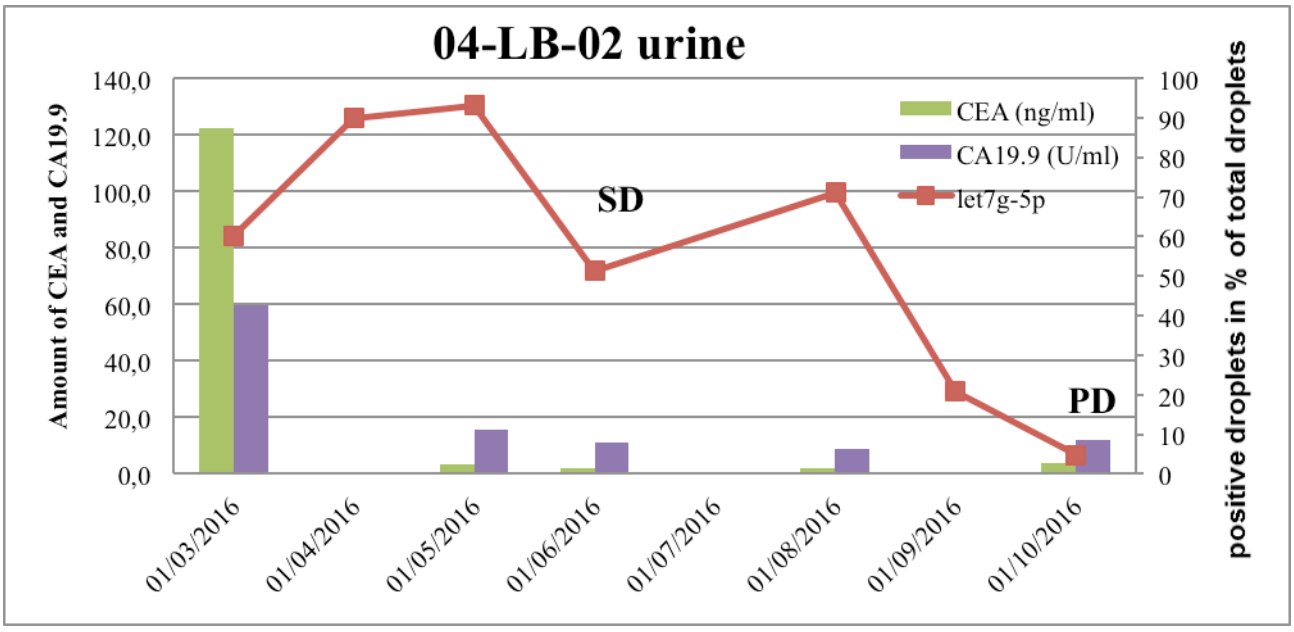
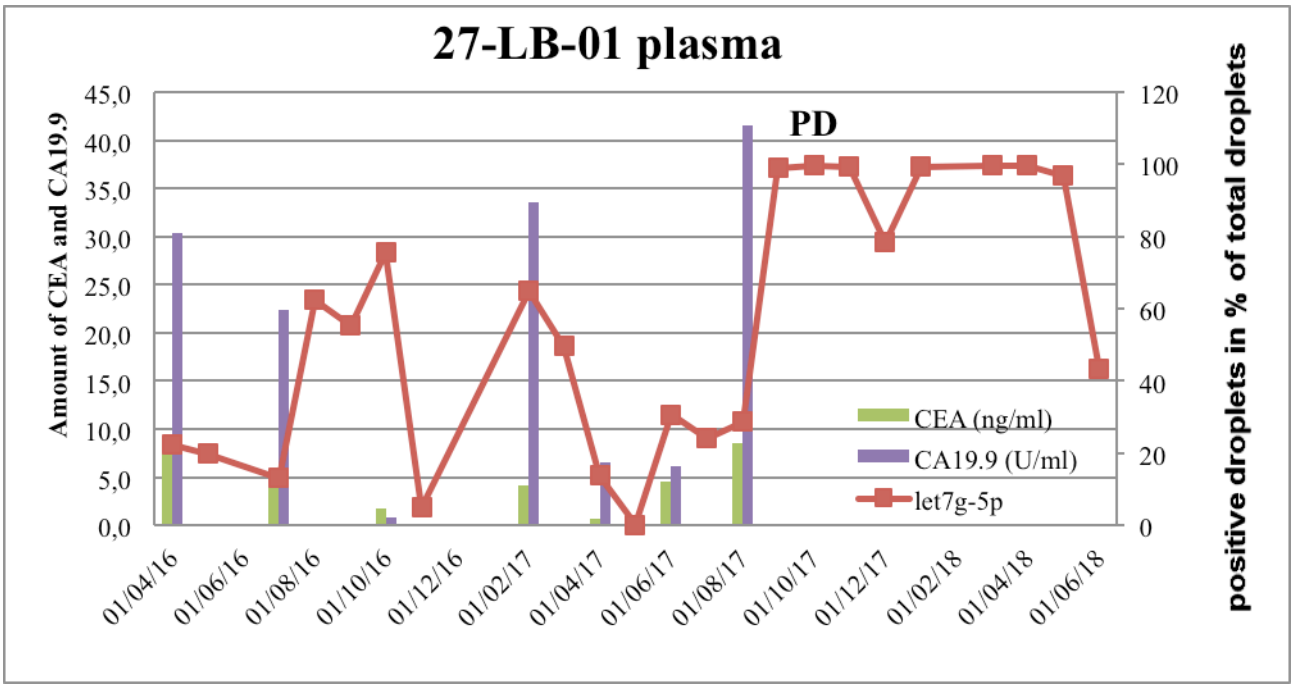


Figure 10. Let-7g-5p expression in plasma (A) and urine (B) samples of patient 04-LB-02.
Radiological PD confirmed in October 2016 with retroperitoneal nodes progression.
After disease stabilization with biochemical response (reduction of marker CEA), miRNA expression increased in plasma in parallel with radiological PD (according to RECIST 1.1). On the contrary, miRNA trend in urine was opposite.
PD: disease progression.

Patient 27-LB-01 was resected for LCC and developed a pulmonary and retroperitoneal nodal relapse (March 2016). The patient started first line with FOLFIRI + cetuximab followed by maintenance treatment with cetuximab only. During treatment history patient experienced severe hand-foot syndrome leading to dose reductions (30% dose reduction of chemotherapy with FOLFIRI) and treatment intervals (September 2016, November 2016 and March 2017). Moreover, the patient had positive anamnesis for systemic lupus erythematosus requiring chronic steroids administration. Both reasons most probably explain the fluctuations observed in the let-7g-5p expression. However, in proximity of radiological PD of disease (October 2017), let-7g-5p expression had a sharp raise, with almost 100% of droplets positive for the miRNA expression (Figure 11A). Surprisingly, in this case expression of let-7g-5p in urine did not follow a specular trend, with an increase concomitant to radiological progression of disease. However, during treatment, let-7g-5p had an increase in blood expression and parallel decrease in urine and viceversa (for example, in the period between April and June 2016, Figure 11A and B) A possible explanation for this variability may be the positive anamnesis for lupus and the chronic steroids intake.

A



B

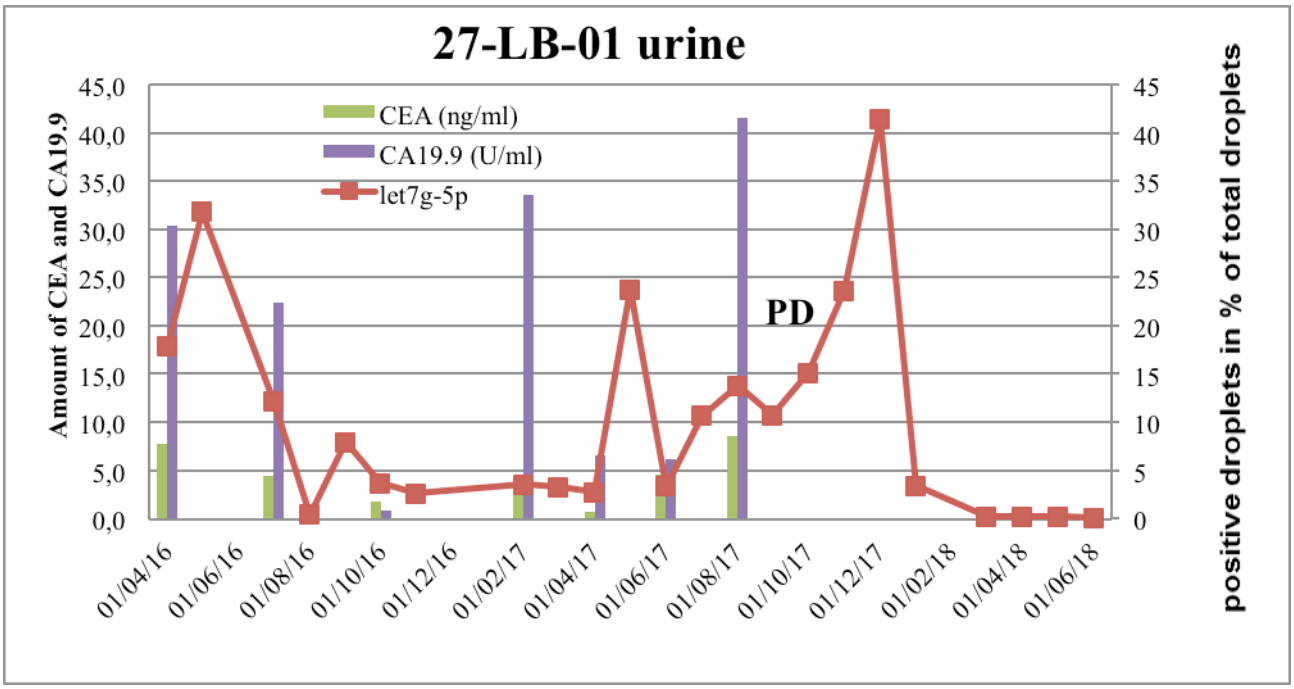
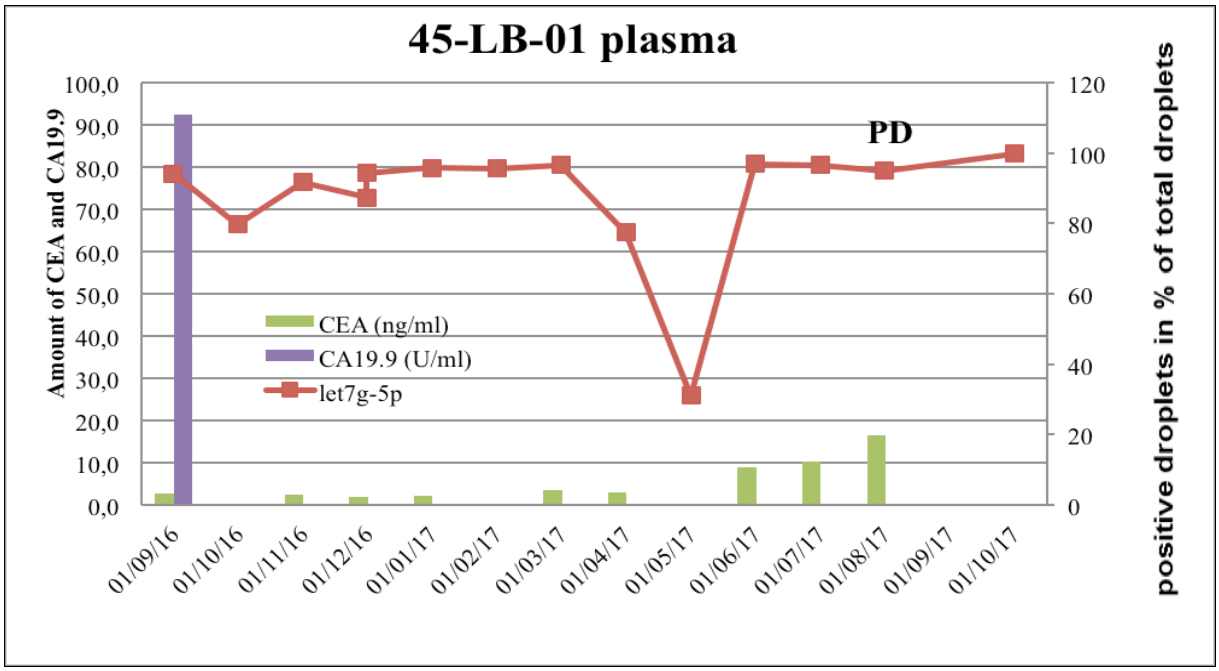


Figure 11. Let-7g-5p expression in plasma (A) and urine (B) samples of patient 27-LB-01.
Radiological PD confirmed in October 2017 with liver and lung progression.
Fluctuating trend may be justified by comorbidities and steroids intake. MiRNA expression increased in plasma in parallel with biochemical (increase of markers CEA and CA19.9) and radiological PD (according to RECIST 1.1). MiRNA trend in urine was similar, with an increase concomitant to biochemical and radiological PD. PD: disease progression.

Patient 45-LB-01 received first line FOLFOX + panitumumab followed by maintenance treatment with panitumumab. After prolonged SD (from August 2016 to July 2017) liver progression and development of peritoneal carcinomatosis occurred in August 2017. Let-7g-5p expression level started to increase from May 2017, three months before the evidence of radiological PD (figure 12A). A similar trend was registered for urine (Figure 12B). It is possible that the first line treatment has not been successful in this patient. Indeed, let-7g-5p had high levels of expression in blood and decreased to a value of zero in urine. Before PD, let-7g-5p expression dropped down in plasma and increased in urine (period between April and June 2017).

A



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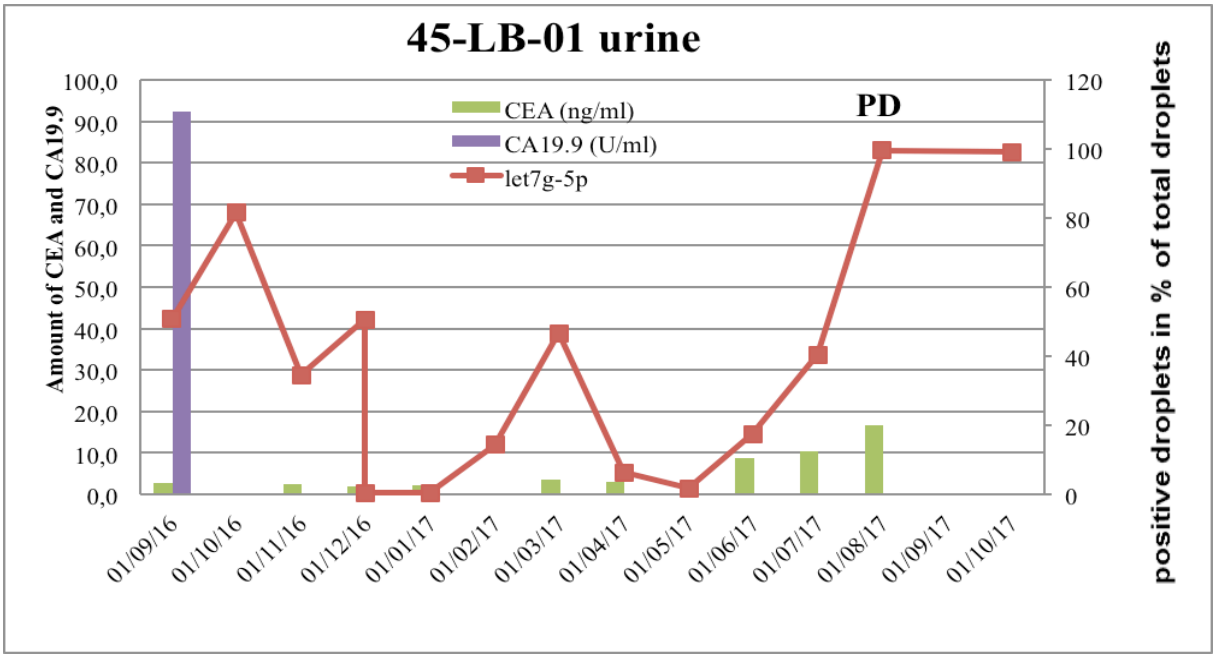
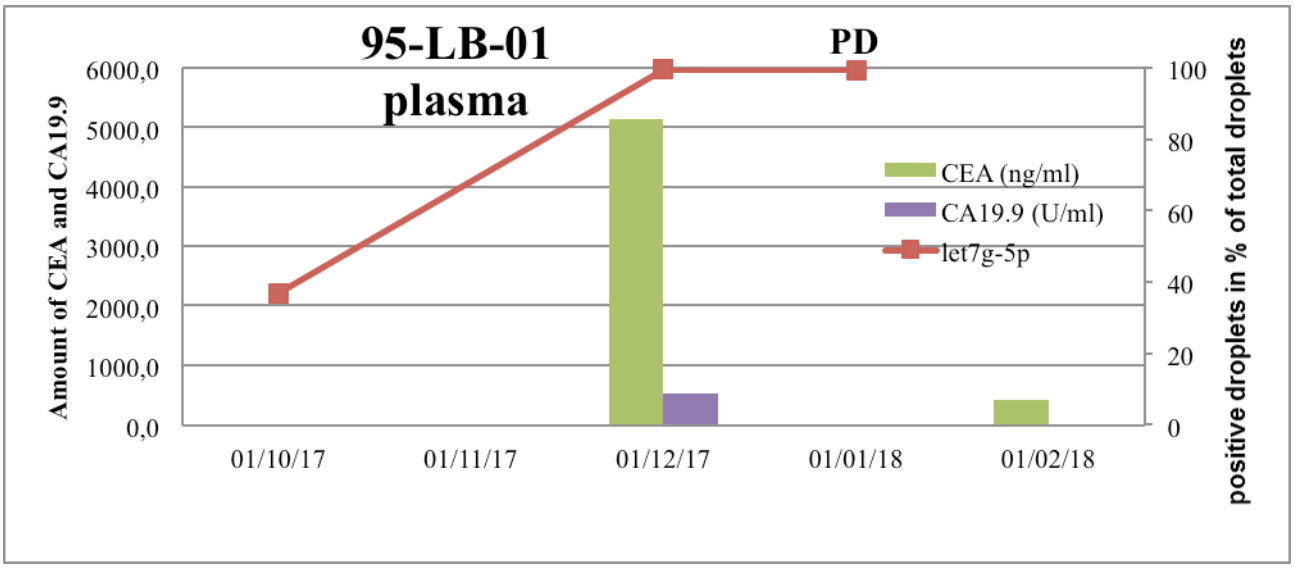


Figure 12. Let-7g-5p expression in plasma (A) and urine (B) samples of patient 45-LB-01.
Radiological PD confirmed in August 2017 with liver progression and development of peritoneal carcinomatosis.
MiRNA expression increased in plasma in parallel with biochemical (increase of marker CEA) and radiological PD (according to RECIST 1.1). MiRNA trend in urine was similar, with an increase concomitant to biochemical and radiological PD. PD: disease progression.

Patient 95-LB-01 received a third-line treatment with single-agent panitumumab following bone progression of LCC. Due to the disease aggressiveness, the patient had a fast progressive disease, proved at the first CT scan evaluation in January 2018. The let-7g-5p expression curve showed a rapid and continuous increase, reaching a plateau at time of radiological PD (Figure 13A). A similar trend was registered for urine (Figure 13B). This unusual correlation between blood and urine let-7g-5p expression may be justified by the atypical pattern of disease progression for LCC with development of bone disease.

A



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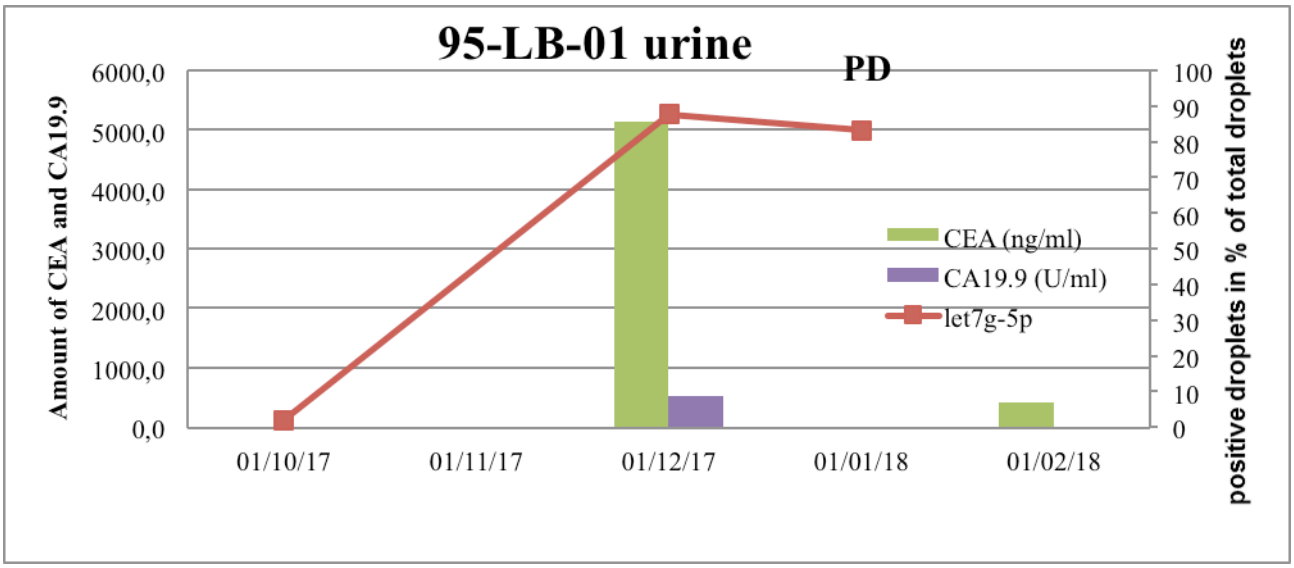

Figure 13. Let-7g-5p expression in plasma (A) and urine (B) samples of patient 95-LB-01.
Radiological PD confirmed in January 2018 with multiple sites of relapse.
MiRNA expression increased in plasma in parallel with biochemical (increase of marker CEA) and radiological PD (according to RECIST 1.1). MiRNA trend in urine was similar, with an increase concomitant to biochemical and radiological PD.
PD: disease progression.

Among the other samples analysed, a direct correspondence between increase in expression level of let-7g-5p and progression was not found; sometimes based on the fact that clinical parameter have not been available. Indeed, some of the patients are still on treatment and have not experienced a disease progression yet, with levels of let-7g-5p fluctuating around similar values in presence of radiological and biochemical SD. For these patients, liquid biopsies are still being collected and analysed. A verification of the prognostic value of let-7g-5p on a larger cohort is intended.

Liquid biopsies of patients treated with bevacizumab (cohort 2)

This sub-part of the PhD work is based on ten patients. For all of them, *RAS* and *BRAF* mutational profile was assessed on tissue samples collected either for primary tumour or metastatic sites at diagnosis and before the start point of treatment. The same mutations were monitored with ddPCR on plasma samples in order to evaluate changes and modification during the course of treatment.

Mutation load and clinics corresponded in four cases, but, surprisingly for six patients, mutational trend did not follow clinical history. In the following only the four cases in which the mutation load correspond to the clinic are discussed in detail (figure 14-17).

Patient 10-LB-01 was diagnosed with RCC containing *BRAF V600E* mutation. This patient was resected and relapsed with liver disease immediately after surgery. The clinical course had aggressive features with a fast PD during first line treatment with FOLFOX + bevacizumab leading to second line treatment with and FOLFIRI + bevacizumab. The mutation rate had a continuous increase from diagnosis to progression and subsequent death (Figure 14).

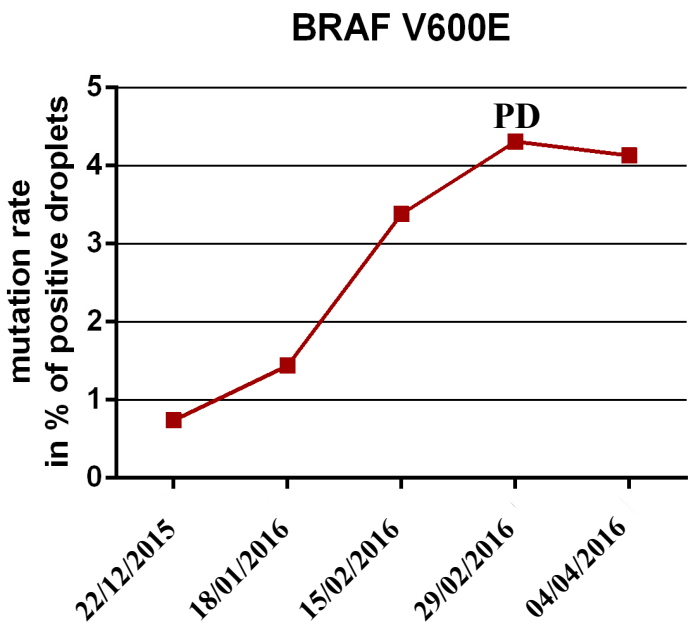

Figure 14: Patient 10-LB-01. *BRAF V600E* mutation. The patient did not have any benefit from treatment and experienced a fast PD.
PD: disease progression.

Patient 25-LB-01 has a *KRAS G12V* mutation. After being diagnosed with RCC and liver metastases, the patient received first line chemotherapy with induction FOLFOXIRI + bevacizumab and maintenance FOLF + bevacizumab. A PR was obtained and the patient underwent a two stage-hepatectomy and right hemicolectomy. Mutation load dropped until it fell below the detection limit of the ddPCR before surgery, with subsequent regrowth in correspondence with clinical relapse of the disease (Figure 15).

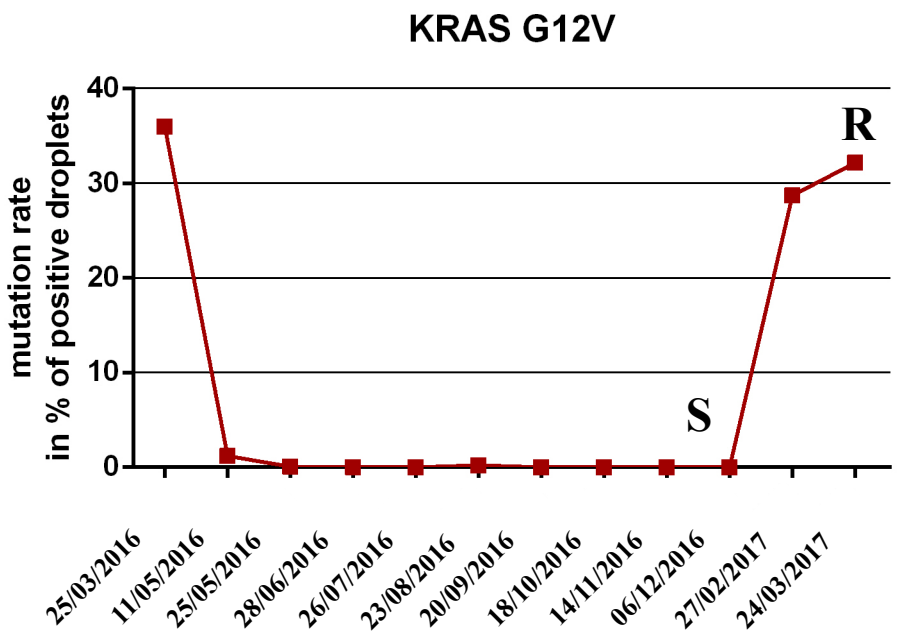

Figure 15: Patient 25-LB-01. *KRAS G12V* mutation. The patient had a negativization of cfDNA mutation before radical surgery, experiencing a fast relapse after operation.
S: surgery; R: relapse

Patient 33-LB-01 harboured *KRAS A146T* mutation. Induction with FOLFOXIRI + bevacizumab and then maintenance therapy with FOLF + bevacizumab for CRC with synchronous liver metastases were administered. After achieving a PR, the patient underwent surgery and remained disease free after radical resection. Mutation rate dropped and reached normal values after 5 cycles, and remained stable afterwards (Figure 16).

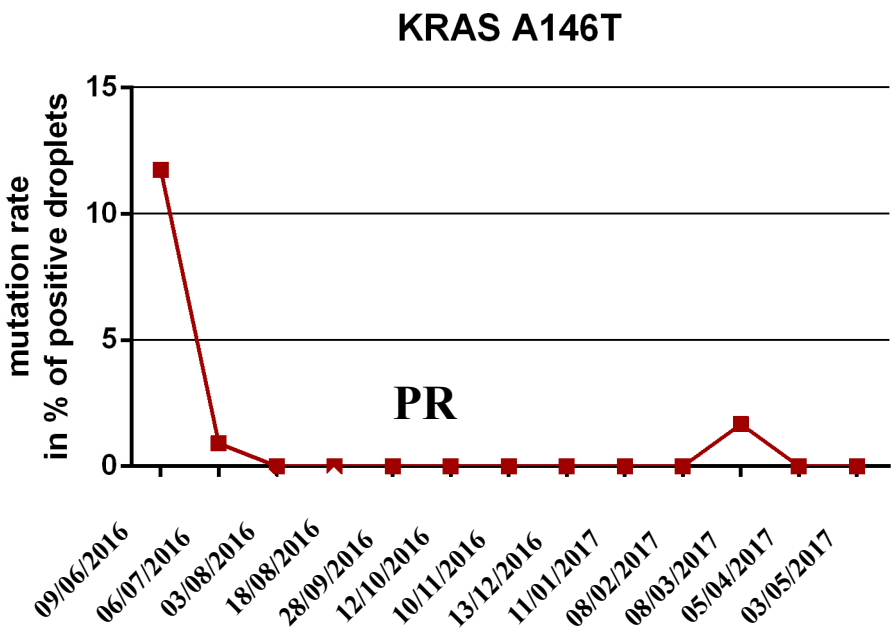

Figure 16: Patient 33-LB-01. *KRAS A146T* mutation. The patient had a disease PR four months after the treatment start and kept further stability.
PR: partial response.

Patient 44-LB-01 was diagnosed with *KRAS G12C* mutated LCC with synchronous liver and lung metastases. After surgery on primary tumour, the patient started first-line with FOLFOX + bevacizumab followed by maintenance therapy with capecitabine + bevacizumab. Reduction in the diameters of metastatic lesion within the limits of SD was observed. Mutation load decreased during the induction phase and had a sharp increase during maintenance treatment, anticipating clinical and radiological PD, which was diagnosed two months later by RECIST 1.1 criteria (Figure 17).

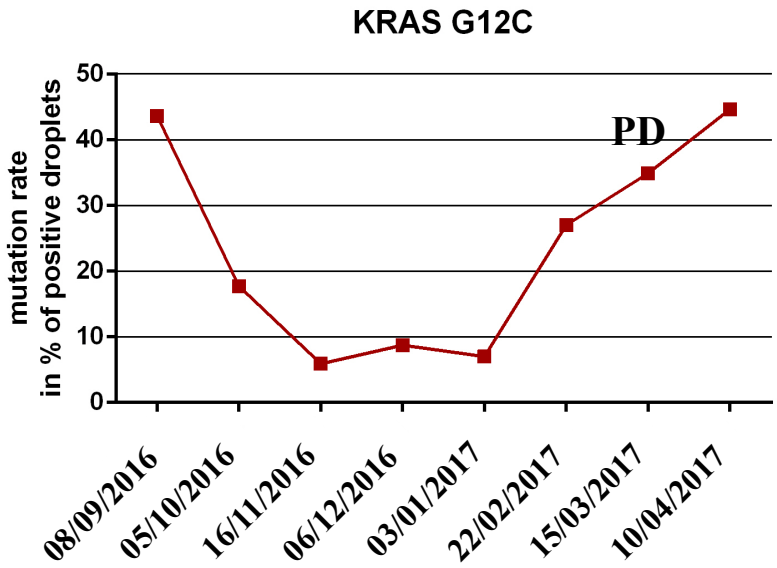

Figure 17: Patient 44-LB-01. *KRAS G12C* mutation. The patient had an increase in the mutation load 2 months before radiological evidence of PD.
PD: disease progression.

Liquid biopsies of long-term responders treated with bevacizumab

Three patients treated with bevacizumab had long and uncommon response period. Liquid biopsies were taken during the course of treatment. NGS analysis was performed on chosen points in time in order to detect possible onset of unknown mutations linked with disease progression.

Patient 01-LB-01, a *RAS* wild-type patient, had nodal retroperitoneal relapse after sigmoidectomy for LCC. Induction chemotherapy with FOLFOX + bevacizumab (eight cycles from October 2015 to February 2016) was administered with SD and then maintenance treatment with FOLF + bevacizumab for more than two years (February 2016 to August 2018, 58 total cycles). After this long period, on August 2018, patient had a nodal PD and started second line with FOLFIRI + bevacizumab. NGS analysis was performed on the sample next to radiological PD. The missense variant c.518 T>A in the gene *TP53* was detected with an allele fraction of 0.67%.

Patient 52-LB-01 was affected by LCC with liver and lung synchronous metastases bearing a *KRAS G12V* mutation. Induction with FOLFOXIRI + bevacizumab (November 2016 to February 2017)

was started. From March 2017, maintenance treatment with FOLF + bevacizumab started and lasted for 19 cycles, until December 2017. Due to hepatic PD (January 2018) the patient rechallenged FOLFOXIRI + bevacizumab as second line treatment. This patient obtained a radiological SD and biochemical response with decrease of both CEA and CA 19.9. However, from May 2018 the patient started to complain a worsening pain and CEA and CA 19.9 level increased. The CT scan performed in June 2018 showed an increase in liver lesions but not a RECIST 1.1 PD, while the evaluation performed in August evidenced hepatic progression. NGS analysis was performed on both progression time points (January and June 2018). In both cases, the same mutations were detected: *KRAS G12V* (documented at the time of diagnosis on tissue biopsy on primary tumour) and *APC* c.3340 C>T (p.Arg 1114*) with stop codon gained. Allele fractions dropped from January to June 2018 (*KRAS G12V* from 5.53 to 1.62% and *APC* c.3340 C>T from 4.68 to 1.39% (Figure 18). This reduction in allele fractions is most probably connected to the second PD. On the other hand, first PD happened after a long maintenance period with FOLF + bevacizumab. Probably, reintroduction of FOLFOXIRI may have contributed to reduce the mutational load more than it was the case for the maintenance treatment.

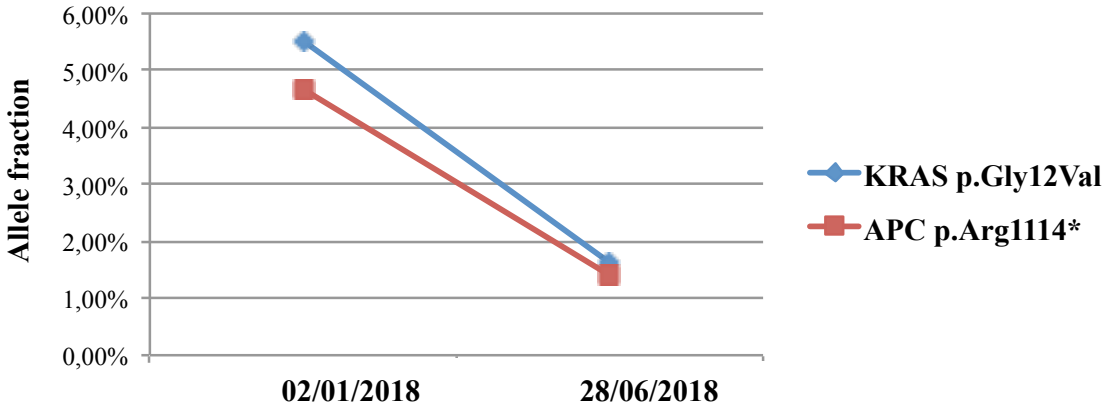

Figure 18. Patient 52-LB-01. Allele fractions between two different progression timepoints. Allele fraction of both mutations was lower at second progression time point (PD).January 2018: first PD, June 2018: second PD.

Patient 02-LB-01 had a surgery for LCC and immediate relapse to lung and Barety nodes. The *RAS* mutation test was positive for *NRAS G12D* mutation. The patient started chemotherapy with FOLFOXIRI + bevacizumab from October 2015 to February 2016 and then induction phase with FOLF + bevacizumab from April 2016 to December 2017. At the end of the year the patient experienced a lung PD and reintroduced FOLFOXIRI + bevacizumab with secondary maintenance after 6 cycles (from February to June 2018). NGS was performed on six different time points: basal (21/12/2015), end of induction treatment (15/03/2016), increase of lesion diameters during maintenance without RECIST 1.1 PD (27/04/2017), further increase of lung lesion diameters without RECIST1.1 PD (06/09/2017), start of rechallenge (25/01/2018) and at the end of rechallenge (12/06/2018). The mutation pattern reproduced the clinical course of the disease, with rising level of *NRAS G12D* mutation from April 2017 until January 2018 (first PD and rechallenge start with FOLFOXIRI + bevacizumab). During rechallenge, a sharp decrease in the same mutation was observed. The same trend was observed for another driving mutation, *TP53* c. 800 C>G (p. Arg267Pro, Figure 19. Furthermore, three other mutations located in *GNAS, APC* and *MET* genes were detected with low allele fraction (Figure 19 and Table 8).

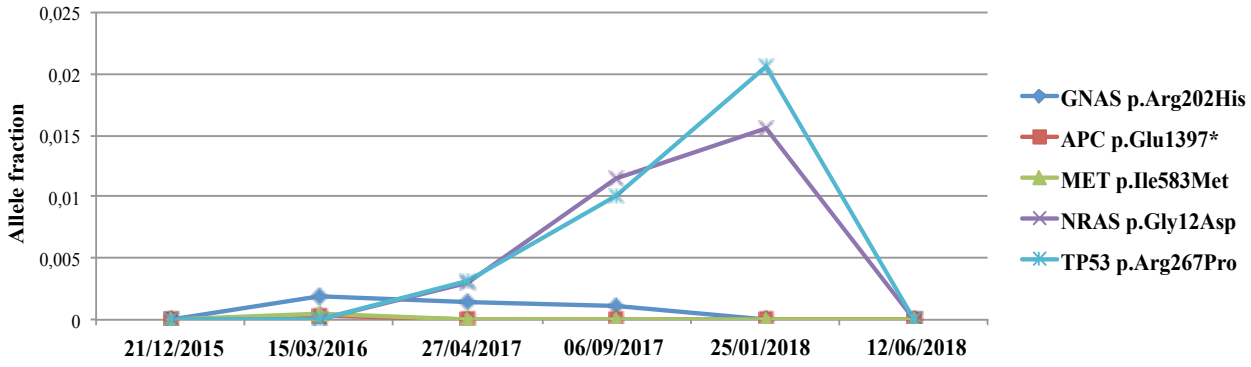

Figure 19. Mutations trend in plasma samples of patient 02-LB-01. Increase in mutation load reached a peak at PD and decreased after reintroduction of FOLFOXIRI + bevacizumab.

Table 8. Allele fractions in plasma samples of patient 02-LB-01.
Yellow highlighted the two driving mutations of genes *NRAS* and *TP53*.

Sample ID	*GNAS* p.Arg202His	*APC* p.Glu1397*	*MET* p.Ile583Met	*NRAS* p.Gly12Asp	*TP53* p.Arg267Pro
21/12/15	0	0	0	0	0
15/03/16	0,19%	0,04%	0,05%	0	0
27/04/17	0,15%	0	0	0,30%	0,32%
06/09/17	0,11%	0	0	1,15%	1,01%
25/01/18	0	0	0	1,56%	2,06%
12/06/18	0	0	0,00%	0	0

Liquid biopsies of resected patients

Among the 16 resected patients, we chose three patients who experienced a PD within four months after surgery. We considered the last sample before surgery in order to evaluate mutation pattern with NGS analysis. Our purpose was to verify the possible prognostic role of mutations identified before surgery, hypothesizing the persistence of mutations despite previous treatment in cases that had a poor clinical trend.

Patient 94-LB-01, diagnosed with a *RAS* wild-type RC with liver and lung metastases, had induction chemotherapy with FOLFOX + panitumumab and maintenance chemotherapy with FOLF + panitumumab. After achieving a PR, the patient became a good candidate for surgery, performed on April 2018. After surgery the patient experienced a rapid disease relapse and died in August 2018. Table 9 and figure 20 show changes in mutation pattern between 21/02/2018 (before surgery) and after surgery (26/06/2018).

Table 9. Mutations and single nucleotide polymorphism (SNP) found in the two analysed samples of patient 94-LB-01. *ABL1* is a SNP present in both samples. This confirms that the two samples belong to the same patient.

Sample ID	*RET* p.Leu746Me	*ABL1* p.Thr762Thr	*TP53* p.Glu204*	*APC* p.Gln1328*
21/02/2018	0,74%	48,54%	0	0
26/06/2018	0,00%	39,03%	57,68%	54,22%

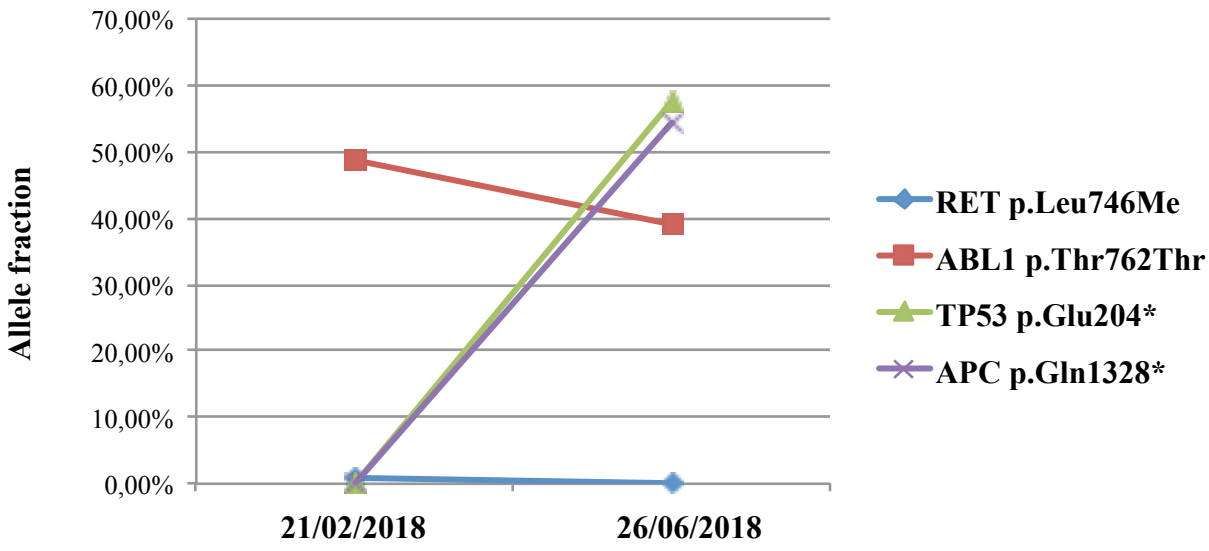

Figure 20. Mutations and single nucleotide polymorphism (SNP) found in the two analysed samples of patient 94-LB-01. *ABL1* is a SNP, present in both samples. This confirms that the two samples belong to the same patient.

Patient 18-LB-01, with a *RAS* wild-type genotype, relapsed four years after LCC resection developing liver disease. He started first line treatment with FOLFIRI + cetuximab and maintenance cetuximab, experiencing a PR and being proposed for liver surgery. Resection happened on July 2016 and the patient remained free from disease until May 2017. NGS analysis on pre-surgical sample identified two mutations: c. 3178 C>T of *FLT1* gene (stop codon gained, allele fraction 0.55%) and c. 329 G>C of *TP53* (missense variant, allele fraction 0.38%).

Patient 25-LB-01, known for *KRAS G12V* mutation at diagnosis, received FOLFOXIRI + bevacizumab. Following maintenance treatment for RCC with synchronous bilobar liver

metastases, the patient underwent two-stage hepatectomy and right hemicolectomy between December 2016 and January 2017 and an immediate relapse on March 2017 (liver and abdominal nodes). An important mutation load was detected with NGS performed on pre-surgical sample, with 7 mutations and one SNP (Table 10).

Table 10. Mutations and single nucleotide polymorphism (SNP) found in the pre-surgical sample of patient 25-LB-01 *MET* is a SNP.

Gene	Coding change	Variant description	Allele fraction
FGFR2	c.607C>T	Missense variant	0,16%
KRAS	c.35G>T	Missense variant	1,48%
MAP2K1	c.332T>A	Missense variant	0,06%
TP53	c.658T>A	Missense variant	0,28%
TP53	c.388C>T	Missense variant	2,05%
PIK3CA	c.113G>A	Missense variant	2,75%
APC	c.4135G>T	Stop gained	3,74%
MET*	c.2962C>T	Missense variant	45,85%

DISCUSSION

Heterogeneity is one of the main mechanisms responsible for tumour resistance to treatment. Therefore, the study and correct assessment of tumour heterogeneity is essential for the development of new and effective therapies in oncology [71]. Heterogeneity is both spatial, with clonal diversity among different disease sites (such as primary tumour and metastases), and temporal, due to continuous changes in the molecular makeup of cancer cells [71].

Liquid biopsies are certainly valid tools for the study of temporal and spatial heterogeneity [55,72]. Differently from tissue biopsies, liquid biopsies allow the contemporary evaluation of genetically different and co-occurring cell clones during the natural history of the disease and are not restricted to one single tumour site [72,73]. Recently, a mathematical model for prediction of resistance to treatment with anti-EGFR agents (cetuximab or panitumumab) in advanced CRC has been proposed [55]. This model relies upon the fact that, even in patients responsive to treatment, rare or slow-growing sub-clones resistant to anti-EGFR are present and detectable at the beginning of treatment. In resistant patients, a substantial amount of resistant clones is already present at baseline. By analysing monthly or be-weekly liquid biopsies, this model was able to predict with high accuracy the time until radiographic evidence of PD [55]. In CRC, the significance of genomic aberrations driving the disease development and progression have pushed to realize that the serial genotyping of both primary tumour and metastases is needed in order to achieve a truly personalized treatment for the patient [74] As far as serial tissue biopsies are not feasible, ctDNA can be used instead as a surrogate of both tumour burden and its heterogeneity [74].

The exploratory prospective analysis presented in this PhD work evaluated the role of liquid biopsies in metastatic CRC in two different cohorts. The *RAS* wild-type cohort of patients (cohort 1) had a better prognosis and prolonged survival in comparison to the second cohort, almost totally composed of *RAS* or *BRAF* mutated patients treated with chemotherapy and the anti-VEGF agent

bevacizumab (cohort 2). Indeed, a statistically significant difference was found in mOS in favour of cohort one (18.9 months *vs* 15 months for cohort 2, p=0.0362), while, on the contrary, no statistical significant difference between the cohorts was noticed in regard to mPFS (39.7 *vs* 22.9 months, p=0.2, figure 6). These results are in line with previous reports as far as the negative prognostic significance of *RAS* and *BRAF* mutations in advanced CRC has been already proven and is well-established [17].

Patients harbouring *RAS* or *BRAF* mutations assessed at time of diagnosis on histologic sample were tested for the same mutations with liquid biopsies using plasma. The mutation trend followed clinical history only in four cases while in six cases no correspondence was found. Liquid biopsies have limitations affecting their applications [75]. A major shortcoming is given by the fact that levels of cfDNA among cancer patients vary, with tumour-derived DNA accounting for only 0.1 to 10% of total DNA [76,77]. Moreover, the level of tumour cfDNA depends on many factors, such as cancer stage, vascularization, tumour burden, metastatic potentials of cancer cells and apoptotic rate [54,76] Although frequencies of genomic alteration detected in cfDNA were shown to be comparable to those observed on CRC tissues [78], different mutations play a role in the course of the disease, with possible onset of new "driver" mutations. Lack of adherence between molecular and clinical course of the disease may be justified by the molecular heterogeneity of the disease, with presence of multiple mutations beyond the one tested playing a role in disease progression [79-81]. Molecular heterogeneity of CRC was documented both in tissue and in cfDNA [82]. In fact, distinct resistance mechanisms and new acquired mutation within different tumour lesions from the same primary site may cause mixed radiological responses (with response of only one or few lesions) [82]. In addition, mutant *RAS* clones raise and decrease in relation with treatment administration or therapeutic intervals due to clonal evolution [83]. Mutant clones increase during treatment developing drug resistance and decrease after drug withdrawal, regaining drug sensitivity [83]. Detection of circulating mutations may happen months before the clinical evidence of PD [83]. In a CRC series, the

emergence of *KRAS* mutant clones happened as early as ten months prior to radiological progression [84]. Even in the study population used in this work, different *KRAS* and *BRAF* mutations showed up or increased their level before radiological diagnosis of PD. This is in agreement with the widely accepted view that CRC molecular progression often happens before clinically evaluable PD [55,85,86]. Therefore, the diagnostic potential of liquid biopsies may help in the early detection of resistance to biological antitumor agents [85,87,88].

Differently from *RAS* mutated CRC disease, the use of liquid biopsies in *RAS* wild-type CRC receiving anti-EGFR treatment has been widely studied [89]. The principal mechanism linked with clinical PD to anti-EGFR is the development of *KRAS* mutations [89]. These mutations can be easily detected in the blood of CRC patients in almost 40% of cases during the course of treatment [89]. At the same time, many other mechanisms can play a role in promoting resistance to anti-EGFR treatment [45]. Indeed, mechanisms of acquired resistance may involve alteration in the *EGFR* [90], *BRAF V600E* [91] and *PIK3CA* exon 20 mutations [92], *PTEN* loss [92]and *STAT3* phosphorylation [93]. Moreover, activation of alternative pathways such as IGF1R [94]and VEGF/VEGFR-pathway [95], *MET* [96] and *HER2* amplification [97]may be mechanisms of resistance, as well.

For patients with absence of *RAS* and *BRAF* mutations, the role of let-7g-5p was evaluated. The human let-7 family consist of 13 miRNAs located on nine different chromosomes [98]. Many human cancers have a deficient expression of let-7 family members (for example, let-7b, let 7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98) with a clear link between loss of miRNA and development of poorly differentiated and aggressive cancers [98-101]. In general, let-7 family members are able to suppress tumour proliferative activities and survival by negatively mediating a number of oncogenes regulating cell cycle, differentiation and apoptotic pathways [102]. Among common targets, let-7 miRNAs family negatively regulates the expression of Ras protein [103], as well as for Myc proto-oncogene and the JAK-STAT3 pathway [102]. However, evidence about the oncogenic role of the same miRNAs is also available. For example, let-7/miR-98 was reported to inhibit the

death receptor Fas [104]. Moreover, let-7 miRNAs family regulates NF-kB pathway [105] and the two well-known onco-suppressors p53 [106] and TGF-β [107]. Small evidence is available about let-7g-5p in the context of CRC. Recent reports included let-7g among the list of onco-suppressor miRs, bringing a negative prognosis when down-regulated in gastrointestinal cancers such as esophageal, colorectal and gastric cancer, both in tissue and in blood [108]. Moreover, let-7g was listed among a signature of six serum miRNAs able to distinguish with high sensitivity and specificity between cases affected by colorectal cancer and healthy controls [109]. The same miRNAs had a strong prognostic meaning being associated with colorectal cancer progression [109]. At the same time, lower let-7g expression in tissue specimens was associated with better survival in patients receiving chemotherapy [110]. This is because of the higher cellular proliferation rate in case of onco-suppressor down-regulation, resulting in increased lethality [110]. Increased levels in colon cancer tissue specimens were associated with a higher response to the 5-fluorouracil-based antimetabolite S-1 [111]. In a previous preclinical study, let-7g-5p level was shown to be higher in cetuximab sensible cells compared to resistant ones. Resistant cells secret a higher amount of let-7g-5p in exosomes compared to sensible cells. (Hahne JC, Valeri N, unpublished). Based on cell culture experiments, a potential mechanism of acquired resistance based on the intra-cellular transport of this miRNAs via exosomes was hypothesized (Hahne JC, Valeri N, unpublished). Even in gastric cell-lines, let-7g was reported to be secreted into the extracellular environment via exosomes as a mechanism of resistance [112]. In the here presented work, let-7g-5p was evaluated both in plasma and urine of *RAS* wild-type patients during anti-EGFR treatments, with contrasting results. Indeed, in five cases, let-7g-5p expression followed and sometimes anticipated radiological PD, while in other cases its expression trend was not adhering to clinical outcome. Despite in most tumours the expression of all members of the let-7 family (including let-7g-5p) was shown down-regulated compared to normal tissue [113], tumour suppressor functions of let-7 miRNAs family were not described under all circumstances and in all tissues [114]. For instance, colon cancer cell lines were shown to have a

higher expression of let-7g compared to normal colon tissue [115]. Moreover, let-7g was found up-regulated in lung cancer cells after radiation. Indeed, only let-7g overexpression caused radioresistance, suggesting a possible different role of let-7g with compared to the other let-7 family members [116]. Previous reports underlined the positive regulation of let-7 in case of anti-EGFR treatment [117-119]. Indeed, high expression of let-7c was found to be associated with longer PFS and OS in *KRAS* wild-type patients receiving an anti-EGFR treatment [117]. In addition, high let-7a levels were associated with better survival outcomes in *KRAS* mutated and chemotherapy refractory patients treated with a cetuximab-based salvage treatment [118]. Then, confirming the correlation between let-7 and *KRAS,* a let-7 binding site polymorphism (LCS6) in *KRAS* predicted a favourable outcome in patients treated with salvage cetuximab/panitumumab single agent [119].

Trend in urine was unpredictable as well, with a trend specular to plasma in some cases and parallel in other situations. A possible explanation for that may be found in the increased turnover of miRNAs by kidneys and reduced stability in urine with respect to blood [120]. Let-7g-5p had a trend towards decrease in the urine and increase in the blood of patients progressing to anti-EGFR therapy. However, its expression could be influenced by different and various factors: interaction with other non-coding RNAs or cfDNA mutations, alterations in exosomes trafficking or affected by standard chemotherapy administered together with anti-EGFR therapy.

By using next generation sequencing on serial plasma samples of three long-term responders treated with bevacizumab, new mutations that were not tested at initial tissue molecular analysis were detected. *TP53* was the acquired mutation promoting progression in two cases, while *APC* was detected together with *KRAS* mutation in a third case. These results confirm the crucial role of acquired mutations different from known *RAS* mutations in driving disease progression [45]. However, in both *RAS*-mutated patients, original *KRAS* mutation had changes in expression level due to the clonal evolution given by treatment administration, with reduction during disease response and increase concomitant to PD.

Furthermore, the prognostic role of liquid biopsies in patients undergoing radical surgery for advanced disease after a variable period of treatment and relapsing after surgery was evaluated. An NGS panel was used to analyse plasma samples collected in all three patients relapsed after being operated. Three patients were found to have one or more mutations expressed before surgery. Interestingly, one patient showed a mutation pattern shift before and after surgery, with acquisition of *APC* and *TP53* mutations at high frequencies and fast disease progression. Once again, acquisition of new mutations has a fundamental role, with mutational activation of oncogenes and inactivation of tumour suppressor genes [121]. It was possible to show in these patients that mutations before surgery can predict disease relapse after operation, and may be responsible for differences in aggressiveness of the disease before and after radical surgery.

This study has some limitations. First of all, it is a small exploratory analysis with the intent to test the possible prognostic and predictive role of liquid biopsies during treatment for advanced disease. Higher numbers of patients are warranted to evaluate the potential predictive role of let-7g-5p in *RAS* wild-type patients. Therefore, the collection and analysis of liquid biopsies based on blood and urine is continued in the case of the already recruited patients that have not progressed until now. Furthermore, based on the results obtained during this work a bigger study is intended for evaluation of let-7g-5p expression level as prognostic marker.

The use of liquid biopsies to track known *RAS* mutations seems promising in order to predict the onset of resistance, while the use of NGS, although limited by economic reasons, may show the emergence of new acquired mutations and is likely to play a bigger role in forecasting disease progression and relapse. Indeed, patients presenting detectable mutations before surgery, and especially a high mutation load, may be relieved from an unnecessary surgical act due to the high risk of relapse.

CONCLUSIONS AND FUTURE PERSPECTIVES

Liquid biopsies may have a prognostic and predictive role in the management of patients with advanced CRC receiving active treatment. Tracking of known starting cfDNA mutations and detection of new acquired mutation as well as evaluation of miRNA expression levels can become of high importance in predicting clinical trend of the disease and may be used in combination with radiological examinations and biochemical analysis in anticipating crucial therapeutic decisions as well as suitable candidates for radical surgery.

Future ongoing studies will include a bigger number of patients in *RAS* wild-type cohort in order to validate the potential role on miRNA let-7g-5p for resistance to anti-EGFR agents. Secondly, NGS analysis will be performed on all 16 resected patients collected in the frame of this study, both relapsed and disease–free after surgery. It is reasonable to expect an absence of trackable mutations in case of non-relapsing patients, while "driver" mutations will be most probably found in pre-surgical samples of relapsed tumour patients.

REFERENCES

1. Shirafkan N, Mansoori B, Mohammadi A, et al. MicroRNAs as novel biomarkers for colorectal cancer: New outlooks. *Biomed Pharmacother.* 2018;97:1319-1330.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 2018;68(1):7-30.
3. Mik M, Berut M, Dziki L, et al. Right- and left-sided colon cancer - clinical and pathological differences of the disease entity in one organ. *Arch Med Sci.* 2017;13(1):157-162.
4. Holch JW, Demmer M, Lamersdorf C, et al. Pattern and Dynamics of Distant Metastases in Metastatic Colorectal Cancer. *Visc Med.* 2017;33(1):70-75.
5. Merkel S, Weber K, Croner RS, et al. Distant metastases in colorectal carcinoma: A proposal for a new M1 subclassification. *Eur J Surg Oncol.* 2016;42(9):1337-1342.
6. Dienstmann R. Tumor Side as Model of Integrative Molecular Classification of Colorectal Cancer. *Clin Cancer Res.* 2018;24(5):989-990.
7. Ghidini M, Petrelli F, Tomasello G. Right Versus Left Colon Cancer: Resectable and Metastatic Disease. *Curr Treat Options Oncol.* 2018;19(6):31.
8. Lee MS, Menter DG, Kopetz S. Right Versus Left Colon Cancer Biology: Integrating the Consensus Molecular Subtypes. *J Natl Compr Canc Netw.* 2017;15(3):411-419.
9. Sugai T, Habano W, Jiao YF, et al. Analysis of molecular alterations in left- and right-sided colorectal carcinomas reveals distinct pathways of carcinogenesis: proposal for new molecular profile of colorectal carcinomas. *J Mol Diagn.* 2006;8(2):193-201.
10. Lim DR, Kuk JK, Kim T, et al. Comparison of oncological outcomes of right-sided colon cancer versus left-sided colon cancer after curative resection: Which side is better outcome? *Medicine (Baltimore).* 2017;96(42):e8241.
11. Powell AG, Wallace R, McKee RF, et al. The relationship between tumour site, clinicopathological characteristics and cancer-specific survival in patients undergoing surgery for colorectal cancer. *Colorectal Dis.* 2012;14(12):1493-1499.
12. Saltzstein SL, Behling CA. Age and time as factors in the left-to-right shift of the subsite of colorectal adenocarcinoma: a study of 213,383 cases from the California Cancer Registry. *J Clin Gastroenterol.* 2007;41(2):173-177.
13. Loree JM, Pereira AAL, Lam M, et al. Classifying Colorectal Cancer by Tumor Location Rather than Sidedness Highlights a Continuum in Mutation Profiles and Consensus Molecular Subtypes. *Clin Cancer Res.* 2018;24(5):1062-1072.

14. Salem ME, Weinberg BA, Xiu J, et al. Comparative molecular analyses of left-sided colon, right-sided colon, and rectal cancers. *Oncotarget.* 2017;8(49):86356-86368.
15. Shimada Y, Kameyama H, Nagahashi M, et al. Comprehensive genomic sequencing detects important genetic differences between right-sided and left-sided colorectal cancer. *Oncotarget.* 2017;8(55):93567-93579.
16. Takahashi Y, Sugai T, Habano W, et al. Molecular differences in the microsatellite stable phenotype between left-sided and right-sided colorectal cancer. *Int J Cancer.* 2016;139(11):2493-2501.
17. Foltran L, De Maglio G, Pella N, et al. Prognostic role of KRAS, NRAS, BRAF and PIK3CA mutations in advanced colorectal cancer. *Future Oncol.* 2015;11(4):629-640.
18. Chang L, Chang M, Chang HM, et al. Expending Role of Microsatellite Instability in Diagnosis and Treatment of Colorectal Cancers. *J Gastrointest Cancer.* 2017;48(4):305-313.
19. Bohn BA, Mina S, Krohn A, et al. Altered PTEN function caused by deletion or gene disruption is associated with poor prognosis in rectal but not in colon cancer. *Hum Pathol.* 2013;44(8):1524-1533.
20. Ross JS, Fakih M, Ali SM, et al. Targeting HER2 in colorectal cancer: The landscape of amplification and short variant mutations in ERBB2 and ERBB3. *Cancer.* 2018;124(7):1358-1373.
21. Pugh SA, Shinkins B, Fuller A, et al. Site and Stage of Colorectal Cancer Influence the Likelihood and Distribution of Disease Recurrence and Postrecurrence Survival: Data From the FACS Randomized Controlled Trial. *Ann Surg.* 2016;263(6):1143-1147.
22. Petrelli F, Tomasello G, Borgonovo K, et al. Prognostic Survival Associated With Left-Sided vs Right-Sided Colon Cancer: A Systematic Review and Meta-analysis. *JAMA Oncol.* 2017;3(2):211-219.
23. Strickler JH, Wu C, Bekaii-Saab T. Targeting BRAF in metastatic colorectal cancer: Maximizing molecular approaches. *Cancer Treat Rev.* 2017;60:109-119.
24. Peeters M, Kafatos G, Taylor A, et al. Prevalence of RAS mutations and individual variation patterns among patients with metastatic colorectal cancer: A pooled analysis of randomised controlled trials. *Eur J Cancer.* 2015;51(13):1704-1713.
25. Rosty C, Young JP, Walsh MD, et al. PIK3CA activating mutation in colorectal carcinoma: associations with molecular features and survival. *PLoS One.* 2013;8(6):e65479.
26. Yaeger R, Cowell E, Chou JF, et al. RAS mutations affect pattern of metastatic spread and

increase propensity for brain metastasis in colorectal cancer. *Cancer.* 2015;121(8):1195-1203.

27. Van Cutsem E, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. *Ann Oncol.* 2016;27(8):1386-1422.
28. de Gramont A, Figer A, Seymour M, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol.* 2000;18(16):2938-2947.
29. Douillard JY, Group VS. Irinotecan and high-dose fluorouracil/leucovorin for metastatic colorectal cancer. *Oncology (Williston Park).* 2000;14(12 Suppl 14):51-55.
30. Ellis LM, Rosen L, Gordon MS. Overview of anti-VEGF therapy and angiogenesis. Part 1: Angiogenesis inhibition in solid tumor malignancies. *Clin Adv Hematol Oncol.* 2006;4(1):suppl 1-10; quz 11-12.
31. Cunningham D, Lang I, Marcuello E, et al. Bevacizumab plus capecitabine versus capecitabine alone in elderly patients with previously untreated metastatic colorectal cancer (AVEX): an open-label, randomised phase 3 trial. *Lancet Oncol.* 2013;14(11):1077-1085.
32. Kabbinavar FF, Wallace JF, Holmgren E, et al. Health-related quality of life impact of bevacizumab when combined with irinotecan, 5-fluorouracil, and leucovorin or 5-fluorouracil and leucovorin for metastatic colorectal cancer. *Oncologist.* 2008;13(9):1021-1029.
33. Saltz LB, Clarke S, Diaz-Rubio E, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol.* 2008;26(12):2013-2019.
34. Passardi A, Nanni O, Tassinari D, et al. Effectiveness of bevacizumab added to standard chemotherapy in metastatic colorectal cancer: final results for first-line treatment from the ITACa randomized clinical trial. *Ann Oncol.* 2015;26(6):1201-1207.
35. Souglakos J, Ziras N, Kakolyris S, et al. Randomised phase-II trial of CAPIRI (capecitabine, irinotecan) plus bevacizumab vs FOLFIRI (folinic acid, 5-fluorouracil, irinotecan) plus bevacizumab as first-line treatment of patients with unresectable/metastatic colorectal cancer (mCRC). *Br J Cancer.* 2012;106(3):453-459.
36. Cremolini C, Loupakis F, Antoniotti C, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. *Lancet Oncol.* 2015;16(13):1306-1315.

37. Giantonio BJ, Catalano PJ, Meropol NJ, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol.* 2007;25(12):1539-1544.
38. Bennouna J, Sastre J, Arnold D, et al. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *Lancet Oncol.* 2013;14(1):29-37.
39. Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med.* 2013;369(11):1023-1034.
40. Petrelli F, Ardito R, Ghidini A, et al. Different Toxicity of Cetuximab and Panitumumab in Metastatic Colorectal Cancer Treatment: A Systematic Review and Meta-Analysis. *Oncology.* 2018;94(4):191-199.
41. Qin S, Li J, Wang L, et al. Efficacy and Tolerability of First-Line Cetuximab Plus Leucovorin, Fluorouracil, and Oxaliplatin (FOLFOX-4) Versus FOLFOX-4 in Patients With RAS Wild-Type Metastatic Colorectal Cancer: The Open-Label, Randomized, Phase III TAILOR Trial. *J Clin Oncol.* 2018:JCO2018783183.
42. Battaglin F, Dadduzio V, Bergamo F, et al. Anti-EGFR monoclonal antibody panitumumab for the treatment of patients with metastatic colorectal cancer: an overview of current practice and future perspectives. *Expert Opin Biol Ther.* 2017;17(10):1297-1308.
43. Wang L, Wei Y, Fang W, et al. Cetuximab Enhanced the Cytotoxic Activity of Immune Cells during Treatment of Colorectal Cancer. *Cell Physiol Biochem.* 2017;44(3):1038-1050.
44. Misale S, Di Nicolantonio F, Sartore-Bianchi A, et al. Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution. *Cancer Discov.* 2014;4(11):1269-1280.
45. Zhao B, Wang L, Qiu H, et al. Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. *Oncotarget.* 2017;8(3):3980-4000.
46. Van Emburgh BO, Arena S, Siravegna G, et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. *Nat Commun.* 2016;7:13665.
47. Van Cutsem E, Kohne CH, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med.* 2009;360(14):1408-1417.
48. Van Cutsem E, Kohne CH, Lang I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol.* 2011;29(15):2011-2019.

49. Van Cutsem E, Lenz HJ, Kohne CH, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J Clin Oncol.* 2015;33(7):692-700.
50. Heinemann V, von Weikersthal LF, Decker T, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol.* 2014;15(10):1065-1075.
51. Venook AP, Tabernero J. Progression-free survival: helpful biomarker or clinically meaningless end point? *J Clin Oncol.* 2015;33(1):4-6.
52. Holch JW, Ricard I, Stintzing S, et al. The relevance of primary tumour location in patients with metastatic colorectal cancer: A meta-analysis of first-line clinical trials. *Eur J Cancer.* 2017;70:87-98.
53. Cremolini C, Antoniotti C, Moretto R, et al. First-line therapy for mCRC - the influence of primary tumour location on the therapeutic algorithm. *Nat Rev Clin Oncol.* 2017;14(2):113.
54. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol.* 2014;32(6):579-586.
55. Khan KH, Cunningham D, Werner B, et al. Longitudinal Liquid Biopsy and Mathematical Modeling of Clonal Evolution Forecast Time to Treatment Failure in the PROSPECT-C Phase II Colorectal Cancer Clinical Trial. *Cancer Discov.* 2018;8(10):1270-1285.
56. Siravegna G, Marsoni S, Siena S, et al. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol.* 2017;14(9):531-548.
57. Zhang W, Xia W, Lv Z, et al. Liquid Biopsy for Cancer: Circulating Tumor Cells, Circulating Free DNA or Exosomes? *Cell Physiol Biochem.* 2017;41(2):755-768.
58. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol.* 2013;200(4):373-383.
59. Yoshioka Y, Katsuda T, Ochiya T. Extracellular vesicles and encapusulated miRNAs as emerging cancer biomarkers for novel liquid biopsy. *Jpn J Clin Oncol.* 2018;48(10):869-876.
60. Hibner G, Kimsa-Furdzik M, Francuz T. Relevance of MicroRNAs as Potential Diagnostic and Prognostic Markers in Colorectal Cancer. *Int J Mol Sci.* 2018;19(10).
61. Peng Y, Croce CM. The role of MicroRNAs in human cancer. *Signal Transduct Target Ther.* 2016;1:15004.
62. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov.* 2017;16(3):203-222.

63. Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metastasis Rev.* 2009;28(3-4):369-378.
64. Janas T, Janas MM, Sapon K, et al. Mechanisms of RNA loading into exosomes. *FEBS Lett.* 2015;589(13):1391-1398.
65. Becker A, Thakur BK, Weiss JM, et al. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell.* 2016;30(6):836-848.
66. Tominaga N, Kosaka N, Ono M, et al. Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. *Nat Commun.* 2015;6:6716.
67. Normanno N, Cervantes A, Ciardiello F, et al. The liquid biopsy in the management of colorectal cancer patients: Current applications and future scenarios. *Cancer Treat Rev.* 2018;70:1-8.
68. Duffy MJ, Sturgeon C, Lamerz R, et al. Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report. *Ann Oncol.* 2010;21(3):441-447.
69. Wang G, Fu Z, Li D. MACC1 overexpression and survival in solid tumors: a meta-analysis. *Tumour Biol.* 2015;36(2):1055-1065.
70. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer.* 2009;45(2):228-247.
71. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol.* 2018;15(2):81-94.
72. Venesio T, Siravegna G, Bardelli A, et al. Liquid Biopsies for Monitoring Temporal Genomic Heterogeneity in Breast and Colon Cancers. *Pathobiology.* 2018;85(1-2):146-154.
73. Hench IB, Hench J, Tolnay M. Liquid Biopsy in Clinical Management of Breast, Lung, and Colorectal Cancer. *Front Med (Lausanne).* 2018;5:9.
74. Khakoo S, Georgiou A, Gerlinger M, et al. Circulating tumour DNA, a promising biomarker for the management of colorectal cancer. *Crit Rev Oncol Hematol.* 2018;122:72-82.
75. Arneth B. Update on the types and usage of liquid biopsies in the clinical setting: a systematic review. *BMC Cancer.* 2018;18(1):527.
76. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med.* 2008;14(9):985-990.
77. Kidess E, Jeffrey SS. Circulating tumor cells versus tumor-derived cell-free DNA: rivals or partners in cancer care in the era of single-cell analysis? *Genome Med.* 2013;5(8):70.
78. Strickler JH, Loree JM, Ahronian LG, et al. Genomic Landscape of Cell-Free DNA in Patients with Colorectal Cancer. *Cancer Discov.* 2018;8(2):164-173.

79. Burrell RA, McGranahan N, Bartek J, et al. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature.* 2013;501(7467):338-345.
80. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature.* 2013;501(7467):328-337.
81. Prasetyanti PR, Medema JP. Intra-tumor heterogeneity from a cancer stem cell perspective. *Mol Cancer.* 2017;16(1):41.
82. Russo M, Siravegna G, Blaszkowsky LS, et al. Tumor Heterogeneity and Lesion-Specific Response to Targeted Therapy in Colorectal Cancer. *Cancer Discov.* 2016;6(2):147-153.
83. Siravegna G, Mussolin B, Buscarino M, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat Med.* 2015;21(7):795-801.
84. Misale S, Yaeger R, Hobor S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature.* 2012;486(7404):532-536.
85. De Figueiredo Barros BD, Kupper BEC, Aguiar Junior S, et al. Mutation Detection in Tumor-Derived Cell Free DNA Anticipates Progression in a Patient With Metastatic Colorectal Cancer. *Front Oncol.* 2018;8:306.
86. Sun Q, Liu Y, Liu B, et al. Use of Liquid Biopsy in Monitoring Colorectal Cancer Progression Shows Strong Clinical Correlation. *Am J Med Sci.* 2018;355(3):220-227.
87. Palmirotta R, Lovero D, Cafforio P, et al. Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology. *Ther Adv Med Oncol.* 2018;10:1758835918794630.
88. Siravegna G, Corcoran RB. Blood-Based Prediction of Tumor Relapse: The cfDNA Forecast. *Cancer Discov.* 2018;8(10):1213-1215.
89. Diaz LA, Jr., Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature.* 2012;486(7404):537-540.
90. Montagut C, Dalmases A, Bellosillo B, et al. Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. *Nat Med.* 2012;18(2):221-223.
91. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol.* 2008;26(35):5705-5712.
92. Markman B, Atzori F, Perez-Garcia J, et al. Status of PI3K inhibition and biomarker development in cancer therapeutics. *Ann Oncol.* 2010;21(4):683-691.
93. Chen Y, Huang R, Ding J, et al. Multiple myeloma acquires resistance to EGFR inhibitor via induction of pentose phosphate pathway. *Sci Rep.* 2015;5:9925.

94. Sclafani F, Kim TY, Cunningham D, et al. A Randomized Phase II/III Study of Dalotuzumab in Combination With Cetuximab and Irinotecan in Chemorefractory, KRAS Wild-Type, Metastatic Colorectal Cancer. *J Natl Cancer Inst.* 2015;107(12):djv258.
95. Ciardiello F, Bianco R, Caputo R, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res.* 2004;10(2):784-793.
96. Gherardi E, Birchmeier W, Birchmeier C, et al. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer.* 2012;12(2):89-103.
97. Bertotti A, Migliardi G, Galimi F, et al. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discov.* 2011;1(6):508-523.
98. Boyerinas B, Park SM, Hau A, et al. The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer.* 2010;17(1):F19-36.
99. Dahiya N, Sherman-Baust CA, Wang TL, et al. MicroRNA expression and identification of putative miRNA targets in ovarian cancer. *PLoS One.* 2008;3(6):e2436.
100. O'Hara AJ, Wang L, Dezube BJ, et al. Tumor suppressor microRNAs are underrepresented in primary effusion lymphoma and Kaposi sarcoma. *Blood.* 2009;113(23):5938-5941.
101. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 2004;64(11):3753-3756.
102. Wang X, Cao L, Wang Y, et al. Regulation of let-7 and its target oncogenes (Review). *Oncol Lett.* 2012;3(5):955-960.
103. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell.* 2005;120(5):635-647.
104. Geng L, Zhu B, Dai BH, et al. A let-7/Fas double-negative feedback loop regulates human colon carcinoma cells sensitivity to Fas-related apoptosis. *Biochem Biophys Res Commun.* 2011;408(3):494-499.
105. Wang T, Wang G, Hao D, et al. Aberrant regulation of the LIN28A/LIN28B and let-7 loop in human malignant tumors and its effects on the hallmarks of cancer. *Mol Cancer.* 2015;14:125.
106. Boominathan L. The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. *PLoS One.* 2010;5(5):e10615.

107. Barh D, Malhotra R, Ravi B, et al. MicroRNA let-7: an emerging next-generation cancer therapeutic. *Curr Oncol.* 2010;17(1):70-80.
108. Zheng Q, Chen C, Guan H, et al. Prognostic role of microRNAs in human gastrointestinal cancer: A systematic review and meta-analysis. *Oncotarget.* 2017;8(28):46611-46623.
109. Wang J, Huang SK, Zhao M, et al. Identification of a circulating microRNA signature for colorectal cancer detection. *PLoS One.* 2014;9(4):e87451.
110. Yang J, Ma D, Fesler A, et al. Expression analysis of microRNA as prognostic biomarkers in colorectal cancer. *Oncotarget.* 2017;8(32):52403-52412.
111. Nakajima G, Hayashi K, Xi Y, et al. Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are Associated with Chemoresponse to S-1 in Colon Cancer. *Cancer Genomics Proteomics.* 2006;3(5):317-324.
112. Ohshima K, Inoue K, Fujiwara A, et al. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. *PLoS One.* 2010;5(10):e13247.
113. Vishnubalaji R, Hamam R, Abdulla MH, et al. Genome-wide mRNA and miRNA expression profiling reveal multiple regulatory networks in colorectal cancer. *Cell Death Dis.* 2015;6:e1614.
114. Qian P, Zuo Z, Wu Z, et al. Pivotal role of reduced let-7g expression in breast cancer invasion and metastasis. *Cancer Res.* 2011;71(20):6463-6474.
115. Chen KJ, Hou Y, Wang K, et al. Reexpression of Let-7g microRNA inhibits the proliferation and migration via K-Ras/HMGA2/snail axis in hepatocellular carcinoma. *Biomed Res Int.* 2014;2014:742417.
116. Weidhaas JB, Babar I, Nallur SM, et al. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. *Cancer Res.* 2007;67(23):11111-11116.
117. Cappuzzo F, Sacconi A, Landi L, et al. MicroRNA signature in metastatic colorectal cancer patients treated with anti-EGFR monoclonal antibodies. *Clin Colorectal Cancer.* 2014;13(1):37-45 e34.
118. Ruzzo A, Graziano F, Vincenzi B, et al. High let-7a microRNA levels in KRAS-mutated colorectal carcinomas may rescue anti-EGFR therapy effects in patients with chemotherapy-refractory metastatic disease. *Oncologist.* 2012;17(6):823-829.
119. Saridaki Z, Weidhaas JB, Lenz HJ, et al. A let-7 microRNA-binding site polymorphism in KRAS predicts improved outcome in patients with metastatic colorectal cancer treated with salvage cetuximab/panitumumab monotherapy. *Clin Cancer Res.* 2014;20(17):4499-4510.

120. Gasparri ML, Casorelli A, Bardhi E, et al. Beyond circulating microRNA biomarkers: Urinary microRNAs in ovarian and breast cancer. *Tumour Biol.* 2017;39(5):1010428317695525.
121. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990;61(5):759-767.

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