



## Circulating biomarkers for early detection and clinical management of colorectal cancer

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### ABSTRACT

New non-invasive approaches that can complement and improve on current strategies for colorectal cancer (CRC) screening and management are urgently needed. A growing number of publications have documented that components of tumors, which are shed into the circulation, can be detected in the form of liquid biopsies and can be used to detect CRC at early stages, to predict response to certain therapies and to detect CRC recurrence in a minimally invasive way. The analysis of circulating tumor DNA (ctDNA), tumor-derived cells (CTC, circulating tumor cells) or circulating microRNA (miRNA) in blood and other body fluids, have a great potential to improve different aspects of CRC management. The challenge now is to find which types of components, biofluids and detection methods would be the most suitable to be applied in the different steps of CRC detection and treatment. This chapter will provide an up to date review on ctDNA, CTCs and circulating miRNAs as new biomarkers for CRC, either for clinical management or early detection, highlighting their advantages and limitations.

### 1. Introduction

Colorectal cancer (CRC) is a major worldwide public health issue being one of the most frequent types of solid cancer in developed countries. Incidence rates of CRC show a strong positive gradient with an increasing level of economic development (Arnold et al., 2016). It is predicted that, by 2040 the number of cases will have risen to 1800 million now to 3093 million in 2040 (<http://gco.iarc.fr/tomorrow/home>). Moreover, despite all the efforts of modern medicine, the

prognosis of CRC patients predominantly depends on the stage of the disease at diagnosis (Norci, 2018). It is well known that early detection of CRC has an impact in decreasing its associated mortality and detection of its precursor lesion can even reduce the incidence, however, current CRC screening strategies still have many limitations (Thorsteinnsson and Jess, 2011). Therefore, it is extremely important to find new non-invasive biomarkers that could be well accepted by the population and that could detect tumor presence in asymptomatic early stages when CRC is still curable.

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On the other hand, the management of CRC patients has relied for decades on rather robust diagnostic methods including biopsy-based techniques, such as colonoscopy and histopathological analysis of tumor tissue, and imaging methods, which comprise x-rays examination, computed tomography and magnetic resonance. However, the clinical disease assessment and tumor stage determination based on these analyses cannot provide clinically useful prognostic and predictive information at the level of each patient (Amin et al., 2017). For this reason, both basic and clinical scientists are constantly searching for more reliable individual biomarkers to prevent the disease relapse, occurrence of severe side effects caused by chemotherapy and the development of treatment resistance which would be finally leading to improving patients' survival and quality of life.

The presence of tumor traits in a patient's bloodstream and other body fluids has been identified as a potential individual biomarker with diagnostic, prognostic and predictive importance. It has been shown in numerous studies that the detection of so-called circulating tumor cells (CTCs) in the peripheral blood of patients evinces a negative impact on their survival (Jia et al., 2017; Rahbari et al., 2010; Tan et al., 2016; Thorsteinsson and Jess, 2011). Due to the similarity with conventional tumor tissue biopsy, the term “liquid biopsy” has been introduced for CTCs detection from blood (Alix-Panabieres and Pantel, 2013). In contrast to tissue biopsy, liquid biopsy provides several advantages, such as quick and easy extraction from patients, low cost and minimal pain and risk for patients due to its minimal invasiveness.

Lately, it was observed that not only the presence of CTCs but also the presence of circulating tumor DNA (ctDNA) is of clinical importance (Barault et al., 2018; Diehl et al., 2008). Furthermore, the biological role of microRNAs (miRNAs) in the pathogenesis of CRC is becoming evident and their presence in the patients' peripheral blood has also been proposed as an important predictive/prognostic biomarker (Herrerros-Villanueva et al., 2019; Kral et al., 2018; Schetter et al., 2012). Therefore, the term “liquid biopsy” has recently been broadened and is currently used for the detection of all circulating tumor traits (mainly CTCs, ctDNA, circulating miRNAs, as well as others such as exosomes, proteins, mRNAs and others ...) in the peripheral blood of patients or other biological fluids (Marrugo-Ramírez et al., 2018). The concept of liquid biopsy/circulating biomarker is slowly approaching clinical practice in the form of numerous clinical studies. In this comprehensive review, the source, characteristics, and detection technologies for ctDNA, CTCs, and circulating miRNAs, as well as their potential use in the diagnosis, recurrence monitoring, and prognostic assessment for CRC tumors are described.

## 2. Circulating tumor cells in colorectal cancer

### 2.1. General introduction to circulating tumor cells (CTCs)

CTCs denote epithelial cancer cells, which gained access to the circulatory system and are detectable in peripheral blood. CTCs are of high interest because they can provide direct access to systemic cancer during all stages of cancer development in the sense of a “liquid biopsy”. This does not only provide the opportunity for better understanding the biology underlying dissemination and metastasis, but also to use these cells as biomarkers to detect, analyse, and treat systemic cancer more effectively. However, because blood concentration of CTCs is extremely low and cancer specific markers are missing, their detection remains challenging and poses some limitations upon their value as diagnostic tool. Addressing these challenges, numerous platforms have been established to detect CTCs in blood samples. The goal of this chapter is to inform the reader about the state of CTC analysis and its clinical value in CRC.

### 2.2. CTC enrichment and detection

A major technological breakthrough to detect the extremely rare

CTCs was described by Racila and colleagues in 1998 (Racila et al., 1998). They applied immunomagnetic CTC enrichment using an antibody directed against the epithelial cell adhesion molecule (EpCAM) coupled to ferrofluids and combined it with flow cytometry for CTC detection. This approach led to the development of the CellSearch® system (CS), which is today the widely-used gold standard and still the only FDA-approved method for CTC-detection. Since then, more than 45 different platforms for CTC-detection became commercially available (technologies reviewed in more detail by Ferreira and colleagues (Ferreira et al., 2016)). While few of them are integrated systems, for enrichment and detection, most only provide tools for CTC enrichment. Generally, CTC-detection is performed in a two-step process consisting of an enrichment step and a detection step. In the absence of cancer-specific markers, CTCs are mainly separated from the excess hematopoietic cells by their (remnant) epithelial features by means of certain physical and biological properties (Joosse et al., 2015; Stoecklein et al., 2016). These physical properties include cell size, density, deformability as well as dielectric characteristics, while biological properties are related to the expression of certain epithelial proteins or the absence of leukocyte markers on the cell surface. Enrichment methods exploiting physical features of CTCs are versatile but currently dominated by size-based separation approaches, e.g. filtration. However, the lack of specificity of the biophysical separation methods poses a certain disadvantage, since these characteristics often overlap, at least in part, between CTCs and leukocytes. For example, filtration devices with 8 µm pore sizes (e.g. ISET - Isolation by Size of Epithelial Tumor cells) will lose obviously all smaller CTCs together with the leukocytes passing through the filter (Stoecklein et al., 2016).

Enrichment methods based on biological properties are less diverse and mainly involve immuno-magnetic separation with antibodies either directed against certain epithelial/cancer-associated proteins for positive enrichment or against hematopoietic cell markers, such as the CD45 leukocyte antigen, for depletion. So far, EpCAM has been the most widely used surface protein for positive selection. EpCAM is specific for epithelial cells and is strongly expressed in most carcinomas, while its expression in non-epithelial cells is restricted to embryonic stem cells and rare subsets of adult stem and progenitor cells (Gires and Stoecklein, 2014). However, it is important to note that EpCAM is not a universal cancer marker: in some cancer types (e.g. squamous cell carcinomas) EpCAM expression is quite heterogeneous/absent or can become down-regulated if cancer cells undergo epithelial-to-mesenchymal transition (EMT) and therefore escape capturing process (Gires and Stoecklein, 2014; Lim et al., 2014). Bottom line for all enrichment methods is that no technique is 100% efficient and that there always will be a cell-loss, which cannot be determined for the individual sample. Moreover, none of the enrichment methods delivers pure CTCs and a background of few, up to several thousand white blood cells are commonly observed (Stoecklein et al., 2016). Thus, a second step for detection of the CTCs is required for which different experimental strategies have been developed. The most widely used approach is multi-marker immunofluorescence staining to identify cells with epithelial character. This is accomplished by the use of antibodies against cytokeratins (CK) as typical epithelial intermediate filaments and against CD45 as exclusion marker labelling leukocytes, as well as DAPI for nuclear staining to identify intact cells. Such immunostainings are also a component of most (semi-)automated CTC-detection platforms, such as CS, or those from Epic Sciences (Werner et al., 2015) and RareCyte (Campton et al., 2015). However, despite the widely accepted phenotype EpCAM+/CK+/CD45-/DAPI+, initially established for CS system, it is important to stress that a commonly defined CTC phenotype is lacking and that different technologies usually use different antibody clones, making comparison and standardization across different platforms challenging.

A second important CTC-detection method is based on nucleic acid identification by PCR methods for DNA mutation analysis, epigenetic analysis, or mRNA profiling for epithelial and/or malignancy-

associated gene expression (Hardingham et al., 2000, 1995; Helo et al., 2009; Lianidou and Markou, 2011; Müller et al., 2012; Pixberg et al., 2015; Sieuwerts et al., 2009; Smirnov et al., 2005; Zieglschmid et al., 2007). In the case of CRC, CK20 mRNA has been frequently used for CTC detection (Hardingham et al., 2015). One of the most prominent commercial kits for CTC-detection by mRNA profiling is the AdnaTest®, a multiplex reverse-transcription (RT)-PCR for a panel of genes offering the possibility for molecular characterization of CTCs in clinical diagnostics (Zieglschmid et al., 2007). While PCR-based CTC detection might be helpful in specific clinical situations (e.g. prognostic test or screening for resistance markers), they do not allow isolation of intact cells for further molecular analysis. Another approach for detection is the Epispot assay, an in vitro functional assay based on immunofluorescence detection of secreted proteins as markers derived from viable cancer cells in short-term culture (Alix-Panabières, 2012).

### 2.3. CTCs as prognostic marker in CRC patients

The initial driver for CTC research in CRC, that already started in the 1950ies, was the question whether tumor cell dissemination into tumor draining veins is already present in operable cancer patients and whether this is associated with increased metastatic risk. In one of the earliest studies, Engell described that CTCs in tumor draining veins as a quite frequent event (> 50%) in a series of over 100 CRC patients (Engell, 1955). These findings were corroborated by other investigators (Pruitt et al., 1958; Roberts et al., 1961) and, although discussed controversially at the time (Swinton, 1956), these pioneering investigations helped to establish the concept of adjuvant therapy in the 1950-60ies (Economou et al., 1958; Fisher et al., 1968; Stoecklein et al., 2016). The potential of detecting micrometastatic disease was of immediate interest to better identify high-risk patients who may benefit from additional systemic therapies. However, these cytologic analyses without effective CTC-enrichment were quite far away from routine use. This changed with the advent of sensitive molecular detection techniques. For example, Hardigan and colleagues pioneered PCR-based CTC-detection of blood samples immuno-enriched for EpCAM-positive cells; initially by PCR-detection of mutated KRAS and later by RT-PCR detection of CK19, CK20 and MUC2 (Hardingham et al., 2000). They observed, with an immunobead RT-PCR assay, that CTC detection (42% positive rate) correlated with shorter disease-free survival (hazard ratio [HR] 2.8, 95% confidence interval [CI] 1.169–6.716) suggesting CTCs as an adjunct for more accurate staging in stage III CRC patients. In fact, a meticulous meta-analysis by Rahbari and colleagues could confirm the overall prognostic impact of CTCs in non-metastatic CRC (Rahbari et al., 2010), which was further substantiated by a recent meta-analysis just focusing on RT-PCR studies (C. C. Yang et al., 2017). This meta-analysis included 12 eligible studies with a total of 2363 non-metastatic CRC patients and revealed a shorter overall survival (HR = 3.07, 95% CI: 2.05–4.624,  $P < 0.001$ ) and disease-free survival (HR = 2.58, 95% CI: 2.00–3.32,  $P < 0.001$ ) for CTC-positive patients determined by RT-PCR assays, regardless of the sampling time, adjuvant therapy and TNM stage. The authors concluded that detection of CTCs by RT-PCR has not only prognostic value for non-metastatic CRC patients but might be also used as an indicator of metastatic disease, prior to routine clinical imaging. However, it is important to stress that despite absent expression of CTC marker-mRNAs in blood cells, increased expression of typical CTC markers such as CK19, CK20 or CEA (carcinoembryonic antigen) was observed repeatedly in blood samples from donors without cancer (Hardingham et al., 2015). It was therefore stated that primer-probe combinations, enrichment strategies (e.g. density gradient centrifugation or immunomagnetic separation) as well as methods for RNA extraction can all influence background expression but are not yet well standardized for CTC detection (Sergeant et al., 2008). A standardized and semi-automated approach for CTC-detection became available with the FDA-cleared CS system, which aims to detect and document intact CTCs. After being extensively tested in healthy subjects, patients with

benign and diverse malignant epithelial tumors (Allard, 2004), the initial prospective multicenter study using this system in CRC was performed in 430 patients with metastatic disease by Cohen and colleagues in patients at baseline and after starting first, second, or third line therapies (Cohen et al., 2008). Patients with  $\geq 3$  CTCs/7.5 mL blood had a shorter median progression-free survival (4.5 v 7.9 months;  $p = 0.0002$ ) and overall survival (9.4 v 18.5 months;  $P = 0.0001$ ). Notably, this prognostic impact persisted at several different follow-up time points after therapy. Conversion of the baseline CTC count from  $\geq 3$  to  $< 3$  at 3–5 weeks was associated with significantly longer survival times compared with patients with  $\geq 3$  CTCs at both time points ( $P = 0.0002$ ). From their data, the authors concluded that CTCs add relevant prognostic information to routine clinical imaging. Several following studies were in line with these initial data, including two prospective studies in over 450 mCRC patients (Aggarwal et al., 2013; Sastre et al., 2012; Sotelo et al., 2015). The prognostic utility was further corroborated by the meta-analysis by Tan and Wu, including 15 studies with 3129 patients (Tan and Wu, 2018). Ten of these studies were performed with the CS system. Their analyses disclosed presence of CTCs to be significantly associated with poor prognosis (overall survival: HR = 2.36, 95% CI: 1.87–2.97;  $P = 0.006$ ) as well as with disease progression (progression-free survival: HR = 1.83, 95% CI: 1.42–2.36;  $P = 0.00001$ ), and confirmed the prognostic significance for the CS group. Also, the meta-analysis of Huang and colleagues (Huang et al., 2014) of 11 studies containing 1847 patients with CRC clearly demonstrated the prognostic significance of CS CTCs in CRC (overall survival: HR = 2.00, 95% CI: 1.49, 2.69,  $P < 0.01$  and progression-free survival: HR = 1.80, 95% CI: 1.52, 2.13,  $P < 0.01$ ). Another interesting meta-analysis including 13 studies with 2388 CRC patients under chemotherapy (Huang et al., 2014) supported the claim that CTCs could be useful as a surrogate marker for the response to chemotherapy and to provide additional prognostic information to radiographic imaging of the tumor. Nevertheless, the authors stressed that well-designed, large-scale multicenter studies are required to explore whether an individualized therapeutic decision based on CTC levels would improve the prognosis of CRC patients (Huang et al., 2014). Conflicting data on the prognostic value of CS CTCs were reported in the non-metastatic situation. While two studies reported a significant prognostic impact of CS CTCs in non-metastatic CRC (Bork et al., 2015; VAN DALUM et al., 2015), Sotelo and colleagues (Sotelo et al., 2015) investigated pre-operative blood samples in 519 stage III CRC patients and did not find any prognostic impact for  $> 1$ ,  $> 2$  or  $> 3$  CTCs/7.5 mL, respectively. Potential reasons for the conflicting results are the relatively short follow-up time of the Sotelo study and the low overall CTC detection frequency with CS in CRC. In fact, even in mCRC detection rates are lower than compared to breast or prostate cancer, with a negativity rate of around 50% for the FDA cleared protocol analysing 7.5 ml blood (Allard, 2004). The low detection rate of CTCs in CRC triggered three strategies to increase sensitivity: one approach aims to detect CRC cells that do not express EpCAM., Since EMT-like processes may impact the expression on disseminating CTCs (Lim et al., 2014) and rapid down-regulation of EpCAM upon entering the blood stream has been reported (Gorges et al., 2012), combining the CS assay with another, EpCAM-independent, assay might be beneficial. This strategy was followed by the Pantel group analysing, in parallel, 47 blood samples from mCRC patients using CS and the AdnaTest® (Gorges et al., 2016). Combined analysis of both assays led to an improved detection CTC rate of 50% compared to 33% with CS alone and CTC-positivity could be correlated with poor survival. A second approach to increase CRC CTC detection rates is to draw blood from the mesenteric vein before first-pass effects in the liver can negatively affect CTC concentration in the periphery. For example, Rahbari and colleagues demonstrated a higher detection frequency (35% vs. 17.5%) and higher mean CTC counts (1.5 vs 0.3 CTC/7.5 mL) in blood taken from the mesenteric vein when compared to a central venous blood draw (Rahbari et al., 2012). A similar result was obtained by the Alix-

Panabières group in mCRC using in parallel the CS assay and the Epi-spot assay (analysis of CK19 secretion in live CTCs) to compare blood samples from the mesenteric vein and the periphery (Denève et al., 2013). Interestingly, measurement of CTCs in pulmonary veins of patients suffering from CRC lung metastasis also revealed higher detection frequencies when measured by a filtration method, underpinning the significance of local tumor compartments when analysing CTCs and the possibility of relevant cancer cell shedding from metastasis (Le et al., 2018). A third approach to augment the detection rate is to increase the blood volume analysed in CS. This has been done by Terstappen group that examined four sequential aliquots of 7.5 mL (30 mL) for the presence of CTCs with the CS system in 183 patients with newly diagnosed non-metastatic CRC (VAN DALUM et al., 2015). Despite this effort, the frequency was only 24% for patients with > 1 CTC and only five patients displayed more than five CTCs in their samples. However, the authors found a significant correlation between CTCs before surgery and a shorter relapse-free and cancer-specific survival. Interestingly, CTCs detected at diverse follow-up time points after surgery were of different prognostic impact. While CTCs detected weeks after the operation were irrelevant, those detected 2–3 years after surgery had a significant prognostic impact.

#### 2.4. CTCs for CRC screening

As outlined above, CTC detection is infrequent and quite challenging in early stage CRC. Therefore, the utility of CTCs for cancer screening or early detection is barely conceivable. Yet, this was claimed by a recent study presented at ASCO 2018 involving a new CTC assay tested in a single Taiwanese center on 620 participants (182 healthy controls, 111 participants with precancerous lesions, and 327 patients with stage I-IV CRC) (Tsai et al., 2018). The study participants received a routine blood draw, which was analysed with the CellMax biomimetic platform (CMx), in which only 2 mL of the blood are passed through a microfluidic anti-EpCAM-antibody coated biochip (Tien et al., 2016). The CMx CTC results were compared to a standard clinical protocol including colonoscopy and biopsy results, revealing an overall accuracy of 88% for all disease stages, including precancerous lesions. The reported false positive rate for healthy controls was low (3.3%) and the false negative rate for cancer patients was at 16% acceptable. Obviously, these promising data need to be interpreted with caution until independent groups validated the CMx method and replicated these data. While CTC-based screening appears rather challenging, tumor-derived circulating endothelial cell clusters in CRC may present an auspicious cell-based liquid biopsy alternative for early detection (Cima et al., 2016). These benign endothelial cell clusters express both epithelial and mesenchymal markers and shared some phenotypic CTC features, but did not display the genetic alterations of their matched tumors. Comprehensive experiments allowed to trace their origin back to the tumor endothelium and indicated a direct release of the clusters from the tumor vasculature. The isolation and enumeration of these benign cell clusters distinguished healthy volunteers from treatment-naïve as well as pathological early-stage CRC patients with high accuracy, indicating that tumor-derived circulating endothelial cell clusters may be used for non-invasive CRC screening.

#### 2.5. Future directions for CTCs in CRC

The available clinical data clearly demonstrate the prognostic significance of CTCs at all stages of CRC. However, it still has not been conclusively demonstrated that the additional prognostic information is of clinical utility compared to the established prognostic variables, and whether it will improve therapeutic decisions. This warrants further investigation.

The most realistic short-term scenarios for routine clinical application of CTC-detection to benefit CRC patients are to support rational decision making for systemic therapies in the adjuvant as well as the

metastatic setting. The greatest potential for CTC-based liquid biopsies in mCRC lies in the direct molecular analysis of CTCs. Several protocols to isolate CTCs for subsequent molecular profiling have been established. This allows to parse (individual) CTCs for clinically relevant mutations, marker expression or even to profile whole genomes/transcriptomes on the single cell level (e.g. reviewed in (Alberter et al., 2016; Alix-Panabières and Pantel, 2017; Stoecklein et al., 2016)). This might not only allow better therapeutic prediction, but may help to tackle acquired therapeutic resistances. A remaining challenge for CTC-based liquid biopsies is the low detection frequency rendering half of the mCRC patients as not informative. Therefore, recent approaches aim to massively augment the analysed blood volume by use of diagnostic leukapheresis (DLA) (Andree et al., 2018; Fehm et al., 2018; Fischer et al., 2013; Lambros et al., 2018) or functionalized catheters for in vivo CTC capturing (Saucedo-Zeni et al., 2012; Vermesh et al., 2018). Since it was predicted that almost every mCRC patient has EpCAM-positive CTCs in the total blood volume (Coumans et al., 2012), such high-volume approaches could enable real liquid-biopsies for comprehensive CTC-diagnostics.

### 3. Circulating tumor DNA as biomarker for colorectal cancer

#### 3.1. General introduction to circulating tumor DNA (ctDNA)

In addition to cell-free DNA (cfDNA) from non-malignant, predominantly hematopoietic cells, blood samples may contain minute amounts of circulating tumor DNA (ctDNA) derived from primary and metastatic lesions. In a heterogeneity perspective, ctDNA may provide a more comprehensive overview of the spectrum of mutations that is present in a patient's tumor rather than providing a snapshot of a single tissue biopsy. Although ctDNA levels in plasma show great variability within and between tumor types (Bettegowda et al., 2014), ctDNA represents an opportunity for minimal-invasive monitoring of the disease courses and assessment of residual disease after curative surgery (Siravegna et al., 2017; Vymetalkova et al., 2018; Wan et al., 2017). “Omics” sciences offer several high-throughput technologies (including Next Generation Sequencing, NGS) for assaying biomarkers in body fluids, cells and tissues. These technologies are constantly being improved enabling specific detection and measurements of low-abundant targets (Roychowdhury et al., 2011). Technological improvements in the recent years enabled applicability of ctDNA also in early stage cancer and might eventually lead to diagnose CRC even in asymptomatic individuals. However, several factors pose challenges for early detection, including the low abundance of tumor derived DNA, the lower frequency of aberrations in early stage tumors, as well as potentially confounding phenomena such as clonal expansions of non-tumorous tissues (e.g. clonal hematopoiesis of indeterminate potential, CHIP) or the accumulation of cancer-associated mutations with age (Heitzer et al., 2017). To overcome these difficulties, research is moving beyond genetic alterations and liquid biopsy strategies are being extended to additional parameters such as fragment size (Mouliere et al., 2018), nucleosome patterns (Ulz et al., 2016b) or by combining analyses of multiple components (Cohen et al., 2018).

#### 3.2. Methodological aspects of ctDNA analysis

Due to the high variability of ctDNA fractions in plasma (Bettegowda et al., 2014), a variety of methodological approaches has been developed. According to their genomic coverage, methods for ctDNA analysis can be divided into targeted and untargeted approaches (Heitzer et al., 2015). The former methods has been limited to evaluation of single or few biomarkers with the aim of guiding the selection of therapies as it focuses on the investigation of *a priori* known genetic alterations from primary tumors, such as hot-spot mutations in *KRAS*, *BRAF* and *EGFR* genes. The latter approaches allow investigating ctDNA without the previous knowledge of any specific mutations in primary



tumor tissue. Initially, the detection of specific mutations in ctDNA relied upon real-time allele-specific PCR (qPCR) method (Diaz Jr. and Bardelli, 2014). Due to the limited sensitivity (Sn) (0.1–1%) and specificity (Sp) of qPCR approaches, these analyses were predominantly performed in patients with advanced stages and thus with higher ctDNA levels (Dawson et al., 2013). However, in many cases - particularly in those with localized tumors - the proportion of ctDNA can be significantly lower than 0.1% (Bettegowda et al., 2014; Heitzer et al., 2018). Digital PCR (dPCR) approaches have overcome the limitations of qPCR and are now being extensively used in biomarker analyses in body fluids (Diaz Jr. and Bardelli, 2014; Wan et al., 2017). Provided there is enough input material, dPCR is capable of detecting mutant alleles with a fractional abundance from 0.1% down to 0.005% (Day et al., 2013; Sanmamed et al., 2015). Further high resolution PCR-based developments which have been successfully applied for ctDNA analyses include Beads-Emulsion-Amplification and Magnetics (BEAMing) (Diehl et al., 2006), Amplification Refractory Mutations Systems (ARMS) (Spindler et al., 2012) or high-throughput multiplex ultrasensitive mutation detection (UltraSEEK) (Mosko et al., 2016). The main disadvantage of PCR-based approaches is, however, the limited capacity to investigate larger numbers and different kinds of genomic alterations (Yi et al., 2017). In contrast, NGS does not require prior knowledge of the nature of potential genetic changes in the tumor. NGS has extensively been applied for molecular characterization of cfDNA in cancer, and its clinical utility has been proven in large clinical cohorts (Bettegowda et al., 2014; Frenel et al., 2015; Lebofsky et al., 2015). Besides the high throughput, the main advantages of NGS approaches are the ability to detect the presence of mutations (both somatic and germline), copy number alterations (CNAs), and other chromosomal rearrangements, including translocations, transversions and inversions (Dawson et al., 2013; Heitzer et al., 2013; Leary et al., 2012). During the last few years, the analytical sensitivity for detecting genetic alterations in ctDNA has significantly improved also for NGS methods, mostly due to the use of unique molecular identifiers (UMI) and sophisticated bioinformatics algorithms (Heitzer et al., 2018). NGS approaches include single target assays (e.g. Safe-Sequencing System (Safe-SeqS)) (Bettegowda et al., 2014), amplicon based hotspot panels or personalized assays (e.g. bi-Tagged-amplicon deep sequencing (TAm-Seq) (Forsheew et al., 2012), or Circulating Single-Molecule Amplification and Resequencing Technology (cSMART) (Lv et al., 2015) and larger hybrid-capture based gene panels such as Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) (Newman et al., 2014). Although these approaches do not necessarily require knowledge about the genetic composition of the tumor, they are limited in scope as they focus only on regions of interest and are thus unable to reveal all genetic alterations in the tumor. Since, in addition to mutations, aneuploidy or chromosomal rearrangements contribute to tumorigenesis, the assessment of these alterations is equally important. Therefore untargeted, comprehensive approaches like whole exome sequencing (WES) (Dawson et al., 2013; Murtaza et al., 2013) and whole genome sequencing (WGS) (Heitzer et al., 2013; Leary et al., 2012) represent important tools in exploring the tumor biology, intra-tumor heterogeneity and tumor evolution. Although less analytically sensitive than targeted NGS approaches, WES and WGS-based analyses of ctDNA throughout the disease course enabled tracking of clonal genomic evolution associated with treatment response or as a consequence of tumor progression (Murtaza et al., 2013; Ulz et al., 2016a). However, tumor heterogeneity is characterized by many genetic variants present at low frequencies. This poses a challenge for WES/WGS analyses, as their reliable identification requires a high read depth often not realistic for WES/WGS due to the high cost. Therefore, these approaches are only amenable to samples from advanced patients with high tumor fractions.

In addition to genetic alterations, epigenetic changes are represented in ctDNA and several methods for examining the methylation patterns in cfDNA have been published (Daniunaite et al., 2018). Similar to the analyses of genetic alterations, either selected candidate

genes or the entire epigenome is assessed. The sequencing-based methods, i.e. direct bisulfite sequencing (BS) or pyrosequencing enable interrogation of single CpGs. However, conventional PCR-based techniques achieve higher analytical sensitivity and might be better suited for a challenging analyte like ctDNA (Daniunaite et al., 2018). These include Methylation-specific PCR (MSP) (Mitchell et al., 2016) or methylation-sensitive high-resolution melting (MS-HRM) (Avraham et al., 2012) and can already detect as little as 0.1% of methylated DNA whereas TaqMan based MethyLight assays have been shown to be even more sensitive (Philipp et al., 2014; Rezvani et al., 2017). Furthermore, MethyL-BEAMing was demonstrated to digitally quantify cancer-derived methylation patterns in plasma DNA (Li et al., 2009). Likewise, droplet digital PCR (ddPCR) was recently demonstrated as a robust method for the detection of minute amounts of methylated ctDNA (Pharo et al., 2018). Moreover, several studies have demonstrated that interrogation of the entire methylome is feasible, either by employing whole bisulfite sequencing (Cheng et al., 2017) or more recently immunoprecipitation-based protocols (Shen et al., 2018).

### 3.3. ctDNA for the diagnosis of early stage CRC

Stage-dependent survival rates in CRC, which are 94, 82, 67, and 11% for stages I, II, III, and IV, respectively (Lansdorp-Vogelaar et al., 2009; Siegel et al., 2018), clearly reflect the importance of screening and early detection. In the United States, colonoscopy is the predominant screening tool. It has high accuracy, but is expensive, invasive, and unpleasant for the patient, contributing to suboptimal patient compliance (Bretthauer et al., 2016; Liang and Dominitz, 2019). In Europe faecal-based occult blood tests (FOBT and FIT) are more commonly used (Schreuders et al., 2015). Randomized controlled trials have indicated that faecal-based tests reduce CRC mortality. The false positive rate is, however, high, and the sensitivity for precancerous lesions called advanced adenomas (AA) is limited (Hewitson et al., 2008; Morikawa et al., 2005). An attractive alternative to these methods are blood-based detection tests, offering the patient a non-invasive, low-risk test that can easily be performed during routine physical examinations. ctDNA has the potential to diagnose early stage CRC as has been shown using a variety of methods (Wang et al., 2018).

Since aberrant DNA methylation is a characteristic of most solid cancers and has been observed in aberrant crypt foci as well as in pre-malignant adenomas, methylation marks represent a promising biomarker for early diagnosis of cancer (Ahlquist et al., 2008; Danese and Montagnana, 2017; Hanley et al., 2017; Lam et al., 2016). A systematic review of the potential of cfDNA for CRC diagnosis also indicates that methylated epigenetic ctDNA markers are perhaps the most promising candidates for a blood-based CRC-screening modality (Petit et al., 2019). In this context, *SEPT9* is one of the most widely studied gene promoters in CRC (Song and Li, 2015). In April 2016 Epi proColon test (Epigenomics AG, Berlin, Germany) that interrogates ctDNA *SEPT9* methylation for cancer screening was approved by the US Food and Drug Administration (FDA) (Payne, 2010). The Sn and Sp in retrospective case control studies have been reported between 52% and 90% and 88% and 95%, respectively (deVos et al., 2009; Grutzmann et al., 2008; Lofton-Day et al., 2008; Warren et al., 2011). Prospective studies have found somewhat lower Sn (48–68%) and Sp (80–92%) in CRC, with Sn of only 11–22% for AA (Church et al., 2014; Potter et al., 2014). A recent meta-analysis of 25 studies including 2613 CRC cases and 6030 controls reported that the use of different analysis algorithms with respect to one or two positive counts in one, two or three PCRs affects sensitivity and specificity (Song et al., 2017). Another meta-analysis confirmed the use of the algorithm with the highest sensitivity for screening, while the algorithm with highest specificity might be used for diagnostic purposes to exclude normal subjects (Sun et al., 2018). Although, the performance of the *SEPT9* assay was shown to be superior to CEA and the FIT tests in the screening of symptomatic populations (Song et al., 2017), *SEPT9* methylation did not distinguish well between

CRC and polyps or adenomas (Sun et al., 2018). Nevertheless, the main advantage of this test relates to patient compliance, which has been reported to be substantially increased compared to colonoscopy (Adler et al., 2014).

The combination of multiple biomarkers is likely to achieve a higher sensitivity and therefore is increasingly being investigated in CRC detection and screening, and several studies have evaluated the diagnostic performance of the *SEPT9* assay along with other candidate methylated genes (He et al., 2010; Rasmussen et al., 2017). Another two-markers panel consisting of *BCAT1* and *IKZF1* methylation reached a Sn of 66% and a Sp of 94% for CRC detection in a prospective study analysing > 2000 individuals, including 129 with CRC (Pedersen et al., 2015). The Sn for AA was, however, low (6%), and a later prospective study reported that methylation was more likely to be present in patients with late stage disease (Symonds et al., 2018). In a study comparing the performance of the *BCAT1/IKZF1* test with FIT, the two-markers panel had somewhat lower Sn (62% vs. 79%) but better Sp (92% vs. 81%) (Symonds et al., 2016). Nevertheless, evaluation of these markers in a representative screening population remains to be performed in order to conclude regarding this clinical utility. Besides, the methylation frequency of these markers was reduced after surgery in several patients (Pedersen et al., 2015; Young et al., 2016), motivating Clinical Genomics to develop the Colvera™ test for monitoring tumor recurrence. Lee et al. specifically looked for markers in plasma that could be used for early detection of CRC (Lee et al., 2009). Among patients with stage I and II disease a panel consisting of *APC*, *MGMT*, *RASSF2A*, and *WIF1* methylation could detect 86% of the cancers at a Sp of 92%. For colorectal adenomas the Sn was 75% (Lee et al., 2009). Similarly, Tänzer et al. demonstrated that combining *SEPT9* and *ALX4* methylation could detect both precancerous lesions as well as early stage cancer (Tänzer et al., 2010). Other promising DNA methylation panels for CRC detection have also been suggested (Lind et al., 2011a, 2011b; Vedeld et al., 2015), but except for the single marker *SPG20* (Rezvani et al., 2017), their suitability in blood remains to be explored. Novel techniques for analysing larger parts/the entire DNA methylome are now being developed and will likely increase the Sn of DNA methylation analysis even further (Shen et al., 2018). Alternative sources of tumor DNA from colorectal tumors are stool samples and one test combining faecal altered ctDNA and occult blood testing is FDA-approved: Cologuard. Cologuard uses a combined analysis of two methylated genes (*NDRG4* and *BMP3*), mutated *KRAS* and the immunochemical assay for human haemoglobin, FIT. It achieves higher sensitivity for advanced neoplasm than FIT, at expenses of more false-positive results (Imperiale et al., 2014). This low specificity along with its high cost and logistic difficulties due to the large amount of fresh faeces needed, difficult its use in a population-based scenario.

In addition, other potential ctDNA biomarkers for CRC detection have been reported. Recently, Cohen et al. described a multi-analyte test (CancerSeek) that identified eight common cancers, including CRC, by determining the levels of circulating proteins and mutations in ctDNA (Cohen et al., 2018). Using analysis of multiple mutations in a total of 16 different cancer genes combined with selected protein biomarkers, the authors detected ctDNA in 43% of stage I CRC compared to a 70% detection rate in stage II–III (Cohen et al., 2018). If more genes were added, the sensitivity rose so that analysing 58 genes could detect ctDNA in 50% of stage I CRC (Phallen et al., 2017). Of note, 84% of all CRC display CNA (Cancer Genome Atlas, 2012; Muller et al., 2016) suggesting that screening for CNA might be possible. Studies including small groups of stage I CRC have demonstrated that this might be feasible (Li et al., 2017; Molparia et al., 2018; Xu et al., 2018).

However, to establish the clinical utility of this kind of tests for CRC detection, cohort studies including individuals that reflect the screening population, are needed. Furthermore, in line with other recently studied ctDNA markers (Lin et al., 2014; Yang et al., 2018), the sensitivity for early-stage disease was lower than for advanced stages (Cohen et al., 2018). In accordance with this observation, a recent joint review by the

American Society of Clinical Oncology (ASCO) and the College American Pathologists (CAP) concluded that there currently is little evidence for the clinical validity of ctDNA in early-stage cancer (Merker et al., 2018). Nevertheless, recent developments in the field, which include the analysis of fragment length (Heitzer and Speicher, 2018; Mouliere et al., 2018) or nucleosome occupancy patterns (Snyder et al., 2016) in combination with machine learning technologies may elucidate novel cancer-specific footprints in plasma. Moreover, companies such as GRAIL or Freenome also pursue such combination strategies including machine learning technologies and sequencing depth or breadth of genomic coverage to develop models based on cell-free DNA for the accurate classification of subjects with and without cancer (Aravani et al., 2017). First data on CRC of such a classifier, achieved a mean area under the ROC curve (AUC) of 0.92 (95% CI 0.91–0.93) with a mean Sn of 85% (95% CI 83–86%) at 85% Sp (Wan et al., 2018).

#### 3.4. ctDNA as a prognostic marker in early-stage CRC

The prognostic value of ctDNA has clearly been demonstrated in a variety of studies and summarised in Basnet et al. (Basnet et al., 2016). Although most studies analysed patients with metastatic CRC (mCRC), a few publications include patients with localised CRC. As early as 2002, a small French study showed that ctDNA might represent a prognostic marker in 25 early stage (I–III) CRC patients. The authors demonstrated that the two-year recurrence-free survival was 66% for the 17 patients with detectable ctDNA pre-operatively (measured using *KRAS* mutation and hypermethylation of *CDKN2A/p16*), compared to 100% for the eight patients with no detectable ctDNA (Lecomte et al., 2002). Similar results were found in another small study with 21 stage I–III CRC patients using dPCR. Patients with localised disease (stage I–III) and with positive ctDNA levels within 3 months postoperatively displayed shorter recurrence-free survival (HR 37.7) and 5-year overall survival (HR 6.7) (Scholer et al., 2017).

In a well-designed large study of 230 patients with resected stage II CRC, ctDNA analysis was performed on sequential plasma samples over a median of 27 months using error-corrected NGS-based sequencing of one selected mutation per patient (Tie et al., 2016). False positive mutations in plasma due to clonal haematopoiesis were excluded by analysis of leukocyte DNA. In those who were not treated with adjuvant chemotherapy, 8% (14/178) had detectable ctDNA after surgery (with allele frequencies of 0.006–1.8%). Eighty % of these ctDNA-positive individuals had a recurrence at a median of 27 months follow-up time compared to 8% recurrence rate in the ctDNA-negative group (HR 18). ctDNA detection was superior to CEA in detecting residual disease and recurrence and also out-performed radiology with a lead time of 167 days (range 81–279) between ctDNA detection and radiologic recurrence. Among the 52 CRC patients receiving chemotherapy after surgery, 11% (6/52) had detectable ctDNA postoperatively. In three of them (50%) the tumor recurred and ctDNA recurrence was detected 5–7 months before clinical diagnosis of detection in two of these patients. Of note, in the third patient with recurrence, the ctDNA levels did not rise, suggesting the selection of a novel clone of cancer cells in the relapse (Tie et al., 2016). Indeed, progression of novel clones in ctDNA carrying partially different single nucleotide variants than those in the ctDNA assayed at diagnosis has been shown to occur in colon cancer metastases in some small studies (Reinert et al., 2016; Vietsch et al., 2017).

#### 3.5. ctDNA as a monitoring tool in metastatic patients

ctDNA has been clinically utilized to improve disease management of mCRC patients: 1) as a predictive biomarker for treatment selection; and 2) as a monitoring tool for treatment response (Siravegna et al., 2017). A prime example concerns stratification for eligibility for anti-EGFR treatment. mCRC patients can be treated with therapeutic antibodies directed against EGFR (cetuximab, panitumumab) and VEGF (bevacizumab), often in combination with chemotherapeutic drugs

such as 5-fluorouracil, oxaliplatin, and irinotecan (Fakih, 2015). However, not all mCRC patients benefit from receiving systemic therapy while many suffer its adverse side-effects. *KRAS* mutations predict resistance to treatment with therapeutic antibodies directed against *EGFR* and occur in approximately 45% of mCRC patients (Linardou et al., 2008). Consequently, mCRC patients are stratified for *KRAS* mutation status as an indication of primary resistance to anti-*EGFR* therapy (Linardou et al., 2008). In addition to *KRAS* mutations, mutations in *BRAF*, *NRAS*, and several other genes mutated at lower frequencies in mCRC patients also predict primary resistance to anti-*EGFR* therapy (Huisken et al., 2015). Therefore, there is an urgent clinical need for better stratification of mCRC patients for optimal treatment and for more accurate monitoring of treatment response. In current clinical practice, DNA mutation analysis is conducted using cancer tissue, mostly primary tumor, obtained as a surgical resection specimen or biopsies. However, metastatic lesions are not readily accessible for performing (burdensome) invasive needle biopsies. With reported concordance rates of ~75%–95% for *KRAS* mutation status between primary cancer and metastatic lesions (Knijn et al., 2011; Mostert et al., 2013), such test results may not accurately reflect the mutation status of the metastases that are treated. Several studies have been performed in which mutation frequencies determined in primary tumor tissue were compared to detectability of these mutations in liquid biopsies, reporting sensitivities of ctDNA analysis of 59% for a mixed group of tumor types (Frenel et al., 2015), 58% in metastatic lung cancer (Couraud et al., 2014), and > 75% in metastatic breast cancer (Rothe et al., 2014). Importantly, mCRC patients have among the highest levels of ctDNA (Bettegowda et al., 2014) and with mutations in *KRAS* being an early event in the pathogenesis of CRC, concordance rates of *KRAS* mutation status between tissue analysis and ctDNA liquid biopsy analysis are high, ranging between 80% and 96% (Demuth et al., 2018). ctDNA analysis of *KRAS* mutations are currently being considered as a clinically applicable alternative to tissue DNA mutation analysis, as a first step towards determination of primary resistance to anti-*EGFR* medication. These ctDNA analyses can be extended to detect mutations in a wider panel of genes that may confer primary resistance to anti-*EGFR* treatment (Demuth et al., 2018) by targeted sequencing approaches (Newman et al., 2014; Phallen et al., 2017; Strickler et al., 2018). Whether this can be performed in a cost-effective manner still needs to be determined by health technology assessment, based on information obtained from clinical trials. Besides prediction of primary resistance to anti-*EGFR* therapy, the early identification of acquired resistance is subject of extensive research. The detection of specific ctDNA mutations or CNAs is indicative for the molecular characteristics of the tumor clones that acquire therapy resistance. In case of anti-*EGFR* treatment, mechanisms of acquired resistance to therapy include the expansion of clones with *KRAS* mutations and amplifications, *MET* amplifications, and mutations in the *EGFR* extracellular domain that is targeted by the therapeutic antibodies (Diaz Jr. et al., 2012; Mohan et al., 2014; Montagut et al., 2018).

ctDNA analysis is also suited to monitor treatment response as in general, quantitative increases of ctDNA reflect disease progression. At present, computed tomography imaging is the gold standard to visualize metastatic lesions and follow response to treatment in mCRC patients. However, volumetric changes in size of lesions take time, with lesions sometimes hardly shrinking even when neoplastic cells are successfully targeted. Compared to CT imaging, cancer-specific ctDNA mutations are a more direct biomarker that represents tumor burden. Changes in the number of tumor cells are reflected by changes in the levels of cell free DNA as well as ctDNA (Newman et al., 2014; Phallen et al., 2017; Strickler et al., 2018). Due to the relatively short half-life of ctDNA, somewhere between 20 and 60 min (Muhanna et al., 2017), the levels of ctDNA rapidly drop after surgical removal of tumor tissue. These biological characteristics allow ctDNA to be applied as a marker for disease monitoring and treatment response, with ctDNA being capable to detect disease recurrence with lead times of 2–15 months

compared to CT imaging (Reinert et al., 2016). These observations warrant validation in larger studies, to confirm that detection of disease progression by ctDNA analyses precedes such detection by CT imaging, and to examine whether CT scans can be replaced, at least in part, by ctDNA analyses.

### 3.6. Conclusion and outlook regarding ctDNA

In summary, ctDNA can be used for the determination of genomic and epigenetic alterations in cancer patients for various applications, such as risk assessment, diagnosis, prognosis, treatment response and recurrence monitoring. With sensitive technologies, it is already possible to detect ctDNA even in asymptomatic patients with very low ctDNA in their body fluids. However, despite these high hopes, the clinical use of ctDNA is still limited as we lack core understanding of the mechanisms and dynamics of ctDNA, but more importantly most of the emerging ctDNA assays have not yet been clinically validated. To this end, large-scale randomized studies are needed to finally demonstrate a clinical benefit for patients. The ctDNA field is rapidly developing and recent studies have been moving beyond mutations and copy number alterations by measuring other parameters such as DNA fragment size, epigenetic modifications, chromatin organization and nucleosome footprints. Moreover, future developments focus on multi-parametric assays which eventually will yield sufficient Sn and Sp to make ctDNA accessible to a broad spectrum of cancer patients.

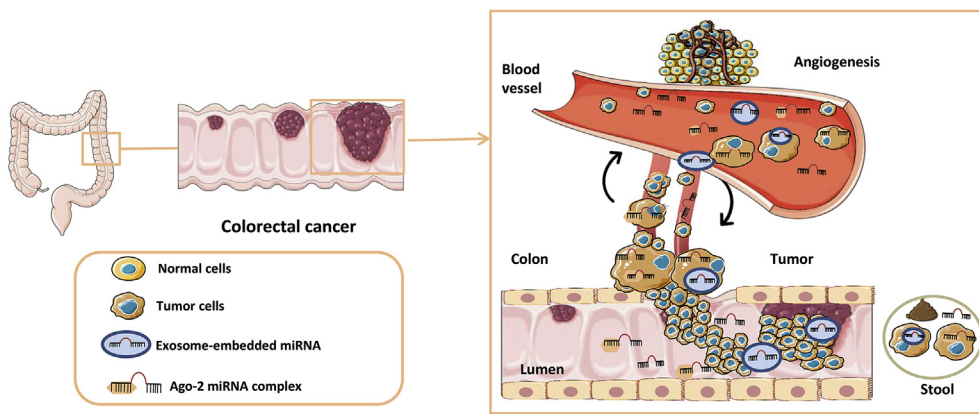
## 4. Circulating micRNAs as new biomarkers for early detection of colorectal cancer

### 4.1. General features of microRNAs

MicroRNAs (miRNAs or miR-) are endogenous, non-coding, single-stranded RNA of 18–25 nucleotides length that can regulate the expression of hundreds of gene targets post-transcriptionally. Briefly, they can negatively regulate gene expression through two mechanisms. The first one, exerting their regulatory function through imperfect complementary binding inside the 3'- untranslated regions (3'-UTRs) of target mRNAs, thus promoting the inhibition at the translation level. The second one, by binding with perfect complementarity to mRNA sequences, thus leading the degradation of target mRNAs (Lau, 2001; Yang et al., 2015). Both mechanisms are effective in a negative regulation way, however there is some evidence that in specific conditions miRNAs can also activate translation of targeted mRNAs (Vasudevan et al., 2007). It is thought that approximately 60% of the human protein-coding genome may be under the regulation of miRNAs (Friedman et al., 2009).

One miRNA can bind to hundreds of target mRNAs and a single mRNA can be targeted by several miRNAs. Therefore, miRNAs are involved in many biological processes such as activation of the immune system, inflammatory responses, cellular proliferation, differentiation, apoptosis (Bartel et al., 2009). Since the first discovery of miRNA in the earlier 1990 in *C. elegans* by Ambross and colleagues (Lee et al., 1993), several efforts have been made in this field to shed light on how they exert their functions as regulators of gene expression, how they are expressed and especially, how they are involved in physiological and pathological events. In 2002, the first association between miRNAs and human cancer was described (Calin et al., 2002). After that, some studies showed that most of the miRNA genes are located in fragile sites of chromosomal regions and in regions of loss of heterozygosity, suggesting that miRNAs might have a relevant role in human cancer pathogenesis (Calin et al., 2004). Indeed, the expression of many miRNAs has been shown to be altered in cancer. A wide range of studies show this deregulation due to their capability to act as oncogenic (oncomiRs) or tumor suppressor miRNAs (Croce and Calin, 2005; Gregory and Shiekhattar, 2005).





**Fig. 1.** Circulating microRNAs as non-invasive biomarkers for colorectal cancer. MicroRNAs (miRNAs) from colorectal tumors can be found in different biological fluids such as blood or stool, coming from different sources. They can be actively secreted into extracellular fluids by different cell types inside the tumor, or coming from a passive release due to apoptosis and necrosis of cancer cells. In addition, circulating tumor cells (blood) or exfoliated tumor cells (stool) also contain intracellular miRNAs that can be finally found in the circulation. Circulating miRNAs travel associated to various carriers, embedded into microvesicles mostly exosomes or forming complexes with proteins such as Ago2 or others.

#### 4.2. Circulating miRNAs as potential non-invasive biomarkers

MiRNAs can be found in the circulation alone or in combination with some proteins like Argonaute. Besides that, they can be secreted directly into extracellular fluids and transported by microvesicles—mainly exosomes (O'Brien et al., 2018) (Fig. 1). The first leading study of miRNAs in biological fluids came from Chim et al., in 2008, where placental miRNAs were detected in the maternal plasma (Chim et al., 2008). Subsequent studies were performed in order to characterize miRNAs in fluids like biomarkers. Lawrie et al. found a group of miRNAs significantly up-regulated in serum of patients with diffuse large-B cell lymphoma compared with healthy controls (Lawrie et al., 2008). MiRNAs hold unique characteristics that herald them as ideal minimally invasive tumor biomarkers. Tumor-specific miRNAs can be found in circulation, they are more stable and resistant to storage and handling than other molecules (Mitchell et al., 2008), their sequences are conserved across species, no previous preparations as bowel preparation and no diet restrictions would be required, they can be detected in low quantity of sample with high specificity and reproducibility by cutting-edge technologies and they are present in several biofluids (e.g. serum, plasma, breast milk, saliva, faeces, amniotic fluid, tears, urine) making their detection non-invasive. Multiple studies have described systemic dysregulated miRNAs in different human diseases and have proposed several circulating miRNAs as potential non-invasive biomarkers for different cancers.

#### 4.3. Blood-based miRNAs as biomarkers for early detection of CRC

Feasibility of using circulating miRNAs in plasma or serum samples to detect CRC in a non-invasive way is nowadays getting closer to reality as has been demonstrated by many groups over the last few years. However, which is the best miRNA or miRNA signature to use in this context, which is the capacity of detecting pre-neoplastic lesions, or which is the best biofluid or methodology to be used is still unknown. Validation studies of those miRNAs currently found in larger cohorts of patients are needed to answer these questions.

CRC survival depends on the stage of the disease at the time of diagnosis, localized disease having a 5-years survival rate of 90%, compared to a 10% survival rate for patients with metastatic disease at the moment of diagnosis. Therefore, CRC is one of the tumors that can most benefit from population screening programs. It is well known that CRC is preceded by development of benign lesions -polyps or adenomas—which can be detected and excised, preventing their progression towards malignant tumor lesions. Thus, precancerous AA represents a pertinent target lesion for a CRC screening test. However, early detection of AA remains an unmet need as there is not any test available with promising Sn and Sp for these lesions becoming cancerous. Although some strategies are available to screen CRC average risk individuals

including colonoscopy or non-invasive faecal occult blood testing alone or combined with stool DNA analysis, each one of them have important disadvantages. Blood-based tests may offer advantages compared to colonoscopy, since they are exempt from serious complications and do not require intensive time commitment (bowel preparation, procedure itself and recovery), and to faecal tests, as the person should not need to handle their faeces at home with logistic difficulties. It is well known that minimally invasive tests will achieve higher adherence over time taking into consideration availability, costs and patient-clinicians preferences. Therefore, we intend to highlight here the main circulating miRNAs that have been most reported to be altered in plasma or serum samples from CRC patients, or even in patients with precancerous AA, which would be more likely to become clinical biomarkers in this setting.

MiR-17-92 miRNA cluster, also called oncomiR-1, is one of the most known oncogenic miRNA cluster that is involved in colorectal carcinogenesis. This cluster maps the human chromosome 13q, and it contains several members such as miR-17, miR-18a, miR19a, miR-20a, miR-19b and miR-92a (Diosdado et al., 2009). Their overexpression and oncogenic role have been described in several cancers including CRC (Concepcion et al., 2012). Therefore, it is not surprising that members of this cluster are found altered in CRC related biofluids in a recurrent way.

For instance, overexpression of miR-17-3p and miR-92a in plasma from CRC patients compared to healthy individuals was firstly described some years ago by Ng et al. (AUC = 0.72 and 0.89, respectively) (Ng et al., 2009). Later, results from our group showed that several members of this cluster together with others miRNAs, selected from a genome-wide microarray performed on plasma samples, were significantly up-regulated in patients with CRC or AA compared to healthy subjects (Giráldez et al., 2013). Subsequent studies have confirmed these results (Herrerros-Villanueva et al., 2019). A recent meta-analysis including 10 studies, with a total of 938 CRC patients and 638 control individuals, has shown that circulating miR-17 presents a summary ROC curve with AUC = 0.76, with a pooled Sn of 76% and a Sp of 68%, to detect CRC (Liu et al., 2018). Furthermore, a panel of 8 plasma miRNAs containing miR-17 could distinguish colorectal adenomas from controls with an AUC = 0.87 (Kanaan et al., 2013). It is also noteworthy miR-92a within this oncogenic cluster, highly abundant in serum and plasma. As it was mentioned before, this miRNA was significantly up-regulated in plasma from CRC patients (Ng et al., 2009), as well as in AA patients (AUC = 0.75) (Giráldez et al., 2013; Huang et al., 2010). Moreover, miR-92a has also been found increased, together with miR-21, in serum from CRC and AA patients compared to healthy individuals (Liu et al., 2013). However, it must be taken into account that miR-92a, as it also happens with others members of this cluster, can be affected by hemolysis of the sample. Other members of the miR-17-92 cluster such as miR-19a and miR-19b have been also



found up-regulated in plasma from CRC patients compared to healthy individuals, combination of both showing AUC = 0.82 (Giráldez et al., 2013) and further combination with four more plasma miRNAs (miR-18a, miR-29a, miR-15b and miR-335) reaching AUC = 0.95 for CRC patients and AUC = 0.91 to distinguish AA patients from controls (Herrerros-Villanueva et al., 2019). Serum from CRC patients also presented significant higher values of miR-19a compared to healthy ones showing AUC = 0.83 (Zekri et al., 2016) or 0.69 (Zhu et al., 2017). MiR-18a, another cluster member, has been found up-regulated in plasma from CRC patients showing AUC = 0.80 (Zhang et al., 2013). In addition to this, it is important to point out the early diagnostic capacity of plasma miR-18a to distinguish AA patients from healthy individuals AUC = 0.84 or 0.64 (Giráldez et al., 2013). Similar results have been obtained in serum where miR-18a levels are also increased in patients with colonic polyp lesions showing AUC = 0.87 (Zekri et al., 2016). Finally, Chen and colleagues found a slight increase in miR-20a plasma levels in CRC patients showing AUC = 0.59 (Chen et al., 2015).

Besides miR-17-92 cluster members, it is important to highlight the importance of miR-29a in this setting. It was already described in 2010 as a novel promising plasma biomarker for CRC early detection by Huang and colleagues (Huang et al., 2010), as it showed AUC = 0.84 to discriminate CRC patients from healthy individuals, and AUC = 0.77 for AA. Further studies showed similar results in plasma (Giráldez et al., 2013; Luo et al., 2013) and serum (Yamada et al., 2015). It is important to point out that miR-29a can have advantages over others miRNAs since it is unaffected by hemolysis of the sample.

It is also worth mentioning miR-21 in this context as it is currently one of the most studied diagnostic circulating miRNA in CRC due to its abundance and oncogenic function in multiple cancers. Kanaan et al. showed how plasma miR-21 could distinguish CRC patients from controls with AUC = 0.91 (90% Sp, 90% Sn) (Kanaan et al., 2012). Besides, Wikberg and colleagues showed that plasma levels of miR-21 not only were up-regulated in CRC patients at the moment of diagnosis but, also, they were elevated several years before diagnosis (Wikberg et al., 2018). Similar results have been found for miR-21 in serum (Zhu et al., 2017). However, we must take into account that it lack of cancer specificity since it is also up-regulated in other conditions such as inflammation and it can be influenced by hemolysis of the sample.

Apart from analysing individual miRNAs, several studies have obtained better results using combinations of miRNAs in order to improve CRC diagnostic capacity. AUC values are higher when several miRNAs are combined (Carter et al., 2016; Giráldez et al., 2013; Herrerros-Villanueva et al., 2019).

More recently, exosome miRNA content has emerged as potential new biomarkers for CRC with more disease specificity. Although there are still very few studies exploring this issue in CRC, serum exosomal miR-21 and miR-23 stood out as the most increased in CRC patients presenting AUC of 0.80 and 0.95, respectively (Ogata-Kawata et al., 2014). In addition, other study revealed that serum exosomal levels of several cluster miR-17-92 members, such as miR-19a and miR-92a, were increased in CRC patients compared to controls, and correlated with CRC recurrence (Matsumura et al., 2015). Further studies exploring this issue are needed to unveil more biomarker candidates, and validation studies in larger cohort of patients will be utterly necessary to see if separation of exosomes before circulating miRNA analysis improves detection performance or it is a technical step that we could just skip.

#### 4.4. Analytical challenges for the clinical use of plasma/serum miRNAs as biomarkers for CRC

The potential clinical use of circulating miRNAs from liquid biopsies as a complementary tool in CRC screening programs is very attractive due to the characteristics of these molecules exposed above. However, it is still hampered by several technical issues that should be overcome (McDonald et al., 2011). Hemolysis, that can occur during sample

extraction or preparation, may influence levels of specific circulating miRNAs present in plasma or serum samples due to the rupture of erythrocytes containing miRNAs (Kirschner et al., 2011). Therefore, it is very important to monitor hemolysis of samples in a pre-analytical phase. Secondly, there is still no consensus on an adequate endogenous normalizer to quantify circulating miRNA expression in CRC. Some authors use miR-16 as a housekeeping in plasma/serum CRC samples (J. J. Yang et al., 2017) although it has been shown that miR-16 is highly affected by hemolysis (Kirschner et al., 2013) and it could introduce bias. Moreover, some studies have shown an increase of miR-16 in several cancers, even CRC. To overcome this problem many studies add, into the same volume of biofluid, synthetic non-human spiked-in miRNAs as exogenous controls that also normalize differences in miRNA recovery between samples (Kroh et al., 2010). Another fact that must be taken into account is the use of poor or rich platelet plasma or serum in order to avoid differences in miRNA levels due to platelet miRNA content contamination (Cheng et al., 2013). Also challenging is the miRNA quantification methodology to use and, finally, it is difficult to determine if aberrantly expressed miRNAs in the circulation are cancer-specific or confounded by co-morbidities (Healy et al., 2012).

#### 4.5. Circulating miRNAs in other biofluids as biomarkers for CRC

To date, faecal immunochemical test (FIT) detecting human haemoglobin in occult blood from faeces is the most recommended test for CRC screening programs (Carroll et al., 2014). Although its high Sn detecting CRC, its Sn for AA is low (20–25%) (Quintero et al., 2012). Addressing this inconvenience, some studies have been focused on the identification of molecular biomarkers in stool that could improve this limitation (Imperiale et al., 2004). Stool based-miRNAs are considered promising biomarkers due to the significant continuous rate of exfoliated tumor cells into colonic lumen (Koga et al., 2010; Matsushita et al., 2005; Yu et al., 2008), their stability and reproducibility when they are extracted from stool (Wu et al., 2012). Table 1 summarizes the main results obtained in this field so far. The majority of faecal miRNA studies have been focused on analysing members of the cluster miR-17-92 due to its involvement in colorectal adenoma to adenocarcinoma progression, as previously mentioned. As expected, all members of the cluster are significantly up-regulated in stools from CRC patients in accordance with the results found in tissue, plasma or serum studies (Rotelli et al., 2015; Yau et al., 2015). For instance, Wu et al. reported that miR-92a had a Sn and Sp of 81% and 73%, respectively, distinguishing CRC from control individuals (Wu et al., 2012). Other studies have combined members of the cluster with some other miRNAs obtaining similar diagnostic performances (Chang et al., 2016b; Koga et al., 2010; Yau et al., 2014). MiR-21 was also described as up-regulated in stool from CRC patients (Link et al., 2010; Rotelli et al., 2015; Wu et al., 2012), as well as, miR-223, miR-221, miR-144\*, miR-141, miR-106a and miR-135b (Chang et al., 2016b; Kalimutho et al., 2011; Koga et al., 2013; Rotelli et al., 2015; Yau et al., 2014). However, there are still few studies including stool from AA patients. To date, stool miR-92a and miR-135b are the ones showing the best accuracy in detecting AA (85% Sn, 73% Sp and 73% Sn, 68% Sp, respectively). More studies with larger cohorts of patients, also including AA, are needed to be able to assess the potential of faecal miRNAs as biomarkers for CRC screening.

Assessment of faecal miRNAs is not exempt from technical limitations. Faeces are a very heterogeneous biological material difficult to normalize between one individual to another in order to perform quantitative analysis of miRNAs. The lack of a good endogenous control produces bias and not comparable results between studies. Standardization of procedures from stool collection and amount of starting material to RNA extraction and detection methods are required to define which faecal miRNAs could help to improve CRC screening.

Although most of the studies are focused on blood or stool, circulating miRNAs have also been found in other non-invasive biofluids

**Table 1**  
Faecal miRNAs as biomarkers for CRC.

Faecal miRNA	Deregulation	Sensitivity (%)	Specificity (%)	AUC	Study samples	References
miR-223 + miR-92a	Up-regulation	71.7	79.9	0.81	N = 447 (138 CRC/309 control)	Chang et al. (2016a)
miR-221 + miR-18a	Up-regulation	CRC: 66	CRC: 75	CRC: 0.75	N = 595 (198 CRC/199 Ad/198 control)	Yau et al. (2014)
miR-20a	Up-regulation	CRC: 55	CRC: 82	CRC: 0.73	N = 595 (198 CRC/199 Ad/198 control)	Yau et al. (2015)
miR-19b-3p	Up-regulation	–	–	–	N = 40 (20 CRC/20 control)	Rotelli et al. (2015)
miR-20a-5p	Up-regulation	–	–	0.90		
miR-21-3p	Up-regulation	–	–	–		
miR-92a-3p	Up-regulation	–	–	–		
miR-141	Up-regulation	–	–	0.97		
miR-144*	Up-regulation	74	87	0.83	N = 75 (35 CRC/40 control)	Kalimutho et al. (2011)
miR-21	Up-regulation	–	–	–	N = 29 (10 CRC/9 Ad/10 control)	Link et al. (2010)
miR-106a	Up-regulation	–	–	–		
miR17-92 cluster <sup>¥</sup>	Up-regulation	69.5	81.5	–	N = 340 (206 CRC/134 control)	Koga et al. (2010)
miR-135	Up-regulation	46	95	–		
miR17-92 + miR-21 + miR-135(a/b)	Up-regulation	74	79	–		
miR-21	Up-regulation	CRC: 56	CRC: 73	CRC: 0.64	N = 239 (88 CRC/44 NAA/13 AA/101 control)	Wu et al. (2012)
miR-92a	Up-regulation	CRC: 72	CRC: 73	CRC: 0.78		
		AA: 85	AA: 73			
miR-144-5p + miR-451a + miR-200b-3p	Up-regulation	CRC: 69	CRC: 85	CRC: 0.89	N = 175 (29 CRC/31 AA/115 control)	Wu et al. (2017)
		AA: 23	AA: 85	AA: 0.58		
miR-135b	Up-regulation	CRC:78	CRC:68	CRC: 0.79	N = 424 (104 CRC/169 AA/42 IBD/109 control)	Wu et al. (2014)
		AA:73	AA: 68	AA: 0.71		
miR-106a	Up-regulation	34	97	–	N = 224 (117 CRC/107 control)	Koga et al. (2013)

–: “Sensitivity and Specificity value not mentioned in the original study; CRC: colorectal cancer; Ad: Adenoma; AA: advanced adenoma; NAA: Non-advanced adenoma; IBD: Inflammatory bowel disease; AUC: Area under the ROC curve.

¥ miR17-92 Cluster contains the follow miRNA: miR-17; miR-18a; miR-19a; miR-20a; miR-19b-1; miR-92a-1.

(Igaz and Igaz, 2015; Weber et al., 2010) highlighting their potential as biomarkers in different diseases (Cortez et al., 2011; Hibner et al., 2018; Ortiz-Quintero, 2016). Concretely, saliva has been suggested as a promising biofluid since it is considered like a mirror of blood content, reflecting deregulation of molecules and thus, different physiological status (Mandel, 1993). Some studies have explored salivary miRNAs as cancer biomarkers on oral squamous cell carcinoma, head and neck, esophageal and pancreatic cancers (Gai et al., 2018; Hizir et al., 2014; Humeau et al., 2015; Salazar et al., 2014; Xie et al., 2013). To date, only one study analyses salivary miR-21 levels in CRC and control individuals (Holten-Andersen et al., 2012; Sazanov et al., 2017). Although with a limited number of samples, this study shows that salivary miR-21 is significantly up-regulated in CRC patients with a very high Sn and Sp of 97 and 91%, respectively. Although this result is encouraging, more studies in this field are needed to uncover if salivary miRNAs can be good biomarker candidates for CRC detection.

#### 4.6. Conclusions regarding circulating miRNAs

Circulating miRNAs show great promise as novel biomarkers for early detection of CRC. The fact that patients with precancerous lesions, such as AA, also show an altered pattern of circulating miRNAs makes them good candidates for cancer prevention. Apart from blood, miRNAs are also found in other biofluids. The challenge now is to find which biofluid and detection method would be the most suitable to be applied in a CRC screening setting and which combination of miRNAs would have the best diagnostic performance.

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