Chapter 2

# Liquid Biopsy for Colorectal Cancer Screening, A Modern Approach for Patients Stratification and Monitoring

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72701

## Abstract

Despite great advances have been made in oncologic approaches, the morbidity and mortality caused by colon cancer are still overwhelming. Particularly, the intra- and inter-tumour heterogeneity makes accurate sampling challenging and often leads to failure of even modern therapeutic strategies. Moreover, tumour molecular genotype can suffer alterations over time, triggering suboptimal therapeutic outcomes as a result of irrelevant information provided by histological biopsies. Daily, tumour cells shed into the bloodstream at the early stages of the disease. These circulating tumour cells (CTCs) can be detected and analysed after enrichment, providing this way valuable information in real time. Furthermore, apoptotic and/or necrotic tumour cells discharge DNA fragments into the circulating bloodstream. Elevated levels of these so-called circulating tumour DNA (ctDNA) fragments can be identified in the peripheral blood of patients as compared to healthy individuals. In this view, the detection and characterization of the CTCs and ctDNA are a real-time "liquid biopsy" that has been developed for accurate tumour monitoring and molecular characterization. This modern non-invasive analytical approach allows consecutive sampling to monitor CTC number and tumour genetic changes over time without the need of tissue biopsy. Consequently, "liquid biopsies" can be used to screen for cancer, stratify patients to the optimum treatment and to monitor the patient's response to treatment or identify treatment resistance. This chapter offers an overview of the following approaches with respect to liquid biopsies: CTCs and ctDNA. Some of the analytical techniques and challenges in the detection of these rare events will also be presented here.

**Keywords:** colorectal cancer, liquid biopsy, circulating tumour cells, ctDNA, tumour heterogeneity

## 1. Introduction

Uncontrolled division and growth of human cells and subsequent invasion to other tissue via the circulatory and the lymphatic systems are commonly known as cancer.

One of the cancer types known to affect the gastrointestinal (GI) tract and rated third most commonly diagnosed form irrespective of gender is colorectal cancer (CRC).

In a context outlined by alarming figures of both prevalence (with a lifetime CRC risk of 5.1%) and high mortality rate, CRC being second in line among cancer-related causes of deaths in both genders, advances in therapy have become particularly significant; thus, in addition to established liver resection, outcomes in survival rate have been greatly improved in recent years due to better means for earlier detection, advances in chemotherapy and therapies based on biological agents.

Largely studied nowadays, factors influencing the likelihood of developing CRC are a close family history of genetic changes in that respect (20% of cases), notably associated with certain genetic syndromes such as the Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) in ca. 3% of cases [1] and familial adenomatous polyposis with its variant the Gardner syndrome (in a further ca. 1% of CRC instances).

HNPCC-related CRC risk factors include early onset (ca. 44 average age) triggered by an autosomal dominant inheritance, development in 70% of cases in splenic flexure proximity as well as a surplus of synchronous (18% of all patients) and metachronous CRC (45% of patients) following segmental resection or hemicolectomy. In addition to a CRC cause, Lynch syndrome frequently also results in other carcinoma types such as ovary and endometrial, other gastrointestinal location (stomach, small intestine), pancreas as well as transitional carcinoma in the renal pelvis and the ureter [2].

This high metastatic disease potential is the main cause of CRC lethal outcome and may be attributed to the contribution of a specific gene (I(MACCI)); already isolated [3], this is a transcriptional factor able to influence the expression of the hepatocyte growth factor and therefore associated with proliferation of CRC cells, scattering and new tissue invasion and further tumour growth and metastasis, as shown in cell cultures and animal studies in mice. MACCI close involvement as contributor to occurrence of metastasis makes it a novel target for CRC approach, which needs confirmation by further studies and clinical trials [4].

Contribution of genetic factors in CRC development combines with action of epigenetic ones at cell level.

However important the role of genetic features, most CRC may rather be the outcome of chronic intestinal inflammation preceding tumour development, gut microbiota and such environmental factors as life style (diet included) and food and environmental-borne mutagens [5, 6].

Among chronic intestinal inflammations responsible for CRC risk, inflammatory bowel disease (under 2% of CRC cases every year [7]), Crohn's disease and ulcerative colitis need special

mention for being the frequent cause of tumour growth [8], the risk growing with the severity of inflammation and the duration of the disorder [9]. Statistically, in that respect, 10 years' duration of Crohn's disease results in CRC in 2% of patients, and the risk increases four times and nine times for 20 and 30 years' durations, respectively [7]. On the other hand, a history of over 30 years of ulcerative colitis results in development of some type of cancer precursor or CRC in ca. 16% patients [7].

## 2. Tumour progression

As regards CRC development, it typically originates in benign, premalignant or malignant polyps occurring at the level of the colon or rectum epithelial lining (as, for instance, hyperplastic polyps, tubular adenoma or colorectal adenocarcinoma, respectively). Such abnormalities are the result of inherited or acquired oncogenic and inactivating mutations revealed by complex genome scale analysis, which has shown the existence of hypermutated and non-hypermutated CRC tumour categories [10].

Among non-hypermutated types, one commonly occurring mutation affects the Wnt signalling pathway, leading to increased signalling activity and emerging at the level of the intestinal crypt stem cell [11].

Most frequently, the mutated CRC-related gene is the *APC* gene. APC protein prevents β-catenin protein accumulation. The absence of APC leads to β-catenin accumulation and translocation to the cell nucleus, where it binds to the DNA, thus activating transcription of proto-oncogenes. Although normally playing an important role in stem cell renewal and differentiation, when inappropriately expressed and highly accumulated, these proto-oncogene products can induce cancer.

In addition to the absence of the APC protein, high β-catenin-related CRC may also be determined by β-catenin (CTNNB1) mutation, blocking its very own breakdown, or occurrence of mutations in other APC similarly operating genes (e.g., AXINI, AXIN2, NKDI, TCF71.2) [12].

Besides deficient Wnt signalling pathways, realization of the cancer potential requires additional mutations. Usually, action of Wnt pathway defects is prevented by intervention of the cell division monitoring p53 protein, a product of the TP53 gene, which normally eliminates flawed cells. Thus, a mutation arising in the TP53 gene may reverse the potential from benign epithelial tumour cell into invasive epithelial cell.

If not affecting the p53-encoding gene, mutations may instead target a different protein playing a protective role, i.e., the BAX12 but also ARDI A, CTNNB I, SOX9, FAM123B and ATM.

On the other hand, hypermutated tumours progress through specific genetic events and display MSH3, MSH6, TGFBR2, ACVR2A, SLC9A9, BRAF and TCF71.2 mutated forms.

Whichever the tumour type, all these genes are involved in the Wnt and TGF-β signalling pathways, leading to higher MYC activity, as major CRC factor [13]. Role of "field defects"/"field cancerisation", the concept first emerged in the early 1950s to refer to an area of the epithelium

featuring a preconditioning responsible for cancer predisposition of the area in question [14]. Despite unclear origins at the time of their introduction, the terms define premalignant tissue as potential sites for new cancer.

In time, research has progressively emphasized the importance of "field defects" in advance to CRC, and the assumption was confirmed by studies showing almost the exclusive use of discrete neoplastic foci for in vitro research and well-defined tumours for in vivo studies [15] in all cancer research.

In addition, as research further indicated, the majority of somatic mutations occurring at tumour level emerged during development of apparently normal cells [16], at the site of "field defects" (and therefore in preneoplastic stage).

A further addition to terminology refers to the term "aetiologic field effect", based on the "field defect" concept and referring to molecular and pathologic changes in preneoplastic cells at molecular level. The term also covers the extent to and manner in which exogenous environmental factors as well as molecular changes in the local microenvironment influence neoplastic progression throughout [17].

## 3. Epigenetic factors in tumour growth

However important mutation-induced genetic alterations, epigenetic alterations are significantly more common in CRC and involve hundreds of genes. As revealed by research, (Vogelstein and colleagues) oncogene mutations and suppressor mutations (both known as "driver mutations") are rather limited in average CRC forms (1–2 and 1–5, respectively), although accompanied by an estimated 60 additional so-called "passenger" mutations [18].

Common types of epigenetic cancer-related alterations modifying gene expression levels by action on the different types of RNA (miRNAs) may involve abnormal methylation of DNA in tumour suppressor promoters [19], such as reduced expression of miR-137 because of methylation of the miR-137-encoding DNA sequence in the CpG island [20]. Altered miR-137 expression triggers drastic (2- to 20-fold) alteration of mRNA expression of the target genes and related slighter changes in expression of proteins produced by the genes.

There are further microRNAs, of comparable numbers of target genes, which undergo even more frequent epigenetic alterations of field defects in the colon, resulting in specific CRC forms [21].

As common is direct hyper-/hypo-methylation of CpG islands of protein-encoding genes as well as histone alterations or modification of chromosomal architecture, with influence on gene expression [22]. Research has recently outlined the potential of early epigenetic decline in expression of DNA repair enzyme as cause of cancer characteristic genomic and epigenomic instability [18].

http://dx.doi.org/10.5772/intechopen.72701

# 4. Colorectal cancer (CRC)

## 4.1. CRC clinical manifestations

Although CRC clinical signs vary with tumour location in the intestine as well as with the presence of metastases, medical practice has outlined certain warning signs and symptoms now considered typical, such as loss of appetite, weight loss, vomiting and/or nausea, rectal bleeding and anaemia in the over 50 age group [23], severe and persistent constipation and modified stools (accompanied by blood elimination and/or diminished thickness) [24]; weight loss and changed bowel habit may be considered a warning only if accompanied by bleeding [22].

## 4.2. CRC diagnosis

### *4.2.1. Diagnostic steps*

Typically, CRC may be diagnosed by the sampling of colon areas suspected of tumour development during procedures suitable for the lesion site, i.e. colonoscopy or sigmoidoscopy.

Once the tumour is confirmed, the level of the disease needs to be determined, which is generally done by a CT scan involving the chest, abdomen and pelvis, but also by position imaging tomography and MRI, for certain cases.

The next diagnostic step is to determine the stage of the tumour, based on the TNM cancer staging system (where T stands for primary tumour stage, N for the presence of regional lymph nodes and M for remote metastasis). Staging criteria include the extent of initial tumour spreading, the presence and site of lymph nodes and the metastasis level [25].

### *4.2.2. Microscopic examination*

Adenocarcinoma is a malignant tumour of the epithelium, whose source lies in the superficial glandular epithelial cells of the colon and cecum lining. This tumour invades the colon/cecum wall and further progressively permeates the respective layers (first the muscularis mucosae, then the submucosa and lastly the muscularis propria). Tumour cells in question are organized as irregular tubular, multistratified structures, featuring multiple lumens and decreased stroma (in a back-to-back growth pattern). In addition, in some cases, tumour cell lacks of cohesion may be observed, as well as a secretion of mucus pervading the interstitium and resulting in extensive mucus/colloid (optically "empty" spaces) pools (forming the so called "mucinous (colloid)"), poorly differentiated adenocarcinoma. Mucus remaining within the walls of the tumour cell drives the nucleus towards the cell membrane, and the "signet-ring cell" emerges.

In fact, differentiation may vary in adenocarcinoma, contingent on cellular pleomorphism, glandular architecture and muco-secretion of the predominant pattern; thus, three variants of adenocarcinoma may be observed as regards the degree of cell differentiation: well differentiated, moderately differentiated and poorly differentiated [26].

CRC cell characteristics may be determined by analysis of tissue samples harvested by biopsy or during surgery. The pathology report provides data on cell type and grade. In CRC, the most common cell (98% of cases) is adenocarcinoma, but other types may also occur in rare cases (squamous cell carcinoma and lymphoma) [27].

*4.2.3. Immunochemistry*

It is generally considered that more than half of CR adenomas and up to 90% of CRC tumours present overexpression of the COX (cyclooxygenase)-2, normally absent from healthy colon tissue but acting as fuel for abnormal cell growth [28].

The cancer variant may be determined by histologic examination.

*4.2.4. Macroscopic examination*

In order to predict the likely course of tumour progression and adequate management, macroscopic examination looks closely at the site of the tumour in the intestine; thus, tumour development on the ascending colon and cecum (the right side of the large intestine) is most often exophytic (growing outwards from the bowel wall), which may in infrequent cases result in faecal obstruction, accompanied by anaemia.

Tumours growing on the left bowel side are largely peripheral and may result in obstruction of the bowel lumen and thinner stools [27].

### 4.3. CRC prevention

One key approach to CRC (as for other cancers, in fact) and unanimously recognized as such is prevention; closer surveillance and healthier lifestyle can essentially contribute to CRC prevention.

Therefore, research has greatly been focused on effective means in that respect, in all areas of intervention.

As regards lifestyle, diets are currently recommended to include more significant amounts of vegetables, fruits and whole grains and decrease consumption of white flour products, sugars and red meat.

As in other areas of healthcare, physical exercise has been proved to be beneficial, though less significant for preventing or reducing colon cancer risk [29, 30]. However, avoiding prolonged sitting as a daily routine is important [31].

Medication has also been the target of research, which has shown the potential of aspirin and celecoxib to reduce CRC danger in high-risk groups as determined by assessment of family medical history and other personal risk factors, though not in average risk ones [32–35].

Calcium supplementation is currently under study as well, not with sufficient evidence yet.

As for protection factors, in vitro studies have shown that intake and blood levels of vitamin D act as one, as have lactic acid bacteria, due to their antioxidant activity, immunomodulation

as well as promotion of programmed cell death, proliferative effects and epigenetic alteration of cancer cells [36].

Screening is an important and effective means for prevention and early detection of cancer in general and CRC in particular, the more so as most CRC cases (>80%) originate in adenomatous polyps [37].

As mentioned above, screening is also a very important means for cancer diagnosis before the emergency of actual symptoms (by 2–3 years) [25].

Close relatives of HNPCC patients need accurate and structured screening, according to a well-designed programme and schedule [38], as in certain countries such as Canada, the United Kingdom, Australia and the Netherlands [39–41].

Therefore, these should undergo a first routine colonoscopy at the age of 25, which, as a routine, should be repeated every 3 years, in the case of negative results, and every year should an adenoma be found. In cases where routine colonoscopy reveals the presence of cancer, subtotal colectomy needs to be performed.

In addition, for women, ovarian ultrasound and endometrial biopsy need to be performed as early as 25 years old.

Screening tests have been devised and researched, current practice now relying mostly on colonoscopy (both standard and virtual via CT scan), faecal occult blood testing, multitarget stool DNA screening and flexible sigmoidoscopy [25].

Although with proven efficacy in other respects, sigmoidoscopy is the only procedure able to provide screening of the right side of the colon, the site for almost half (42%) of malignancies [42].

Equally effective, standard colonoscopy is less costly than virtual colonoscopy via CT scan and avoids the additional risk of exposure to radiation and also able to eliminate any potential abnormal growth found [25].

In the 50–75 age group with standard risk factors, screening should include faecal occult blood testing or immunochemical testing every 2 years; an alternative is performance of sigmoidoscopy every 10 years, to the detriment of colonoscopy [43].

For patients with familial adenomatous polyposis, the high-malignancy risk may be offset by total proctocolectomy, ensuring elimination of the risk of both colon and rectal cancers [44].

### 4.4. CRC management

Given CRC's incurable character, therapeutic decisions in that respect can only be directed to either cure or as a palliative, largely depending on tumour stage [45] but also on other factors as well, such as the patient's health status and even preferences.

#### *4.4.1. Therapy*

Surgery can be a means leading to cure but in early stages only, whereas at later stages, when the metastatic disease has also been initiated, the curative potential of surgery decreases, and

palliation (alleviation of tumour-related symptoms and patient's comfort and quality of life) becomes prevalent [25].

For the very first stage, one colonoscopy intervention can suffice to eliminate cancer [46], while the curative potential of surgery decreases with the tumour stage.

Therefore, one stage further, in localized cancers, cure may still be attempted through ample removal associated with ensuring adequate margins, which can be achieved laparoscopically or more often by open laparotomy [25], with colon reconnection, or by colostomy [46].

In the stage of a few emerging metastases, those in the lungs or liver may be eliminated.

In certain cases at this stage, surgery may be preceded by chemotherapy, in an attempt to minimize the tumour before removal.

If recurrence occurs, this mainly involves the lungs and liver [25].

#### *4.4.2. Chemotherapy*

This is administered in cases beyond stage 1 CRC, given the curing potential of surgery. No chemotherapy is also customary in CRC stage II; on condition no such risk factors as threats from negative lymph node sampling or the presence of a T4 tumour are present.

Chemotherapy is also not feasible in patients with identified abnormal mismatched repair genes.

On the contrary, chemotherapy is a must and an integral therapy component in stage II and stage IV CRC25, characterized by cancer spreading to remote organs or the lymph nodes; the use of the chemotherapeutic agents oxaliplatin, fluorouracil or capecitabine is instrumental in increasing life expectancy, with the disadvantage of debatable chemotherapy benefits in the case of cancer-free lymph nodes.

Turn to palliative care becomes necessary where CRC has become extensively metastatic or may not be resected, opening the alternative for several different chemotherapy medications [25], including, oxaliplatin, fluorouracil, capecitabine, irinotecan and tegafur/uracil [47, 48].

#### *4.4.3. Radiotherapy*

Given bowel sensitivity to radiation, patient with colon cancer treatment cannot benefit from addition of radiation to chemotherapy, although this may be effective for rectal cancer [25]. The same was for chemotherapy; radiotherapy may be used as neoadjuvant and adjuvant in certain rectal cancer stages only [49].

#### *4.4.4. Palliation*

For patients with incurable CRC forms, palliative care, though not a promising cure, may bring the benefit of better quality of the patient's life both directly and indirectly, via the life of their families, lessening symptoms and anxiety and also reducing the need of hospital admission [50].

Palliation is typically symptom directed and consists of procedures designed to improve symptoms or minimize the possibility of complications such as abdominal pain, tumour bleeding and/or bowel obstruction [51], thus contributing to improved quality of life.

Such procedures may include surgery, for elimination of cancer tissue to some extent, without attempting to cure, placement of a stent or performing a bypass of part of the bowel.

Non-surgical palliative care approaches include pain medication and/or radiotherapy aiming to reduce the tumour size [52].

*4.4.5. Follow-up*

The main purpose of follow-up is to obtain the earliest identification of later metachronous lesions, i.e., metastases or tumours not originating from the initial cancer [53].

As an underlying measure for cancer survivors, exercise as a mainstay of lifestyle may be useful as secondary therapy, as shown by results indicating important reduction in 8-oxo-dG in the urine of patients after taking moderate exercise for 2 weeks of following primary therapy [54].

### 4.5. CRC prognosis

The most commonly used prognosis criterion is the 5-year survival rate, which is under 60% for CRC in Europe, whereas this is the cause of death for one third of CRC patients [25] in most developed countries. The reason for these unexpectedly low outcomes despite evident progress in new therapeutic means and their improved availability worldwide is mainly CRC late identification (stage IV already present in 20% of patients seeking medical attention), with potentially resectable isolated liver metastasis in ca. 25% of these patients. Of these 25%, one third of patients undergoing resection achieve 5-year survival [55, 56].

## 5. Liquid biopsy

Despite the major advances in cancer therapies, the morbidity and mortality associated with this disease are still enormous. Tumour heterogeneity holds the main responsibility underlying inefficient treatment and failure of current therapeutic strategies, including the targeted therapies. For efficiency reasons, the molecular targeted therapies require constant monitoring of the tumour genome, but harvesting consecutive tissue biopsies is very difficult and inconvenient for medical and economic reasons. Therefore, the lack of real-time information regarding tumour heterogeneity during the disease evolution most commonly results in the treatment failure and requires the development of novel approaches. In this view, liquid biopsies offer a tool for real-time screening of disease particularities, stratify patients for the best treatment and also monitor the response of the treatment. Due to their non-invasive nature, liquid biopsies can be used for repeated sampling to monitor tumour genetic alterations over time, avoiding this way consecutive tissue biopsies. Liquid biopsies analyse circulating tumour cells, cell-free tumour DNA and/or exosomes, known as tumour-circulating markers.

### 5.1. Circulating tumour cells (CTCs)

Circulating tumour cells (CTCs) have been identified during the 1800s and presumed responsible for the metastatic process [57]. These cells are of epithelial origin and shed from the tumours in the peripheral blood of patients where they can be enriched, detected and analysed.

The detection of CTCs in the peripheral blood of patients with cancer holds a great promise for the future development of efficient anticancer therapies. However, due to the very low concentrations of CTCs in the peripheral blood (one tumour cell for millions of normal blood cells), their detection and identification still remain challenging and require high analytical sensitivity and specificity methods, which usually consist in a combination of enrichment and detection [58].

CTC enrichment strategies include a wide range of technologies based on those CTC particularities that can discriminate them out of the normal haematopoietic cells. Concrete CTCs can be detected based on physical properties such as size, density, electric charges, deformability or biological properties such as cell surface marker expression and viability. CTC separation based on their physical properties holds the great advantage of being done without labelling the cells. Some of these methods include density gradient centrifugation, filtration, photoacoustic flow cytometry, microfluidics, etc. [59, 60].

Nevertheless, the biological properties of the CTCs hold a major role in their identification, mainly based on immunobead assays. These assays use antibodies targeting tumour-associated antigens (positive selection) or leukocyte-specific antigens such as CD45 (negative selection) in order to detect and separate CTCs from the blood cells. The positive selection usually targets the epithelial cell adhesion molecule (EpCAM). Subsequently, CTCs are confirmed with antibodies against cytokeratins (CKs) [59]. Among the current EpCAM-based technologies, the US Food and Drug Administration approved CellSearch® system (Veridex) which is the current "gold standard" for all new CTC-detection methods. According to this standard, CTCs are nucleated cells that express the epithelial cell adhesion molecule and cytokeratins but lack the expression of the common leukocyte CD45 marker ($EpCam^+$_$CK18/19^+$_$DAPI^+$_$CD45^-$ cells).

Interestingly, some CTCs undergo the epithelial to mesenchymal transition (EMT) and loose critical epithelial markers. Capturing CTCs' lacking EpCAM expression requires the use of antibody cocktails against a panel of epithelial cell surface antigens such as HER2, MUC-1, EGFR and folate-binding protein receptor and against mesenchymal or stem cell antigens such as c-MET, N-cadherin and CD318 [61].

Regardless of the enrichment method, the isolated CTCs still contain a significant number of normal blood cells, and therefore CTCs should be next identified by a method that can discriminate between malignant cells and normal blood cells. The CellSearch® system as well as other assays is based on the fluorescent staining of the cells for the following markers: CKs (positive marker), the common leukocyte antigen CD45 (negative marker) and a nuclear dye (4,6-diamidino-2-phenylindole, DAPI).

Functional EPISPOT (for EPithelial ImmunoSPOT) assay has been introduced for CTC analysis in order to detect only the viable CTCs, able to produce metastases [62].

Other alternatives to immunologic assays of viable CTC-detection target specific mRNAs. A commercially available RNA-based CTC assay is the AdnaTest™ (AdnaGen), which uses nonquantitative RT-PCR to identify cells that express the transcripts of tumour-specific genes after immunomagnetic capture of MUC-1, HER2 and EpCAM cells [63].

### 5.2. Circulating tumour DNA (ctDNA)

Cell-free DNA (cfDNA) is a powerful tool for its potential use in a wide range of clinical fields such as cancer research [64, 65], non-invasive prenatal testing [66] and transplant rejection diagnostics [67]. Most cfDNA in plasma is highly fragmented (150–180 bp) [68] with a higher prevalence of tumour-associated mutations in the shorter fragments [69]. In patients suffering from cancer, a fraction of the cfDNA is tumour-derived and is known as circulating tumour DNA (ctDNA).

cfDNA reaches the systemic circulation by various pathologic or normal physiologic mechanisms [70]. However, with respect to solid tumours, the ctDNA is usually released as a result of necrosis or autophagy [71]. Notably, unlike apoptosis, necrosis generates larger DNA fragments [72]. Cancer patients generally have much higher levels of cfDNA than healthy individuals [73, 74]. ctDNA carries genomic and epigenomic alterations according to the tumour genomic alterations (copy number variation, point mutations, microsatellite instability, degree of integrity, loss of heterozygosity, rearranged genomic sequences, DNA methylation, etc.) [75]. Only on the basis of these biological characteristics, ctDNA can be discriminated from normal cfDNA. Consequently, after its validation ctDNA could be used as a specific biomarker that provides personalized information to detect residual disease or monitor tumour progression during therapy.

Due to the high degree of fragmentation as well as the small fraction of ctDNA within the cfDNA, the analysis of ctDNA is challenging and requires highly sensitive techniques. Classical methods of analysis include qRT-PCR, fluorescence and spectrophotometric approaches [76–78]. Digital droplet PCR has been developed as a high sensitive tool to detect ctDNA [79]. This technique consists in a droplet-based system [80, 81], a microfluidic platform [82, 83] and the so-called BEAMing strategy [84, 85]. Additionally, next-generation sequencing technology is currently used in plasma DNA analysis in order to identify ctDNA alterations [86–88].

## 6. Conclusions

There is increasing evidence that circulating tumour markers such as CTCs and ctDNA offer real-time information regarding cancer progression and tumour genotype in the view of a better systemic therapy management with direct impact on patient's disease prognosis. Additionally, future characterization of these circulating markers could contribute to approach-specific-targeted therapies to a certain population of cancer patients.

## Acknowledgements

This work was done under the project PN-IIIP2-2.1-PTE-2016-0149/19PTE - TUMFLOW, financed by UEFISCDI.

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