# British Journal of Pharmacy

*Critical Review*

# Circulating Tumour DNA: a Minimally Invasive Biomarker for Tumour Detection and Stratification

Arif Surani[b*], Krzysztof Poterlowicz[a]

[a]Centre for Skin Sciences, [b]School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK

ARTICLE INFO

ABSTRACT

Genetic and epigenetic alterations significantly contribute to development of human cancer. Genotyping tumour tissue in search for these actionable genetic and epigenetic changes has become routine practice in oncology. However, sampling tumour tissue has significant inherent limitations. It provides only a single snapshot in time, prone to selection bias due to intra-tumour heterogeneity, and cannot always be performed owing to its invasive nature. Circulating tumour DNA (ctDNA) based liquid biopsy provides an effective alternative to invasive tissue sampling and have emerged as a minimally invasive, real-time biomarker. Recent advancements in DNA sequencing technologies have revealed enormous potential of ctDNA to improve tumour detection and stratification. In this review, we critically appraise the role of ctDNA as a liquid biopsy for cancer and evaluate the role of circulating tumour DNA as a diagnostic, prognostic and predictive biomarker. We also highlight some technical challenges and constraints associated with circulating DNA analysis.

## INTRODUCTION

The advent of personalised medicine has revolutionised cancer management. Advancements in genomics and transcriptomics have enabled identification of gene signatures and targets that can be used for diagnostics, prognostics, predictive and therapeutic purposes (Gonzalez de Castro et al. 2013). Analysis of tumour-linked genetic alterations has become routine practice in oncology. Genetic profiling of solid tumours requires the sample of the abnormal tissue (mass, lesions or tumour) which is usually obtained by invasive biopsy (Amalou and Wood 2012). However, the invasive tissue biopsy is associated with significant risks, complications and limitations described blow.

## Intra-tumour heterogeneity and tumour evolution

It has been shown that a tumour consists of a variety of sub-clones that harbour different genetic and epigenetic alterations, a phenomena termed as intra-tumour heterogeneity (ITH) (Marusyk et al. 2012; Swanton 2012). A study by Gerlinger et al. (2012) showed that biopsy samples taken from different regions of Renal Cell Carcinoma displayed heterogeneous somatic mutations and were not detectable in every region of the tumour sequenced. Similarly, Bashashati et al. (2013) have observed regional diversity in mutations, copy numbers and gene expression profiles in primary ovarian cancers prior to therapeutic intervention. Therefore, biopsy of a small region of tumour might not account for ITH and could not accurately characterise distinct molecular alterations present within a single tumour.

It is also widely accepted that tumour development follows a process of Darwinian-like clonal evolution (Gerlinger and Swanton 2010; Gerlinger et al. 2012). High throughput sequential analysis of tumour samples has revealed that ITH evolves temporally under selection pressures imposed by the microenvironment and/or the cancer therapeutics (Anderson et al. 2006; Hunter et al. 2006; Edwards et al. 2008). It implies that the historical tissue biopsy taken at the time of diagnosis might not effectively guide clinical decisions after some passage of time and should be supplemented by serial tissue sampling to account for new mutations.

**Limitations in tissue processing**

Processing of tumour tissue sample affects the quality of DNA obtained. Studies have shown that formalin fixation causes DNA denaturation, base alteration and production of sequence artefact (Douglas and Rogers 1998; Oh et al. 2015). Gallegos Ruiz and colleagues (2007) have observed mutational artefacts in epidermal growth factor receptor (*EGFR*) gene resulting from formalin fixation and paraffin embedding of lung tissue sample. Therefore, formalin-processed tissue specimens might not accurately reflect the genetic alterations present in the tumour tissue. The use of fresh frozen tissue for processing might overcome these limitations, but it is restricted by logistic issues such as availability of liquid nitrogen and requirement of specialised equipment for processing and storage of frozen tissue (Budczies et al. 2011; Oh et al. 2015).

**Associated risks and complications**

Tissue sampling is invasive and is associated with significant risks and complications depending on the type and site of the biopsy. A systematic review on complications of the prostate biopsy by Loeb et al. (2013) showed that it is associated with complications such as pain, haematuria, haematospermia and urinary tract infection. Although most of these complications were mild and self-limiting, longer hospital stay and serious adverse events were reported in patients undergoing surgical biopsy (Lebofsky et al. 2015). Additionally, it is speculated that certain biopsies are associated with dislocation and seeding of malignant cells along the needle track leading to local recurrence and/or systemic spread (Shyamala et al. 2014).

**Liquid biopsy - a paradigm shift in cancer testing:**

The liquid biopsy is a minimally invasive blood test that detects tumour biomarkers (DNA, RNA, protein) in the circulation. It has emerged as an effective alternative to traditional tumour tissue sampling (Karachaliou et al. 2015). Being a simple blood test, it can be repeated to account for the changing genomic landscape of a tumour to track tumour evolution and monitor response to therapy. Additionally, it has the potential to interrogate genetic profiles of primary and metastatic tumours in the body which overcome intra-tumour heterogeneity and sampling bias. One of the most promising application of liquid biopsy is cell-free circulating tumour DNA (ctDNA).

**THE BIOLOGY OF ctDNA**

ctDNA, as the name implies, is the presence of tumour-DNA fragments in the circulation that shows similar molecular alterations present in the corresponding tumour in the body. The presence of cell-free nucleic acids in the human blood was first reported by Mandel and Metais (1948). It was subsequently found that the concentration of cell-free DNA is significantly higher in the circulation of cancer patients compared to healthy controls (Leon et al. 1977). This pioneering seminal work did not attract much interest until Stroun and colleagues (1989) showed that circulating DNA demonstrate similar properties such as decrease strand stability that were found in neoplastic cells. Subsequently, several proof of concept studies showed various tumour specific aberrations in the circulation such as mutation in oncogene and tumour suppressor gene (Sorenson et al. 1994; Silva et al. 1999b), microsatellite instability (Chen et al. 1996) and aberrant DNA methylation (Silva et al. 1999a). In recent years, studies are directed towards evaluating the clinical utility of ctDNA in the management of cancer (Takai et al. 2015).

**Origin of ctDNA**

ctDNA is the proportion of cell-free DNA (cfDNA) that originates from tumour cells and carry tumour-specific alterations. They are short fragments of double stranded DNA that measures between 70 to 200 base pairs but larger fragments of up to 21 kilobases were also recorded (Jahr et al. 2001). The percentage of cfDNA that is derived from tumour varies from patient to patient, ranging from as low as

0.01% to a mutant allelic fraction as high as 93% (Jahr et al. 2001; Diehl et al. 2005; Forshew et al. 2012). It has been estimated that a patient with a tumour load of 100g releases up to 3.3% of tumour-DNA into the circulation on a daily basis (Diehl et al. 2005). Despite numerous studies describing cfDNA, the mechanism of the origin of these circulating DNA in the blood remains ambiguous. Two possible, mutually non-exclusive, mechanisms by which cfDNA enters into the circulation include passive release and active secretion (*Figure 1*). The passive release is mediated through the cellular destruction (both healthy and tumour cells) by apoptosis and necrosis (Jahr et al. 2001). Additionally, cells may actively secrete DNA into the blood in the form of nucleoprotein complex (Stroun et al. 2001). A low proportion of cfDNA is also contributed by the lysis of circulating tumour cells that have been shed from the primary tumour (Stroun et al. 2000).

Controversy exists in the literature regarding the contribution of necrosis and apoptosis in the origin of ctDNA. Some studies advocate that necrotic cells engulfed by the macrophages are the major source of ctDNA fragments (Diehl et al. 2005). However, recent studies have shown that these larger necrotic-derived DNA fragments were barely detectable in patients with pancreatic cancer (Sikora et al. 2015). Alternatively, many studies have suggested apoptosis as the main driver of release (reviewed in Vietsch et al. 2015). These observations were supported by the fact that ctDNA shows fragmentation pattern similar to nucleosomal DNA (~ 180 bp) (reviewed in Heitzer et al. (2015). Further studies are therefore required to elaborate the complex mechanism of origin of ctDNA and contribution of necrosis and apoptosis.
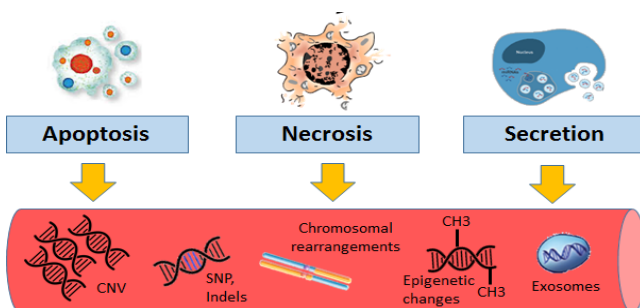


**Fig 1:** Mechanisms of release of cfDNA from tumour cells. Cancer-associated genetic alterations such as single nucleotide variants, copy number variations, methylation changes and chromosomal rearrangements can be detected in ctDNA

## Tumourgenic potential of ctDNA: The Genometastasis Hypothesis

Interestingly, studies have shown that tumour-derived circulating DNA might transfect susceptible cells in the distinct target organs resulting in metastases. This hypothesis was proposed by GarcÍA-Olmo and GarcÍA-Olmo (2001) and have been supported by a number of experimental studies. A study by Garcia-Olmo et al. (2010) have demonstrated the potential of plasma DNA to transfect and oncogenically transform susceptible cells, and showed that plasma of colon cancer patient selectively transforms NIH/3T3 mouse cells while having no effect on human adipose-derived stem cells. Similar findings were also reported by Trejo-Becerril et al. (2012) in an *in vivo* immunocompetent colon-carcinogenesis rat model. However, a number of observations have limited the validity of this hypothesis. In the studies cited above plasma was used as the source of ctDNA. Studies have shown that plasma also contains different types of cell-derived particles carrying nucleic acids, for example, exosome, apoptotic bodies and microvesicles (Belting and Wittrup 2008), and they have been shown to play an important role in tumorigenesis (Azmi et al. 2013; Martins et al. 2013). Therefore, the oncogenic transformations observed by authors could have been mediated by these extracellular cell-derived particles as opposed to cell-free nucleic acid. Moreover, the organ-specific tropism of metastases could not be explained by this hypothesis (Hunter et al. 2008).

### Kinetics of ctDNA

Little is known about the elimination of circulating DNA from blood. Studies on circulating foetal DNA from maternal plasma after delivery displays a half-life between 4 and 30 minutes with an initial rapid phase followed by a slower phase of clearance (Lo et al. 1999). Different studies have speculated that the clearance is mainly mediated by liver, kidney, spleen and other physiological factors in blood such as plasma deoxyribonuclease (Botezatu et al. 2000; Minchin et al. 2001; Cherepanova et al. 2007). However, the contribution of these mechanisms in the clearance of ctDNA is still controversial. A study by Lo et al. (1999) showed that plasma nucleases plays a limited role in the clearance of circulating foetal DNA from maternal plasma. On the contrary, other studies

have identified leading contribution of plasma nucleases and showed that low DNase activity in the plasma of cancer patients resulted in the high level of circulating DNA (Tamkovich et al. 2006; Cherepanova et al. 2008).

## DETECTION OF ctDNA IN BLOOD

### Methodological aspects

Two approaches are used to analyse circulating DNA as a biomarker for carcinogenesis: quantitative analysis of cfDNA and detection of tumour-specific alterations.

*Quantitative analysis of total cfDNA*
Studies have shown that the concentration of cfDNA is significantly higher in cancer patients compared to healthy controls owing to increased release from tumour cell turnover (Chang et al. 2002; Kamat et al. 2010). Review of published articles by van der Vaart and Pretorius (2010) reported that yields of circulating DNA in the plasma of cancer patients were on average 137 ng/ml, which is nine times greater than the concentration observed in the healthy individual (average 15 ng/ml).

Various methods are available to purify circulating DNA from serum or plasma. The classic DNA isolation procedure is based on phenol-chloroform method, a liquid-liquid extraction method that form a biphasic emulsion with aqueous layer containing DNA and organic layer containing precipitated proteins (Sengüven et al. 2014). Other commercially available kits using silica based technology such as Purelink Genomic DNA extraction kit (Invitrogen), and magnetic separation such as Agencourt DNAdvance Kit (Beckman Coulter) are also available (as reviewed in Dhaliwal 2013). The isolated DNA is then quantified using different techniques, including DNA dipsticks (Invitrogen), nick translation, qPCR and fluorometry with SYBR green (Xue et al. 2006; van der Vaart and Pretorius 2010).

Studies have demonstrated that quantitative analysis of cfDNA facilitates early detection of prostate, lung and breast cancer (Sozzi et al. 2003; Altimari et al. 2008; Kohler et al. 2009). A study by Sozzi et al. (2003) has shown eight-fold increase in the concentration of cfDNA in lung cancer patient compared to controls. However, conflicting results were observed in different studies. In a study by Schmidt et al. (2008), no significant differences were observed in the amount of cfDNA in the tumour and non-tumour populations. Additionally, the concentration of cfDNA is also influenced by various physiological and pathological conditions such as pregnancy, exercise, heavy smoking, trauma, inflammatory diseases, premalignant conditions and chronic illnesses in elderly (reviewed in Fleischhacker and Schmidt 2007; Heitzer et al. 2015). Therefore, simple quantitative assessment has limited potential as a standalone biomarker, but it can be used as an adjunct with other conventional markers.

*Characterising tumour-specific alterations in circulating DNA*
Detecting tumour-specific genetic aberrations is a more sensitive and specific biomarker compared to the quantitative measurement of cfDNA (Jung et al. 2010). These genetic alterations include point mutations, insertions and deletions, multi-nucleotide polymorphisms, loss of heterozygosity, microsatellite alterations, copy number variations, chromosomal rearrangements and epigenetic alterations (reviewed in Fleischhacker and Schmidt 2007; Chen et al. 2016) (*Figure 1*).

Different technological platforms are available to detect these alterations. Broadly, they can be divided into two group i.e. targeted and untargeted approaches. The former includes the detection of known genetic changes in circulating DNA based on primary tumour genotype or analysing frequently occurring "driver" mutations in particular cancer. The untargeted approach includes techniques which do not require any prior knowledge of the mutation type. Techniques employed by different studies to detect ctDNA are summarised in *Table 1*.

The targeted approach is mainly based on PCR-based assays. Earlier techniques employed for detecting known minority (mutant) alleles mainly focused on allele specific PCR-based assays (AS-PCR) also called amplification-refractory mutation system (ARMS) (Reviewed in Diaz and Bardelli 2014). Although this technique is comparatively inexpensive and does not require any specialised instruments, the results of AS-PCR are semi-quantitative and provide only relative quantification to the control (Luke et al. 2014). This limitation has been overcome by combining ASPCR

with quantitative methods, for example, fluorescently labelled primer and probe (Taqman, scorpion-ARMS) and peptide nucleic acid (PNA) which have improved interpretation of PCR results (Board et al. 2008). Other modifications of PCR-based assays have also been reported such as co-amplification at lower denaturation temperature-PCR (COLD-PCR) and competitive allele-specific hydrolysis probes (TaqMan) PCR (CAST-PCR) (*Table 1*). Although AS-PCR and its different technical modifications are sensitive in detecting mutant DNA, cross-reactivity has been observed between the probes. In a study by Didelot et al. (2012) using CAST-PCR, cross-reactivity was observed between mutant and wild-type probes for *EGFR* resistant mutation (T790M) and it impacted assay's sensitivity.

**Table 1.** Overview of selected techniques used for detection of ctDNA.

| Technique | Sensitivity[a] | Tumour type | Gene assessed for mutations | Selected studies using the technique |
|---|---|---|---|---|
| Allele-specific PCR | 0.5% - 1% | NSCLC | *EGFR* | (Kimura et al. 2006; Maheswaran et al. 2008; Goto et al. 2012) |
| | | Breast | *PIK3CA* | (Board et al. 2010) |
| | | CRC | *KRAS, BRAF* | (Spindler et al. 2012) |
| Cold-PCR | 0.01% - 0.1% | NSCLC | *EGFR* | (Li et al. 2008) |
| | | CRC | *KRAS, BRAF* | (Mancini et al. 2010) |
| CAST-PCR | 0.1–1 % | Melanoma | *BRAF* | (Ashida et al. 2016) |
| | | Ovarian | *Beta-globin* | (Kamat et al. 2010) |
| PNA- PCR | 0.1%–1% | CRC | *KRAS* | (Xu et al. 2014) |
| | | Pancreatic | *KRAS* | (Tjensvoll et al. 2016) |
| Droplet-based digital PCR | 0.005% - 0.01% | CRC | *KRAS* | (Taly et al. 2013) |
| | | Melanoma | *BRAF, NRAS* | (Tsao et al. 2015) |
| | | Breast | *PIK3CA* | (Oshiro et al. 2015) |
| | | NSCLC | *EGFR* | (Zhu et al. 2015) |
| BEAMing-Digital PCR | 0.01%–1.7% | Breast | *PIK3CA* | (Higgins et al. 2012) |
| | | NSCLC | *EGFR* | (Thress et al. 2015) |
| | | Melanoma | *BRAF, cKIT, NRAS, TERT* | (Lipson et al. 2014) |
| Whole genome sequencing | 1-5 % | Prostate | | (Heitzer et al. 2013) |
| | | CRC | | (Diaz et al. 2013) |
| Whole exome sequencing | 1% | Breast, ovarian and lung | | (Murtaza et al. 2013) |
| TAm-Seq | 2% | Ovarian and Breast | Gene Panel | (Forshew et al. 2012) |
| | | Breast | *PIK3CA, TP53* | (Dawson et al. 2013) |
| Safe-SeqS | | CRC | *KRAS, BRAF, TP53, SMAD4, PIK3CA, APC* | (Bettegowda et al. 2014) |
| CAPP-Seq | 0.02% | NSCLC | Gene panel | (Newman et al. 2014) |
| Ion-AmpliSeq | 0.5% | Breast | Gene Panel | (Rothe et al. 2014) |

[a] *Estimated detectable fraction of mutated alleles (%)*

*Abbreviations: BEAMing Digital PCR = Beads, Emulsions, Amplification and Magnetics; CAPP-Seq = Cancer personalized profiling by deep sequencing; CAST-PCR = Competitive allele-specific hydrolysis probes (TaqMan); CRC = colorectal carcinoma; NSCLC = non-small cell lung cancer; PCR; COLD-PCR = coamplification at lower denaturation temperature-PCR; PIK3CA = phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene; PNA-PCR = peptide-nucleic-acid-mediated-polymerase chain reaction clamping; Safe-SeqS = Safe-sequencing system; TAm-Seq = Tagged amplicon deep sequencing; TERT = Telomerase Reverse Transcriptase gene.*

Digital PCR (dPCR) based technologies effectively address limitations in earlier PCR based approaches. It separates the sample DNA molecules across a very large number of individual compartments such that each chamber has either one target temple or none. This technique was developed by Vogelstein and Kinzler (1999) and has been subsequently optimised using droplet-based (emulsion) or chip-based (nano-fluidic) systems (Baker 2012). BEAMing PCR technology (Beads, Emulsions, Amplification, Magnetics) combines emulsion PCR with magnetic beads and flow-cytometry which enable highly sensitive mutational analysis and quantification of ctDNA (Diehl et al. 2006; Richardson and Iglehart 2012). Studies have used dPCR based system to sensitively detect mutations in ctDNA at a very low allelic frequency (0.005% - 0.01%) in various tumour type (*Table 1*). However, dPCR is expensive, time consuming, require optimisation for each patient, and, like other targeted-based approaches described above, require prior knowledge of specific mutations to be analysed (reviewed in Luke et al. 2014).

The untargeted approach using next generation sequencing (NGS) also known as massively parallel sequencing offer promising potential to identify genome-wide tumour-derived alterations in circulating DNA. NGS-based platforms have been used by different studies to perform whole genome and whole-exome analysis of circulating DNA, and capture known cancer gene panel (*Table 1*). However, there is a trade-off between increasing the portion of the genome to be sequenced and loss of coverage (depth) which limits the ability to detect the variant of low allelic fraction and differentiate it from a technical artefact (Gagan and Van Allen 2015). Although genome-wide analysis of ctDNA (whole genome and exome sequencing) have promising applications, it is currently expensive and have lower analytical sensitivity (>1%) compared to dPCR-based approaches (*Table 1*) (Leary et al. 2012; Murtaza et al. 2013; Ma et al. 2015).

On the other hand, sequencing based on gene panel approach using hybrid capture (CAPP-Seq) or amplicon sequencing (TAm-Seq and Safe-SeqS) facilitate low-cost, high throughput and targeted deep sequencing of ctDNA (Forshew et al. 2012; Newman et al. 2014). In a study by Newman et al. (2014), cancer personalized profiling by deep Sequencing (CAPP-

Seq) was able to detect mutant allelic fraction as low as 0.02% which highlights the high analytical sensitivity of gene panel-based NGS approaches. However, these techniques require advanced bioinformatics platform and are unable to detect translocation and rearrangements without prior knowledge of breakpoint information in case of amplicon-based sequencing (Bratman et al. 2015; Gagan and Van Allen 2015).

**Technical aspects**

ctDNA is a technically challenging analyte owing to lower concentration of tumour-specific DNA within the background of cfDNA derived from non-tumorous cells. Pre-analytical and analytical issues related to ctDNA measurement hinders consistency and reliability of the results obtained. Studies have shown that concentration of cfDNA is affected by various pre-analytical factors related to blood sampling and processing such as use of different anticoagulants for sample collection, time interval between sample collection and processing, centrifugation conditions and whether serum or plasma is used for ctDNA analysis (reviewed in Jung et al. 2010). The use of serum or plasma as an optimal sampling specimen is controversial (Schwarzenbach et al. 2011). Although the amount of circulating DNA in serum is about 3 to 24 fold higher than those found in plasma (Jung et al. 2003), it is considered a less suitable material because of DNA contamination from leukocyte during the clotting process (Lee et al. 2001). However, a study by Umetani et al. (2006) showed that increased concentration of circulating DNA observed in serum might not be due to extraneous DNA contamination and could occur due to unequal distribution of tumour-related cfDNA during serum separation from whole blood. Nonetheless, plasma should be preferred over the serum as a sampling specimen to obtain reliable results due to a lower level of background wild type extraneous DNA. Different studies have also made similar recommendation (Park et al. 2012; Heitzer et al. 2015). Standardisation and methodological harmonisation of pre-analytical variables are important to achieve reliable, consistent and comparative results.

Analysis of ctDNA using different assay platforms and DNA isolation techniques are also responsible for the inconsistencies in the results obtained by various

studies. As demonstrated in *Table 1*, analytical approaches and assay sensitivity varies between different platforms. A recent study by Thress et al. (2015) compared the ability of different technological platforms to detect EGFR mutation (T790M) from ctDNA. The study has showed that digital PCR-based methods (droplet digital-PCR and BEAMing digital-PCR) were purportedly more sensitive than non-digital platforms (ARMS) for detection of mutant sequences. Additionally, as reviewed by Jung et al. (2010), a large number of alternative protocols for DNA isolation is also responsible for variability in circulating DNA concentration observed by different studies. Therefore, it is imperative to develop consensus on reliable and efficient methods to achieve standardisation.

**CLINICAL IMPLEMENTATION OF ctDNA**

ctDNA has emerged as a promising biomarker with various potential applications in precision oncology.

**Applicability of ctDNA as a diagnostic biomarker**

Analysis of tumour-specific mutations as a diagnostic biomarker have been demonstrated by different studies (*Table 2*). Detection of corresponding mutations in various genes including Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*), Tumor Protein P53 (*TP53*) and Adenomatous Polyposis Coli (*APC*) have been found in patients with pancreatic carcinoma (Kinugasa et al. 2015), colorectal cancer (Tie et al. 2015) and ovarian cancer (Forshew et al. 2012), to name a few (See *Table 2* for selected studies assessing ctDNA in different tumour type). In an exemplary paper, Bettegowda et al. (2014) have demonstrated the ability of ctDNA to detect the tumour in a large cohort of patients with different tumour type. They have shown that detectable levels of ctDNA were found in 49% to 78% of patients with localised tumours. However, as indicated in the same study, patient with stage 1 cancer and neoplasm of CNS (e.g. Glioma) showed a very low level of ctDNA. Moreover, Trombino et al. (2005) found no concordance between *KRAS* mutations found in circulating DNA and tumour tissue from patients with NSCLC. Ramirez et al. (2003), on the other hand, found more mutations in *KRAS* gene in the serum of patient compared to the primary NSCLC.

These contradictory findings question the validity of tumour-specific mutations as a diagnostic biomarker.

Another approach to facilitate cancer diagnostics using ctDNA is detection of epigenetic alterations particularly methylation changes in the circulating DNA. Aberrant DNA methylation in the numerous gene such as *APC*, Glutathione S-Transferase Pi 1 (*GSTP1*), Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*), *p16*, *SEPT9*, O-6-Methylguanine-DNA Methyltransferase (*MGMT*), Retinoic Acid Receptor Beta (*RARβ2*) and Ras Association Domain Family Member 1A (*RASSF1A*) have been analysed in cfDNA extracts (reviewed in Jung et al. 2010; Schwarzenbach et al. 2011). The diagnostic sensitivity of methylation markers in different cancers varies from 2% to 75% using a single methylation marker (Jung et al. 2010). Combining methylation markers remarkably improves the diagnostic sensitivity. As shown in a study by Skvortsova et al. (2006), combining methylation markers *RARβ2* and *RASSF1A* provide 95% diagnostic coverage (sensitivity) for detection of breast cancer. However, Fujiwara et al. (2005), reported cases where no concordance was observed between circulating DNA methylation and corresponding tumour tissue. The authors have speculated that it might be due to undetected pre-cancerous lesions or caused by environmental factors such as smoking. Additionally, the altered methylation patterns in circulating DNA are also age-dependent (Fleischhacker and Schmidt 2007). Therefore, these factors should be taken into account before using methylation status as a tumour-specific biomarker.

**Applicability of ctDNA as a prognostic biomarker**

Stratification of cancer patients based on clinical prognosis is very important to precisely tailor the treatment according to the need of individual patients. In this regard, ctDNA plays a very important role to stage cancer, monitor tumour burden, predict survival and detect disease recurrence.

Molecular staging of the tumour is one of the reliable predictors of prognosis (Eschrich et al. 2005). Studies have shown a significant correlation between disease stage and the level of tumour-associated genetic alterations found in the circulation (reviewed in

Crowley et al. 2013). A study by Diehl et al. (2005) conclusively demonstrated that the fraction of mutant molecules (*APC*) found in the plasma of patients with CRC were significantly associated with tumour stage. A recent study by Bettegowda et al. (2014) also supports this conclusion and showed that the concentration of ctDNA in the plasma increases with tumour stage across various tumour type. They found that the fraction of patients with detectable ctDNA was 47, 55, 69, and 82% in stage I, II, III, and IV cancers, respectively. However, conflicting results were obtained by some earlier studies in patients with pancreatic and CRC, showing no correlation between *KRAS* gene mutations in circulating DNA and clinicopathological parameters including tumour stage (Yamada et al. 1998; Frattini et al. 2008). Controversies associated with these studies might be due to the limited sample size and selection of appropriate patient cohort, for example, in the above-mentioned study by Frattini et al. (2008), there was considerable variability in certain demographics characteristics of patients such as tumour size and tumour-grade (moderate or poor).

Although ctDNA shows a great promise in staging tumour as described above, it might not be superior to current clinical methods (radiology, histopathology and clinical observation) to stage a tumour. As the concentration of ctDNA depends on the cellular turnover which increase with the volume of the tumour (Schwarzenbach et al. 2011), it might not be effective to stage early-cancer when the tumour volume is significantly low.

On the other, ctDNA has emerged as a superior biomarker to predict survival, determine the risk of recurrence and detect minimal residual disease. As shown in *Table 2*, different studies have demonstrated the remarkable potential of ctDNA to determine prognosis in breast, lung, CRC and ovarian cancer. Especially in the setting of advance-stage, non-resectable tumour, ctDNA offers an effective alternative to identify tumour-specific mutations in plasma that hold prognostic and predictive information. In a study by Nygaard et al. (2013), *KRAS* mutations in plasma were found to be an independent prognostic marker in patients with NSCLC. The authors have suggested that the detection of plasma mutated *KRAS* could serve as an alternative to

invasive tissue biopsy in establishing prognosis in these patients. However earlier study by Camps et al. (2011) using the same technique (qPCR) to detect plasma mutated *KRAS* showed no significant relationship with overall survival and progression-free survival in patients with advance-stage NSCLC. The opposing outcome between these two studies might be due to methodological differences. Both of these used qPCR to detect mutation, but the earlier study by Camps et al. (2011) targeted only two *KRAS* mutations in codon 12, while the later study interrogated six *KRAS* mutations in codon 12 thus resulting in increased detection of plasma mutated *KRAS* (17.5% compared to 8.8% in earlier study). However, the prognostic value and clinical utility of ctDNA in the context of a resectable tumour is very limited. Since the tumour sample is itself available, conventional tests such as histology, immunohistochemistry and molecular analysis can be performed to determine prognosis (Crowley et al. 2013).

Early identification of disease recurrence is important to stratify patients, so that aggressive and multidisciplinary approaches could be adopted in patients with a limited disease which might improve disease control and offer survival benefit (Pagani et al. 2010). In this regard, ctDNA has proved to be a useful, minimally invasive biomarker in setting of adjuvant therapy. In a recent study by Olsson et al. (2015), the eventual clinically detected recurrence (metastasis) was accurately determined by serial monitoring of ctDNA in patients with primary breast cancer who underwent potentially curative surgery. The study also showed that in 86% of the patients, ctDNA-based detection of occult metastasis preceded the clinical diagnosis with a mean lead time of 11 months. However, the current study was limited by small sample size (20 patients) and retrospective study design. Other studies have also highlighted the role of ctDNA as a predictor of poor outcome and showed a consistent relationship between disease recurrence and reappearance of tumour-specific aberrations in circulating DNA in various cancer type (Diehl et al. 2008; Kuhlmann et al. 2012; Kinugasa et al. 2015), also see *Table 2*. Moreover, studies have shown that the ability of ctDNA to predict recurrence were superior to standard biomarker (CEA) (Diehl et al. 2008; Sato et al. 2016).

**Table 2.** Selected studies of ctDNA detection in various tumour types.

| Tumour type | Patients number | Genetic alterations | Source | Analytical Platform | Clinical Utility | Reference |
|---|---|---|---|---|---|---|
| Colorectal Cancer | 18 | Mutations APC, APC, KRAS, PIK3CA, TP53 | Plasma | BEAMing Digital PCR | Prognostic (Tumour burden) | (Diehl et al. 2008) |
| | 53 | Mutations in panel of 15 genes | Plasma | Safe-SeqS | Prognostic (Tumour response) | (Tie et al. 2015) |
| | 64 | Mutations in KRAS, BRAF, TP53, SMAD4, PIK3CA, APC | Plasma | Safe-SeqS | Diagnostic, Prognostic (Tracking resistance) | (Bettegowda et al. 2014) |
| | 29 | Mutations in KRAS, Methylation RASSF2A promoter | Serum | PCR, MS-PCR | Prognostic (Clinical outcome) | (Lefebure et al. 2010) |
| Breast Cancer | 30 | Mutations in PIK3CA, TP53, and Structural variation | Plasma | TAm-Seq and digital PCR | Prognostic (Tumour burden) | (Dawson et al. 2013) |
| | 20 | Chromosomal rearrangements | Plasma | droplet digital PCR | Prognostic (Poor outcome, eventual clinical recurrence) | (Olsson et al. 2015) |
| | 171 | Mutations in ESR1 | Plasma | Digital PCR | Predictive (Predict for resistance to Aromatase inhibitors therapy) | (Schiavon et al. 2015) |
| | 72 | Mutations in PIK3CA | Plasma and Serum | ARMS-Scorpion, PCR | Predictive (Predict response to PI3K inhibitor) | (Board et al. 2010) |
| Non-small-cell lung cancer | 246 | Mutations in KRAS | Plasma | ARMS-qPCR | Prognostic (poor outcome) | (Nygaard et al. 2013) |
| | 803 | Mutations in EGFR | Plasma | Allele-specific PCR | Predictive (predict response to Gefitinib treatment) | (Douillard et al. 2014) |
| | 13 | Multiple somatic mutations and fusions | Plasma | CAPP-Seq | Diagnostic and Prognostic | (Newman et al. 2014) |
| Ovarian cancer | 38 | Mutations in TP53, PTEN, EGFR, BRAF, KRAS | Plasma | TAm-Seq, digital PCR | Prognostic (metastatic relapse) | (Forshew et al. 2012) |
| | 137 | Mutations in TP53 | Plasma/serum | PCR | Prognostic (Reduced Survival) | (Swisher et al. 2005) |
| Pancreatic cancer | 121 | Mutations in KRAS | Plasma | Safe-SeqS | Diagnostic and Prognostic | (Bettegowda et al. 2014) |
| | 75 | Mutations in KRAS | Serum | Droplet digital PCR | Diagnostic and Prognostic (Predicting survival) | (Kinugasa et al. 2015) |
| | 14 | Mutations in KRAS | Plasma | PNA-PCR | Prognostic (predict survival) | (Tjensvoll et al. 2016) |
| Oesophageal cancer | 39 | Promoter hypermethylation in TP53 | Serum | MS-PCR | Diagnostic (screening patients) | (Hibi et al. 2001) |
| Hepatocellular carcinoma | 26 | Hypomethylation and copy number aberrations | Plasma | Bisulfite sequencing (WGS) | Diagnostic and Prognostic | (Chan et al. 2013) |
| Oral squamous-cell carcinoma | 20 | Microsatellite loci | Serum | PCR | Prognostic | (Kakimoto et al. 2008) |

*Abbreviations: ARMS-PCR = Amplification-refractory mutation system; BEAMing Digital PCR = Beads, Emulsions, Amplification and Magnetics; CAPP-Seq = Cancer personalized profiling by deep sequencing; ESR1 = Estrogen Receptor 1; MS-PCR = Methylation specific PCR; PNA-PCR = peptide-nucleic-acid-mediated-polymerase chain reaction clamping; PTEN = Phosphatase and Tensin Homolog; Safe-SeqS = Safe-sequencing system; TAm-Seq = Tagged amplicon deep sequencing; WGS = Whole Genome Sequencing*

ctDNA is also superior to radiological studies in detecting disease recurrence. A recent study by Tie et al. (2015) showed that early changes in ctDNA in CRC patients receiving chemotherapy predict later radiological response. The resolution limit of radiological imaging studies to detect tumour is a tumour-size of approx. 7–10 mm which contain about 1 billion cells (Francis and Stein 2015). In comparison, tumour containing about 50 million malignant cells releases sufficient DNA which can be detected in circulation (Diaz et al. 2012). Monitoring tumour progression and recurrence requires selection of suitable mutations which are present from the initiation of tumourigenesis and is less responsive to therapeutic interventions (Crowley et al. 2013). However, selection of these candidate mutations is not a simple task because as cancer evolve, it acquires a plethora of somatic mutations (Bardelli et al. 2003), and therefore, detailed knowledge of different key mutations in different cancers is required. Multiplexed mutation detection across a panel of gene holds better clinical utility as a compared to detecting frequently mutated gene (Kidess et al. 2015).

**Applicability of ctDNA as a predictive biomarker**

The presence or absence of actionable oncogenic mutations in the archived tumour tissue sample is currently being used to guide clinical management using a number of available targeted therapies such as gefitinib, cetuximab, everolimus etc. (reviewed in Tu et al. 2016). As described above, archived tissue specimen does not effectively reflect the genomic landscape of a tumour. These genetic alterations have also been determined in ctDNA which suggests that it could be used as a viable alternative to invasive tissue analysis. In an elegant study by Thierry et al. (2014), authors have compared *KRAS* and *BMRAF* mutations in tumour tissue obtained by routine methods and plasma DNA and demonstrated a very high concordance (96%) between these two methods. Spindler et al. (2012) showed that quantitative analysis of plasma mutated *KRAS* helps in stratification of patients with metastatic CRC, and identify patients who could potentially benefit from anti-*EGFR* therapy.

ctDNA analysis has also emerged as a tool for monitoring therapeutic response and early detection of acquired resistance (*Table 2*). In a study by Gray et al. (2015), the plasma concentration of *BRAF* and *NRAS* mutations in patients with advanced metastatic melanoma were found to be associated with treatment response. Likewise, a study by Dawson et al. (2013) showed that the trend of serial ctDNA levels correlated with radiologic response to therapy in patients with metastatic breast cancer. These studies highlighted the potential of ctDNA analysis in monitoring tumour dynamics following treatment. Additionally, ctDNA analysis has been used to detect resistance-associated mutations which were previously detected by tumour re-biopsy using invasive clinical procedures. In a study by Zheng et al. (2016), *EGFR* T790M mutation which is associated with acquired resistance to TKI therapy in patients with NSCLC has been demonstrated in ctDNA and has shown to be a minimially invasive alternative to guide clinical management in these patients.

**CONCLUSIONS**

ctDNA has emerged as a minimally invasive substitute to traditional tumour tissue biopsy. Growing evidence have highlighted the promising role of ctDNA as a prognostic and predictive biomarker and demonstrated that it could be used as a surrogate for tumour tissue to predict the outcome, monitor tumour burden, detect recurrence, and identify genetic determinants for therapy. However, despite great potential of this technique, it has not been approved for routine clinical use.

Many hurdles should be overcome before ctDNA analysis can be adopted into routine clinical practices. One of the major challenges is the lack of standardisation in technical approaches. Inconsistencies are observed in preferable sample type (plasma or serum), sample processing and techniques employed for genotyping ctDNA. Therefore, methodological harmonisation, standardisation and consensus on technical approaches are required to achieve reliable, consistent and comparative results. Another major challenge is related to the specificity of molecular alterations. Individual tumour-derived mutations are never 100% specific for particular cancer and significant overlap

has been observed in different cancers. Thus, ctDNA analysis using a single gene-marker is of limited value and might lead to false-positive results. On this account, ctDNA analysis using a multi-gene panel may increase the test specificity. Additionally, although promising results have been shown by different proof-of-concept studies, prospective, well-designed and adequately powered validation studies in a large cohort and multiple tumour types are required to establish the clinical validity and utility of ctDNA analysis.

## REFERENCES

Altimari, A., Grigioni, A. D., Benedettini, E., Gabusi, E., Schiavina, R., Martinelli, A., Morselli-Labate, A. M., Martorana, G., Grigioni, W. F. and Fiorentino, M. (2008) Diagnostic role of circulating free plasma DNA detection in patients with localized prostate cancer. Am J Clin Pathol 129 (5) 756-62.

Amalou, H. and Wood, B. J. (2012) Biopsy and personalized medicine. Nature reviews. Gastroenterology & hepatology 9 (11) 683-683.

Anderson, A. R. A., Weaver, A. M., Cummings, P. T. and Quaranta, V. (2006) Tumor Morphology and Phenotypic Evolution Driven by Selective Pressure from the Microenvironment. Cell 127 (5) 905-915.

Ashida, A., Sakaizawa, K., Mikoshiba, A., Uhara, H. and Okuyama, R. (2016) Quantitative analysis of the BRAF mutation in circulating tumor-derived DNA in melanoma patients using competitive allele-specific TaqMan PCR. Int J Clin Oncol.

Azmi, A. S., Bao, B. and Sarkar, F. H. (2013) Exosomes in Cancer Development, Metastasis and Drug Resistance: A Comprehensive Review. Cancer metastasis reviews 32 (0) 10.1007/s10555-013-9441-9.

Baker, M. (2012) Digital PCR hits its stride. Nat Meth 9 (6) 541-544.

Bardelli, A., Parsons, D. W., Silliman, N., Ptak, J., Szabo, S., Saha, S., Markowitz, S., Willson, J. K. V., Parmigiani, G., Kinzler, K. W., Vogelstein, B. and Velculescu, V. E. (2003) Mutational Analysis of the Tyrosine Kinome in Colorectal Cancers. Science 300 (5621) 949-949.

Bashashati, A., Ha, G., Tone, A., Ding, J., Prentice, L. M., Roth, A., Rosner, J., Shumansky, K., Kalloger, S., Senz, J., Yang, W., McConechy, M., Melnyk, N., Anglesio, M., Luk, M. T. Y., Tse, K., Zeng, T., Moore, R., Zhao, Y., Marra, M. A., Gilks, B., Yip, S., Huntsman, D. G., McAlpine, J. N. and Shah, S. P. (2013) Distinct evolutionary trajectories of primary high-grade serous ovarian cancers revealed through spatial mutational profiling. The Journal of Pathology 231 (1) 21-34.

Belting, M. and Wittrup, A. (2008) Nanotubes, exosomes, and nucleic acid–binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease. The Journal of Cell Biology 183 (7) 1187-1191.

Bettegowda, C., Sausen, M., Leary, R. J., Kinde, I., Wang, Y., Agrawal, N., Bartlett, B. R., Wang, H., Luber, B., Alani, R. M., Antonarakis, E. S., Azad, N. S., Bardelli, A., Brem, H., Cameron, J. L., Lee, C. C., Fecher, L. A., Gallia, G. L., Gibbs, P., Le, D., Giuntoli, R. L., Goggins, M., Hogarty, M. D., Holdhoff, M., Hong, S. M., Jiao, Y., Juhl, H. H., Kim, J. J., Siravegna, G., Laheru, D. A., Lauricella, C., Lim, M., Lipson, E. J., Marie, S. K., Netto, G. J., Oliner, K. S., Olivi, A., Olsson, L., Riggins, G. J., Sartore-Bianchi, A., Schmidt, K., Shih l, M., Oba-Shinjo, S. M., Siena, S., Theodorescu, D., Tie, J., Harkins, T. T., Veronese, S., Wang, T. L., Weingart, J. D., Wolfgang, C. L., Wood, L. D., Xing, D., Hruban, R. H., Wu, J., Allen, P. J., Schmidt, C. M., Choti, M. A., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., Papadopoulos, N. and Diaz, L. A., Jr. (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6 (224) 224ra24.

Board, R. E., Thelwell, N. J., Ravetto, P. F., Little, S., Ranson, M., Dive, C., Hughes, A. and Whitcombe, D. (2008) Multiplexed assays for detection of mutations in PIK3CA. Clin Chem 54 (4) 757-60.

Board, R. E., Wardley, A. M., Dixon, J. M., Armstrong, A. C., Howell, S., Renshaw, L., Donald, E., Greystoke, A., Ranson, M., Hughes, A. and Dive, C. (2010) Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Res Treat 120 (2) 461-7.

Bratman, S. V., Newman, A. M., Alizadeh, A. A. and Diehn, M. (2015) Potential clinical utility of ultrasensitive circulating tumor DNA detection with CAPP-Seq. Expert Rev Mol Diagn 15 (6) 715-9.

Budczies, J., Weichert, W., Noske, A., Müller, B. M., Weller, C., Wittenberger, T., Hofmann, H.-P., Dietel, M., Denkert, C. and Gekeler, V. (2011) Genome-wide Gene Expression Profiling of Formalin-fixed Paraffin-Embedded Breast Cancer Core Biopsies Using Microarrays. Journal of Histochemistry and Cytochemistry 59 (2) 146-157.

Camps, C., Jantus-Lewintre, E., Cabrera, A., Blasco, A., Sanmartín, E., Gallach, S., Caballero, C., del Pozo, N., Rosell, R., Guijarro, R. and Sirera, R. (2011) The identification of KRAS mutations at codon 12 in plasma DNA is not a prognostic factor in advanced non-small cell lung cancer patients. Lung Cancer 72 (3) 365-369.

Chan, K. C. A., Jiang, P., Chan, C. W. M., Sun, K., Wong, J., Hui, E. P., Chan, S. L., Chan, W. C., Hui, D. S. C., Ng, S. S. M., Chan, H. L. Y., Wong, C. S. C., Ma, B. B. Y., Chan, A. T. C., Lai, P. B. S., Sun, H., Chiu, R. W. K. and Lo, Y. M. D. (2013) Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proceedings of the National Academy of Sciences of the United States of America 110 (47) 18761-18768.

Chang, H. W., Lee, S. M., Goodman, S. N., Singer, G., Cho, S. K., Sokoll, L. J., Montz, F. J., Roden, R., Zhang, Z., Chan, D. W., Kurman, R. J. and Shih Ie, M. (2002) Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. J Natl Cancer Inst 94 (22) 1697-703.

Chen, K. Z., Lou, F., Yang, F., Zhang, J. B., Ye, H., Chen, W., Guan, T., Zhao, M. Y., Su, X. X., Shi, R., Jones, L., Huang, X. F., Chen, S. Y. and Wang, J. (2016) Circulating

Tumor DNA Detection in Early-Stage Non-Small Cell Lung Cancer Patients by Targeted Sequencing. Sci Rep 6 31985.

Chen, X. Q., Stroun, M., Magnenat, J. L., Nicod, L. P., Kurt, A. M., Lyautey, J., Lederrey, C. and Anker, P. (1996) Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 2 (9) 1033-5.

Cherepanova, A. V., Tamkovich, S. N., Bryzgunova, O. E., Vlassov, V. V. and Laktionov, P. P. (2008) Deoxyribonuclease Activity and Circulating DNA Concentration in Blood Plasma of Patients with Prostate Tumors. Annals of the New York Academy of Sciences 1137 (1) 218-221.

Crowley, E., Di Nicolantonio, F., Loupakis, F. and Bardelli, A. (2013) Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 10 (8) 472-484.

Dawson, S. J., Tsui, D. W., Murtaza, M., Biggs, H., Rueda, O. M., Chin, S. F., Dunning, M. J., Gale, D., Forshew, T., Mahler-Araujo, B., Rajan, S., Humphray, S., Becq, J., Halsall, D., Wallis, M., Bentley, D., Caldas, C. and Rosenfeld, N. (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 368 (13) 1199-209.

Dhaliwal A. DNA extraction and purification. Mater Methods 2013;3:191.

Diaz, L. A., Jr. and Bardelli, A. (2014) Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 32 (6) 579-86.

Diaz, L. A., Sausen, M., Fisher, G. A. and Velculescu, V. E. (2013) Insights into therapeutic resistance from whole-genome analyses of circulating tumor DNA. Oncotarget 4 (10) 1856-1857.

Diaz, L. A., Williams, R., Wu, J., Kinde, I., Hecht, J. R., Berlin, J., Allen, B., Bozic, I., Reiter, J. G., Nowak, M. A., Kinzler, K. W., Oliner, K. S. and Vogelstein, B. (2012) The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486 (7404) 537-540.

Didelot, A., Le Corre, D., Luscan, A., Cazes, A., Pallier, K., Emile, J.-F., Laurent-Puig, P. and Blons, H. (2012) Competitive allele specific TaqMan PCR for KRAS, BRAF and EGFR mutation detection in clinical formalin fixed paraffin embedded samples. Experimental and Molecular Pathology 92 (3) 275-280.

Diehl, F., Li, M., Dressman, D., He, Y., Shen, D., Szabo, S., Diaz, L. A., Goodman, S. N., David, K. A., Juhl, H., Kinzler, K. W. and Vogelstein, B. (2005) Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proceedings of the National Academy of Sciences of the United States of America 102 (45) 16368-16373.

Diehl, F., Li, M., He, Y., Kinzler, K. W., Vogelstein, B. and Dressman, D. (2006) BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Meth 3 (7) 551-559.

Diehl, F., Schmidt, K., Choti, M. A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S. A., Kinzler, K. W., Vogelstein, B. and Diaz, L. A., Jr. (2008) Circulating mutant DNA to assess tumor dynamics. Nat Med 14 (9) 985-90.

Douglas, M. P. and Rogers, S. O. (1998) DNA damage caused by common cytological fixatives. Mutat Res 401 (1-2) 77-88.

Douillard, J. Y., Ostoros, G., Cobo, M., Ciuleanu, T., Cole, R., McWalter, G., Walker, J., Dearden, S., Webster, A., Milenkova, T. and McCormack, R. (2014) Gefitinib treatment in EGFR mutated caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. J Thorac Oncol 9 (9) 1345-53.

Edwards, S. L., Brough, R., Lord, C. J., Natrajan, R., Vatcheva, R., Levine, D. A., Boyd, J., Reis-Filho, J. S. and Ashworth, A. (2008) Resistance to therapy caused by intragenic deletion in BRCA2. Nature 451 (7182) 1111-1115.

Eschrich, S., Yang, I., Bloom, G., Kwong, K. Y., Boulware, D., Cantor, A., Coppola, D., Kruhoffer, M., Aaltonen, L., Orntoft, T. F., Quackenbush, J. and Yeatman, T. J. (2005) Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 23 (15) 3526-35.

Fleischhacker, M. and Schmidt, B. (2007) Circulating nucleic acids (CNAs) and cancer — A survey. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1775 (1) 181-232.

Forshew, T., Murtaza, M., Parkinson, C., Gale, D., Tsui, D. W., Kaper, F., Dawson, S. J., Piskorz, A. M., Jimenez-Linan, M., Bentley, D., Hadfield, J., May, A. P., Caldas, C., Brenton, J. D. and Rosenfeld, N. (2012) Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 4 (136) 136ra68.

Francis, G. and Stein, S. (2015) Circulating Cell-Free Tumour DNA in the Management of Cancer. International Journal of Molecular Sciences 16 (6) 14122-14142.

Frattini, M., Gallino, G., Signoroni, S., Balestra, D., Lusa, L., Battaglia, L., Sozzi, G., Bertario, L., Leo, E., Pilotti, S. and Pierotti, M. A. (2008) Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Lett 263 (2) 170-81.

Fujiwara, K., Fujimoto, N., Tabata, M., Nishii, K., Matsuo, K., Hotta, K., Kozuki, T., Aoe, M., Kiura, K., Ueoka, H. and Tanimoto, M. (2005) Identification of epigenetic aberrant promoter methylation in serum DNA is useful for early detection of lung cancer. Clin Cancer Res 11 (3) 1219-25.

Gagan, J. and Van Allen, E. M. (2015) Next-generation sequencing to guide cancer therapy. Genome Medicine 7 (1) 1-10.

Gallegos Ruiz, M. I., Floor, K., Rijmen, F., Grünberg, K., Rodriguez, J. A. and Giaccone, G. (2007) EGFR and K-ras Mutation Analysis in Non-Small Cell Lung Cancer: Comparison of Paraffin Embedded versus Frozen Specimens. Cellular Oncology : the Official Journal of the International Society for Cellular Oncology 29 (3) 257-264.

GarcÍA-Olmo, D. and GarcÍA-Olmo, D. C. (2001) Functionality of Circulating DNA. Annals of the New York Academy of Sciences 945 (1) 265-275.

Garcia-Olmo, D. C., Dominguez, C., Garcia-Arranz, M., Anker, P., Stroun, M., Garcia-Verdugo, J. M. and Garcia-Olmo, D. (2010) Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Res 70 (2) 560-7.

Gerlinger, M., Rowan, A. J., Horswell, S., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., Varela, I., Phillimore, B., Begum, S., McDonald, N. Q., Butler, A., Jones, D., Raine, K., Latimer, C., Santos, C. R., Nohadani, M., Eklund, A. C., Spencer-Dene, B., Clark, G., Pickering, L., Stamp, G., Gore, M., Szallasi, Z., Downward, J., Futreal, P. A. and Swanton, C. (2012) Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. New England Journal of Medicine 366 (10) 883-892.

Gerlinger, M. and Swanton, C. (2010) How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. Br J Cancer 103 (8) 1139-1143.

Gonzalez de Castro, D., Clarke, P. A., Al-Lazikani, B. and Workman, P. (2013) Personalized Cancer Medicine: Molecular Diagnostics, Predictive biomarkers, and Drug Resistance. Clinical Pharmacology and Therapeutics 93 (3) 252-259.

Goto, K., Ichinose, Y., Ohe, Y., Yamamoto, N., Negoro, S., Nishio, K., Itoh, Y., Jiang, H., Duffield, E., McCormack, R., Saijo, N., Mok, T. and Fukuoka, M. (2012) Epidermal growth factor receptor mutation status in circulating free DNA in serum: from IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non-small cell lung cancer. J Thorac Oncol 7 (1) 115-21.

Gray, E. S., Rizos, H., Reid, A. L., Boyd, S. C., Pereira, M. R., Lo, J., Tembe, V., Freeman, J., Lee, J. H., Scolyer, R. A., Siew, K., Lomma, C., Cooper, A., Khattak, M. A., Meniawy, T. M., Long, G. V., Carlino, M. S., Millward, M. and Ziman, M. (2015) Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 6 (39) 42008-18.

Heitzer, E., Ulz, P., Belic, J., Gutschi, S., Quehenberger, F., Fischereder, K., Benezeder, T., Auer, M., Pischler, C., Mannweiler, S., Pichler, M., Eisner, F., Haeusler, M., Riethdorf, S., Pantel, K., Samonigg, H., Hoefler, G., Augustin, H., Geigl, J. B. and Speicher, M. R. (2013) Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Medicine 5 (4) 30-30.

Heitzer, E., Ulz, P. and Geigl, J. B. (2015) Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 61 (1) 112-23.

Hibi, K., Taguchi, M., Nakayama, H., Takase, T., Kasai, Y., Ito, K., Akiyama, S. and Nakao, A. (2001) Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res 7 (10) 3135-8.

Higgins, M. J., Jelovac, D., Barnathan, E., Blair, B., Slater, S., Powers, P., Zorzi, J., Jeter, S. C., Oliver, G. R., Fetting, J., Emens, L., Riley, C., Stearns, V., Diehl, F., Angenendt, P.,

Huang, P., Cope, L., Argani, P., Murphy, K. M., Bachman, K. E., Greshock, J., Wolff, A. C. and Park, B. H. (2012) Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 18 (12) 3462-9.

Hunter, C., Smith, R., Cahill, D. P., Stephens, P., Stevens, C., Teague, J., Greenman, C., Edkins, S., Bignell, G., Davies, H., O'Meara, S., Parker, A., Avis, T., Barthorpe, S., Brackenbury, L., Buck, G., Butler, A., Clements, J., Cole, J., Dicks, E., Forbes, S., Gorton, M., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jenkinson, A., Jones, D., Kosmidou, V., Laman, R., Lugg, R., Menzies, A., Perry, J., Petty, R., Raine, K., Richardson, D., Shepherd, R., Small, A., Solomon, H., Tofts, C., Varian, J., West, S., Widaa, S., Yates, A., Easton, D. F., Riggins, G., Roy, J. E., Levine, K. K., Mueller, W., Batchelor, T. T., Louis, D. N., Stratton, M. R., Futreal, P. A. and Wooster, R. (2006) A hypermutation phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. Cancer Res 66 (8) 3987-91.

Hunter, K. W., Crawford, N. P. S. and Alsarraj, J. (2008) Mechanisms of metastasis. Breast Cancer Research : BCR 10 (Suppl 1) S2-S2.

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D. and Knippers, R. (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61 (4) 1659-65.

Jung, K., Fleischhacker, M. and Rabien, A. (2010) Cell-free DNA in the blood as a solid tumor biomarker--a critical appraisal of the literature. Clin Chim Acta 411 (21-22) 1611-24.

Jung, M., Klotzek, S., Lewandowski, M., Fleischhacker, M. and Jung, K. (2003) Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem 49 (6 Pt 1) 1028-9.

Kakimoto, Y., Yamamoto, N. and Shibahara, T. (2008) Microsatellite analysis of serum DNA in patients with oral squamous cell carcinoma. Oncol Rep 20 (5) 1195-200.

Kamat, A. A., Baldwin, M., Urbauer, D., Dang, D., Han, L. Y., Godwin, A., Karlan, B. Y., Simpson, J. L., Gershenson, D. M., Coleman, R. L., Bischoff, F. Z. and Sood, A. K. (2010) Plasma cell-free DNA in ovarian cancer: an independent prognostic biomarker. Cancer 116 (8) 1918-25.

Karachaliou, N., Mayo-de-las-Casas, C., Molina-Vila, M. A. and Rosell, R. (2015) Real-time liquid biopsies become a reality in cancer treatment. Annals of Translational Medicine 3 (3) 36.

Kidess, E., Heirich, K., Wiggin, M., Vysotskaia, V., Visser, B. C., Marziali, A., Wiedenmann, B., Norton, J. A., Lee, M., Jeffrey, S. S. and Poultsides, G. A. (2015) Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget 6 (4) 2549-2561.

Kimura, H., Fujiwara, Y., Sone, T., Kunitoh, H., Tamura, T., Kasahara, K. and Nishio, K. (2006) High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. Cancer Sci 97 (7) 642-8.

Kinugasa, H., Nouso, K., Miyahara, K., Morimoto, Y., Dohi, C., Tsutsumi, K., Kato, H., Matsubara, T., Okada, H. and Yamamoto, K. (2015) Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. Cancer 121 (13) 2271-80.

Kohler, C., Radpour, R., Barekati, Z., Asadollahi, R., Bitzer, J., Wight, E., Burki, N., Diesch, C., Holzgreve, W. and Zhong, X. Y. (2009) Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors. Mol Cancer 8  105.

Kuhlmann, J. D., Schwarzenbach, H., Wimberger, P., Poetsch, M., Kimmig, R. and Kasimir-Bauer, S. (2012) LOH at 6q and 10q in fractionated circulating DNA of ovarian cancer patients is predictive for tumor cell spread and overall survival. BMC Cancer 12  325-325.

Leary, R. J., Sausen, M., Kinde, I., Papadopoulos, N., Carpten, J. D., Craig, D., O'Shaughnessy, J., Kinzler, K. W., Parmigiani, G., Vogelstein, B., Diaz, L. A. and Velculescu, V. E. (2012) Detection of Chromosomal Alterations in the Circulation of Cancer Patients with Whole-Genome Sequencing. Science translational medicine 4 (162) 162ra154-162ra154.

Lebofsky, R., Decraene, C., Bernard, V., Kamal, M., Blin, A., Leroy, Q., Rio Frio, T., Pierron, G., Callens, C., Bieche, I., Saliou, A., Madic, J., Rouleau, E., Bidard, F. C., Lantz, O., Stern, M. H., Le Tourneau, C. and Pierga, J. Y. (2015) Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol 9 (4) 783-90.

Lee, T. H., Montalvo, L., Chrebtow, V. and Busch, M. P. (2001) Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion 41 (2) 276-82.

Lefebure, B., Charbonnier, F., Di Fiore, F., Tuech, J. J., Le Pessot, F., Michot, F., Michel, P. and Frebourg, T. (2010) Prognostic value of circulating mutant DNA in unresectable metastatic colorectal cancer. Ann Surg 251 (2) 275-80.

Leon, S. A., Shapiro, B., Sklaroff, D. M. and Yaros, M. J. (1977) Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 37 (3) 646-50.

Li, J., Wang, L., Mamon, H., Kulke, M. H., Berbeco, R. and Makrigiorgos, G. M. (2008) Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 14 (5) 579-84.

Lipson, E. J., Velculescu, V. E., Pritchard, T. S., Sausen, M., Pardoll, D. M., Topalian, S. L. and Diaz, L. A. (2014) Circulating tumor DNA analysis as a real-time method for monitoring tumor burden in melanoma patients undergoing treatment with immune checkpoint blockade. Journal for Immunotherapy of Cancer 2  42.

Lo, Y. M., Zhang, J., Leung, T. N., Lau, T. K., Chang, A. M. and Hjelm, N. M. (1999) Rapid clearance of fetal DNA from maternal plasma. American Journal of Human Genetics 64 (1) 218-224.

Loeb, S., Vellekoop, A., Ahmed, H. U., Catto, J., Emberton, M., Nam, R., Rosario, D. J., Scattoni, V. and Lotan, Y. (2013) Systematic review of complications of prostate biopsy. Eur Urol 64 (6) 876-92.

Luke, J. J., Oxnard, G. R., Paweletz, C. P., Camidge, D. R., Heymach, J. V., Solit, D. B., Johnson, B. E. and Group, f. t. C. F. D. W. (2014) Realizing the Potential of Plasma Genotyping in an Age of Genotype-Directed Therapies. Journal of the National Cancer Institute 106 (8).

Ma, M., Zhu, H., Zhang, C., Sun, X., Gao, X. and Chen, G. (2015) "Liquid biopsy"—ctDNA detection with great potential and challenges. Annals of Translational Medicine 3 (16) 235.

Maheswaran, S., Sequist, L. V., Nagrath, S., Ulkus, L., Brannigan, B., Collura, C. V., Inserra, E., Diederichs, S., Iafrate, A. J., Bell, D. W., Digumarthy, S., Muzikansky, A., Irimia, D., Settleman, J., Tompkins, R. G., Lynch, T. J., Toner, M. and Haber, D. A. (2008) Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 359 (4) 366-77.

Mancini, I., Santucci, C., Sestini, R., Simi, L., Pratesi, N., Cianchi, F., Valanzano, R., Pinzani, P. and Orlando, C. (2010) The use of COLD-PCR and high-resolution melting analysis improves the limit of detection of KRAS and BRAF mutations in colorectal cancer. J Mol Diagn 12 (5) 705-11.

Mandel, P. and Metais, P. (1948) Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 142 (3-4) 241-3.

Martins, V. R., Dias, M. S. and Hainaut, P. (2013) Tumor-cell-derived microvesicles as carriers of molecular information in cancer. Curr Opin Oncol 25 (1) 66-75.

Marusyk, A., Almendro, V. and Polyak, K. (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12 (5) 323-334.

Murtaza, M., Dawson, S. J., Tsui, D. W., Gale, D., Forshew, T., Piskorz, A. M., Parkinson, C., Chin, S. F., Kingsbury, Z., Wong, A. S., Marass, F., Humphray, S., Hadfield, J., Bentley, D., Chin, T. M., Brenton, J. D., Caldas, C. and Rosenfeld, N. (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 497 (7447) 108-12.

Newman, A. M., Bratman, S. V., To, J., Wynne, J. F., Eclov, N. C. W., Modlin, L. A., Liu, C. L., Neal, J. W., Wakelee, H. A., Merritt, R. E., Shrager, J. B., Loo, B. W., Alizadeh, A. A. and Diehn, M. (2014) An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nature medicine 20 (5) 548-554.

Nygaard, A. D., Garm Spindler, K.-L., Pallisgaard, N., Andersen, R. F. and Jakobsen, A. (2013) The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. Lung Cancer 79 (3) 312-317.

Oh, E., Choi, Y.-L., Kwon, M. J., Kim, R. N., Kim, Y. J., Song, J.-Y., Jung, K. S. and Shin, Y. K. (2015) Comparison of Accuracy of Whole-Exome Sequencing with Formalin-Fixed Paraffin-Embedded and Fresh Frozen Tissue Samples. PLoS ONE 10 (12) e0144162.

Olsson, E., Winter, C., George, A., Chen, Y., Howlin, J., Tang, M.-H. E., Dahlgren, M., Schulz, R., Grabau, D., van Westen, D., Fernö, M., Ingvar, C., Rose, C., Bendahl, P.-O., Rydén, L., Borg, Å., Gruvberger-Saal, S. K., Jernström, H. and Saal, L. H. (2015) Serial monitoring of circulating tumor DNA in patients with primary breast cancer for

detection of occult metastatic disease. EMBO Molecular Medicine 7 (8) 1034-1047.

Oshiro, C., Kagara, N., Naoi, Y., Shimoda, M., Shimomura, A., Maruyama, N., Shimazu, K., Kim, S. J. and Noguchi, S. (2015) PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat 150 (2) 299-307.

Pagani, O., Senkus, E., Wood, W., Colleoni, M., Cufer, T., Kyriakides, S., Costa, A., Winer, E. P. and Cardoso, F. (2010) International Guidelines for Management of Metastatic Breast Cancer: Can Metastatic Breast Cancer Be Cured? JNCI Journal of the National Cancer Institute 102 (7) 456-463.

Park, J.-L., Kim, H. J., Choi, B. Y., Lee, H.-C., Jang, H.-R., Song, K. S., Noh, S.-M., Kim, S.-Y., Han, D. S. and Kim, Y. S. (2012) Quantitative analysis of cell-free DNA in the plasma of gastric cancer patients. Oncology Letters 3 (4) 921-926.

Ramirez, J. L., Sarries, C., de Castro, P. L., Roig, B., Queralt, C., Escuin, D., de Aguirre, I., Sanchez, J. M., Manzano, J. L., Margelí, M., Sanchez, J. J., Astudillo, J., Taron, M. and Rosell, R. (2003) Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. Cancer Letters 193 (2) 207-216.

Richardson, A. L. and Iglehart, J. D. (2012) BEAMing Up Personalized Medicine: Mutation Detection In Blood. Clinical Cancer Research 18 (12) 3209-3211.

Rothe, F., Laes, J. F., Lambrechts, D., Smeets, D., Vincent, D., Maetens, M., Fumagalli, D., Michiels, S., Drisis, S., Moerman, C., Detiffe, J. P., Larsimont, D., Awada, A., Piccart, M., Sotiriou, C. and Ignatiadis, M. (2014) Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol 25 (10) 1959-65.

Sato, K. A., Hachiya, T., Iwaya, T., Kume, K., Matsuo, T., Kawasaki, K., Abiko, Y., Akasaka, R., Matsumoto, T., Otsuka, K. and Nishizuka, S. S. (2016) Individualized Mutation Detection in Circulating Tumor DNA for Monitoring Colorectal Tumor Burden Using a Cancer-Associated Gene Sequencing Panel. PLoS One 11 (1) e0146275.

Schiavon, G., Hrebien, S., Garcia-Murillas, I., Cutts, R. J., Pearson, A., Tarazona, N., Fenwick, K., Kozarewa, I., Lopez-Knowles, E., Ribas, R., Nerurkar, A., Osin, P., Chandarlapaty, S., Martin, L. A., Dowsett, M., Smith, I. E. and Turner, N. C. (2015) Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med 7 (313) 313ra182.

Schmidt, B., Weickmann, S., Witt, C. and Fleischhacker, M. (2008) Integrity of cell-free plasma DNA in patients with lung cancer and nonmalignant lung disease. Ann N Y Acad Sci 1137 207-13.

Schreuer, M., Meersseman, G., Van Den Herrewegen, S., Jansen, Y., Chevolet, I., Bott, A., Wilgenhof, S., Seremet, T., Jacobs, B., Buyl, R., Maertens, G. and Neyns, B. (2016) Quantitative assessment of BRAF V600 mutant circulating cell-free tumor DNA as a tool for therapeutic monitoring in metastatic melanoma patients treated with BRAF/MEK inhibitors. J Transl Med 14 (1) 95.

Schwarzenbach, H., Hoon, D. S. B. and Pantel, K. (2011) Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 11 (6) 426-437.

Sengüven, B., Baris, E., Oygur, T. and Berktas, M. (2014) Comparison of Methods for the Extraction of DNA from Formalin-Fixed, Paraffin-Embedded Archival Tissues. International Journal of Medical Sciences 11 (5) 494-499.

Shyamala, K., Girish, H. C. and Murgod, S. (2014) Risk of tumor cell seeding through biopsy and aspiration cytology. Journal of International Society of Preventive & Community Dentistry 4 (1) 5-11.

Sikora, K., Bedin, C., Vicentini, C., Malpeli, G., D'Angelo, E., Sperandio, N., Lawlor, R. T., Bassi, C., Tortora, G., Nitti, D., Agostini, M., Fassan, M. and Scarpa, A. (2015) Evaluation of cell-free DNA as a biomarker for pancreatic malignancies. Int J Biol Markers 30 (1) e136-41.

Silva, J. M., Dominguez, G., Villanueva, M. J., Gonzalez, R., Garcia, J. M., Corbacho, C., Provencio, M., España, P. and Bonilla, F. (1999a) Aberrant DNA methylation of the p16(INK4a) gene in plasma DNA of breast cancer patients. British Journal of Cancer 80 (8) 1262-1264.

Silva, J. M., Gonzalez, R., Dominguez, G., Garcia, J. M., Espana, P. and Bonilla, F. (1999b) TP53 gene mutations in plasma DNA of cancer patients. Genes Chromosomes Cancer 24 (2) 160-1.

Siravegna, G. and Bardelli, A. (2014) Genotyping cell-free tumor DNA in the blood to detect residual disease and drug resistance. Genome Biol 15 (8) 449.

Skvortsova, T. E., Rykova, E. Y., Tamkovich, S. N., Bryzgunova, O. E., Starikov, A. V., Kuznetsova, N. P., Vlassov, V. V. and Laktionov, P. P. (2006) Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. Br J Cancer 94 (10) 1492-5.

Sorenson, G. D., Pribish, D. M., Valone, F. H., Memoli, V. A., Bzik, D. J. and Yao, S. L. (1994) Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 3 (1) 67-71.

Sozzi, G., Conte, D., Leon, M., Ciricione, R., Roz, L., Ratcliffe, C., Roz, E., Cirenei, N., Bellomi, M., Pelosi, G., Pierotti, M. A. and Pastorino, U. (2003) Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol 21 (21) 3902-8.

Spindler, K. L., Pallisgaard, N., Vogelius, I. and Jakobsen, A. (2012) Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clin Cancer Res 18 (4) 1177-85.

Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C. and Beljanski, M. (1989) Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 46 (5) 318-22.

Stroun, M., Lyautey, J., Lederrey, C., Olson-Sand, A. and Anker, P. (2001) About the possible origin and mechanism of circulating DNA: Apoptosis and active DNA release. Clinica Chimica Acta 313 (1–2) 139-142.

Stroun, M., Maurice, P., Vasioukhin, V., Lyautey, J., Lederrey, C., Lefort, F., Rossier, A., Chen, X. Q. and Anker, P. (2000) The Origin and Mechanism of

Circulating DNA. Annals of the New York Academy of Sciences 906 (1) 161-168.

Swanton, C. (2012) Intratumor heterogeneity: evolution through space and time. Cancer Res 72 (19) 4875-82.

Swisher, E. M., Wollan, M., Mahtani, S. M., Willner, J. B., Garcia, R., Goff, B. A. and King, M. C. (2005) Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer. Am J Obstet Gynecol 193 (3 Pt 1) 662-7.

Takai, E., Totoki, Y., Nakamura, H., Morizane, C., Nara, S., Hama, N., Suzuki, M., Furukawa, E., Kato, M., Hayashi, H., Kohno, T., Ueno, H., Shimada, K., Okusaka, T., Nakagama, H., Shibata, T. and Yachida, S. (2015) Clinical utility of circulating tumor DNA for molecular assessment in pancreatic cancer. Scientific Reports 5 18425.

Taly, V., Pekin, D., Benhaim, L., Kotsopoulos, S. K., Le Corre, D., Li, X., Atochin, I., Link, D. R., Griffiths, A. D., Pallier, K., Blons, H., Bouche, O., Landi, B., Hutchison, J. B. and Laurent-Puig, P. (2013) Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 59 (12) 1722-31.

Tamkovich, S. N., Cherepanova, A. V., Kolesnikova, E. V., Rykova, E. Y., Pyshnyi, D. V., Vlassov, V. V. and Laktionov, P. P. (2006) Circulating DNA and DNase activity in human blood. Ann N Y Acad Sci 1075 191-6.

Thierry, A. R., Mouliere, F., El Messaoudi, S., Mollevi, C., Lopez-Crapez, E., Rolet, F., Gillet, B., Gongora, C., Dechelotte, P., Robert, B., Del Rio, M., Lamy, P.-J., Bibeau, F., Nouaille, M., Loriot, V., Jarrousse, A.-S., Molina, F., Mathonnet, M., Pezet, D. and Ychou, M. (2014) Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 20 (4) 430-435.

Thress, K. S., Brant, R., Carr, T. H., Dearden, S., Jenkins, S., Brown, H., Hammett, T., Cantarini, M. and Barrett, J. C. (2015) EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer 90 (3) 509-515.

Tie, J., Kinde, I., Wang, Y., Wong, H. L., Roebert, J., Christie, M., Tacey, M., Wong, R., Singh, M., Karapetis, C. S., Desai, J., Tran, B., Strausberg, R. L., Diaz, L. A., Jr., Papadopoulos, N., Kinzler, K. W., Vogelstein, B. and Gibbs, P. (2015) Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol 26 (8) 1715-22.

Tjensvoll, K., Lapin, M., Buhl, T., Oltedal, S., Steen-Ottosen Berry, K., Gilje, B., Søreide, J. A., Javle, M., Nordgård, O. and Smaaland, R. (2016) Clinical relevance of circulating KRAS mutated DNA in plasma from patients with advanced pancreatic cancer. Molecular Oncology 10 (4) 635-643.

Trejo-Becerril, C., Pérez-Cárdenas, E., Taja-Chayeb, L., Anker, P., Herrera-Goepfert, R., Medina-Velázquez, L. A., Hidalgo-Miranda, A., Pérez-Montiel, D., Chávez-Blanco, A., Cruz-Velázquez, J., Díaz-Chávez, J., Gaxiola, M. and Dueñas-González, A. (2012) Cancer Progression Mediated by Horizontal Gene Transfer in an In Vivo Model. PLoS ONE 7 (12) e52754.

Trombino, S., Neri, M., Puntoni, R., Angelini, C., Loprevite, M., Cesario, A., Granone, P., Imperatori, A., Dominioni, L., Ardizzoni, A., Filiberti, R. and Russo, P. (2005) Mutations in K-ras codon 12 detected in plasma DNA are not an indicator of disease in patients with non-small cell lung cancer. Clin Chem 51 (7) 1313-4.

Tsao, S. C., Weiss, J., Hudson, C., Christophi, C., Cebon, J., Behren, A. and Dobrovic, A. (2015) Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. Sci Rep 5 11198.

Tu, M., Chia, D., Wei, F. and Wong, D. (2016) Liquid Biopsy for Detection of Actionable Oncogenic Mutations in Human Cancers and Electric Field Induced Release and Measurement Liquid Biopsy (eLB). The Analyst 141 (2) 393-402.

Umetani, N., Hiramatsu, S. and Hoon, D. S. (2006) Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation. Ann N Y Acad Sci 1075 299-307.

van der Vaart, M. and Pretorius, P. J. (2010) Is the role of circulating DNA as a biomarker of cancer being prematurely overrated? Clin Biochem 43 (1-2) 26-36.

Vietsch, E. E., van Eijck, C. H. J. and Wellstein, A. (2015) Circulating DNA and Micro-RNA in Patients with Pancreatic Cancer. Pancreatic disorders & therapy 5 (2) 156.

Vogelstein, B. and Kinzler, K. W. (1999) Digital PCR. Proceedings of the National Academy of Sciences of the United States of America 96 (16) 9236-9241.

Xu, J.-M., Liu, X.-J., Ge, F.-J., Lin, L., Wang, Y., Sharma, M. R., Liu, Z.-Y., Tommasi, S. and Paradiso, A. (2014) KRAS mutations in tumor tissue and plasma by different assays predict survival of patients with metastatic colorectal cancer. Journal of Experimental & Clinical Cancer Research 33 (1) 1-8.

Xue, X., Zhu, Y. M. and Woll, P. J. (2006) Circulating DNA and Lung Cancer. Annals of the New York Academy of Sciences 1075 (1) 154-164.

Yamada, T., Nakamori, S., Ohzato, H., Oshima, S., Aoki, T., Higaki, N., Sugimoto, K., Akagi, K., Fujiwara, Y., Nishisho, I., Sakon, M., Gotoh, M. and Monden, M. (1998) Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: correlation with clinicopathological features. Clin Cancer Res 4 (6) 1527-32.

Zheng, D., Ye, X., Zhang, M. Z., Sun, Y., Wang, J. Y., Ni, J., Zhang, H. P., Zhang, L., Luo, J., Zhang, J., Tang, L., Su, B., Chen, G., Zhu, G., Gu, Y. and Xu, J. F. (2016) Plasma EGFR T790M ctDNA status is associated with clinical outcome in advanced NSCLC patients with acquired EGFR-TKI resistance. Scientific Reports 6 20913.

Zhu, G., Ye, X., Dong, Z., Lu, Y. C., Sun, Y., Liu, Y., McCormack, R., Gu, Y. and Liu, X. (2015) Highly Sensitive Droplet Digital PCR Method for Detection of EGFR-Activating Mutations in Plasma Cell-Free DNA from Patients with Advanced Non-Small Cell Lung Cancer. J Mol Diagn 17 (3) 265-72.first new gold open access journal. Br J Pharm, 1, 1-13.