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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1728467 since 2020-02-19T11:11:19Z

Published version:

DOI:10.1093/annonc/mdz227

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18 July 2024

How liquid biopsies can change clinical practice in oncology

Siravegna G.[1,2,#], Mussolin B.[1], Venesio T.[1], Marsoni S.[3], Seoane J.[4,5], Dive C.[6], Papadopoulos N.[7,8], Kopetz S.[9], Corcoran R. B.[10], Siu L. L.[11], and Bardelli A.[1,2,#]

[1]Candiolo Cancer Institute, FPO-IRCCS, Candiolo (TO), Italy; [2]Department of Oncology, University of Torino, Candiolo (TO); [3] IFOM, Istituto FIRC di Oncología Molecolare, Milan, Italy; [4]Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital and Universitat Autonoma de Barcelona, CIBERONC, Barcelona, Spain; [5]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain; [6]Clinical and Experimental Pharmacology Group and Manchester Centre for Cancer Biomarker Sciences, Cancer Research UK Manchester Institute, University of Manchester, Manchester, UK; [7]Ludwig Center for Cancer Genetics and Therapeutics, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [8]Sidney Kimmel Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [9]Division of Cancer Medicine, Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, USA; [10]Massachusetts General Hospital Cancer Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA; [11]Princess Margaret Cancer Centre, University of Toronto, Toronto, Canada;

[#] co-senior authors

Corresponding author:
Prof. Alberto Bardelli
Department of Oncology, University of Torino, SP 142 Km 3.95, 10060 Candiolo (TO)
+39 0119933235
alberto.bardelli@unito.it

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Abstract

Cell-free DNA fragments are shed into the bloodstream by tumor cells. The analysis of circulating tumor DNA (ctDNA), commonly known as liquid biopsy, can be exploited for a variety of clinical applications. ctDNA is being used to genotype solid cancers non-invasively, to track tumor dynamics and to detect the emergence of drug resistance. In a few settings, liquid biopsies have already entered clinical practice. For example, ctDNA is used to guide treatment in a subset of lung cancers. In this review, we discuss how recent improvements in the sensitivity and accuracy of ctDNA analyses have led to unprecedented advances in this research field. We further consider what is required for the routine deployment of liquid biopsies in the clinical diagnostic space. We pinpoint technical hurdles that liquid biopsies have yet to overcome, including pre-analytical and analytical challenges. We foresee how liquid biopsies will transform clinical practice: by complementing (or replacing) imaging to monitor treatment response and by detecting minimal residual disease after surgery with curative intent.

Key words:
liquid biopsy, circulating free DNA, cancer diagnosis, resistance, minimal residual disease, clonal evolution

Key message:
A few liquid biopsy-based, circulating tumor DNA (ctDNA) analysis have already entered clinical practice. Improvements in the sensitivity and accuracy of ctDNA analyses led to unprecedented advances in this research field. We foresee that liquid biopsies could transform clinical practice by complementing imaging to monitor treatment response and by detecting minimal residual disease after surgery.

Introduction

Cancer represents the second leading cause of death worldwide [1, 2]. Tumor molecular profiling is essential to optimize treatment in clinical practice [3]. Diagnostic strategies currently employ tissue samples to assess specific biomarkers. Genotyping tumor tissue requires invasive procedures with inherent risks [4]. Up to 25% of lung cancer biopsies fail

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to obtain enough tissue for assessment [5]. Moreover, it has been shown that the genomic profiles of primary tumors and metastases are not always concordant owing to intrinsic molecular cancer heterogeneity [3, 6-8]. Furthermore, the genomic landscape can change during the course of therapy [3]. For all these reasons, tissue biopsies cannot be used to provide up-to-date profiles of the inherent evolving nature of individuals with solid cancers. To overcome these limitations, many groups have successfully used analysis of circulating tumor DNA (ctDNA), a procedure known as liquid biopsy [9-15]. The latter involves the study of body fluids (primarily blood but also saliva, urine and cerebrospinal fluid) which have been found to carry nucleic acid fragments originating from cancer cells. Liquid biopsies allow for repeat sampling due to their minimally-invasive nature. A tissue biopsy can be compared to a *'snapshot'* of the cancer, whereas the analysis of circulating tumor DNA is a '*screenshot*' of the primary and metastatic tumor [16] and longitudinal LBs can be viewed as a *'motion picture'* of tumor evolution. Indeed, liquid biopsies can interrogate clonally divergent, distant lesions without sampling bias, thus allowing longitudinal monitoring of patients during treatment [17]. It is also true that in some instances, liquid biopsies can fail to detect gene alterations which can be otherwise identified in tumor tissue, as it might happen in early stage patients due to a very small amount of circulating tumor material which presently restricts sensitivity.

The biology of circulating tumor DNA

Historically, liquid biopsies can be traced back to 1948, when Mandel and Métais described the existence of circulating cell-free DNA (cfDNA) in the blood of healthy individuals [18]. But it was only in 1977 that the presence of differential levels of cfDNA, with increased levels in the blood of patients with various types of cancer, was demonstrated [19]. In 1994, the clinical potential of cfDNA was recognized as a result of the detection of mutated RAS gene fragments in the blood of cancer patients [20, 21]. It should be noted that the term cfDNA refers to both encapsulated (from circulating vesicles) and non-encapsulated free DNA in the bloodstream or other body fluids and the fraction of cfDNA originated from tumor cells is named cell-free circulating tumor DNA (ctDNA) [11].

The precise mechanism of cfDNA release from non-cancer cells into the peripheral blood and other body fluids, as well as its biological properties remains unclear. ctDNA may originate from tumor cells through multiple mechanisms, including apoptosis, necrosis and

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active secretion from extracellular vesicle and circulating tumor cells (CTCs) [22-24]. The latter carries the same genetic and epigenetic alterations of the primary tumor or metastases and also provides an opportunity for a non-invasive evaluation of cancer. However, only a subset of cancers shed significant number of CTCs in the circulation [25, 26]. Virtually all molecular alterations present in cancer cells can be detected in ctDNA including both coding and non-coding regions, microsatellite loci, loss of heterozygosis (LOH), mutations, polymorphisms, methylation and copy number variations [27, 28].

The realization that cancer patients had greater levels of plasma cfDNA than tumor-free controls [19, 29] led to the clinical applications of liquid biopsies. However, high levels of cfDNA are not specific to cancer as they are also observed in several other pathologies, including pro-inflammatory diseases and autoimmune disorders, such as liver cirrhosis, hepatitis, systemic lupus erythematous or rheumatoid arthritis, as well as pregnancy and intense physical exercise [30, 31].

The size of DNA fragments circulating in blood is relevant and related to its biology. The length of cfDNA and its highly fragmented status was previously revealed by gel electrophoresis in plasma from pancreatic cancer patients and healthy controls [32]. Massively parallel sequencing has shown that ctDNA has a size distribution of 130–170 bp (predominant peak around 166 bp and a series of smaller peaks occurring at 10 bp periodicity at sizes of approximately 143 bp and shorter) [33-36], which is equivalent to the size of nuclease-cleaved nucleosomes. This size distribution suggests that a significant amount of this DNA likely originates from apoptotic processes [32]. In addition, DNA molecules larger than 10.000 bp have also been described, suggesting other release mechanisms such as necrosis or phagocytosis of necrotic tumor cells by macrophages [23, 37].

ctDNA levels are also influenced by disease burden and many other factors such as tumor location, vascularity and cellular turnover [15, 23, 38]. In this regard, Bettegowda and colleagues reported how ctDNA detectability varies among different tumor types [15]. Indeed, most patients with stage III and metastatic cancer of the liver, ovaries, colon, stomach, breast, esophagus, pancreas, bladder, and head and neck as well as patients with neuroblastoma and melanoma, harbor detectable ctDNA levels. In contrast, tumors localized in the central nervous system or those with mucinous features (like prostate and

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thyroid) frequently show low or undetectable ctDNA levels [15, 39]. Notably, in the case of brain tumors, including brain metastatic lesions, ctDNA can be found in the cerebrospinal fluid providing an alternative source for liquid biopsies [40, 41]. ctDNA has a variable half-life in the circulation, with a reported average around 15 min [11], due to rapid clearance by lymphatic circulation via the kidneys, liver and spleen [42]. Tumors are often comprised of multiple cell clones (which account for intra-tumor heterogeneity) and normal/healthy cells, such as hematopoietic and stromal cells. Thus, during tumor progression and turnover both tumor-derived and wild-type (normal) cfDNA can be released into the blood.

Accordingly, the proportion of ctDNA that originates from individual cancer lesions depends on the anatomic location and size of the tumor; It has been estimated that, for a patient with a tumor of 100 g in weigh (3×10^{10} tumor cells), 3.3% of its DNA can be released in the blood circulation every day [43]. Also, the presence of CTCs and metastatic lesions can contribute to ctDNA yields [44].

Technical challenges associated with the collection and processing of ctDNA

Although cancer patients have higher cfDNA levels than healthy donors, the concentrations of overall cfDNA vary considerably [45, 46]. Multiple studies have shown that cfDNA ranges between 0 and >1,000 ng per mL of blood, with an average of 180 ng of cfDNA per mL [11, 47-50]. This amount would, however, represent only a tiny fraction of the total cell turnover per day in the human body. By comparison, in healthy subjects the concentration of plasma cfDNA ranges from less than 10 ng/ml to more than 100 ng/ml, with an average of 30 ng per mL cfDNA [11]. Therefore, in patients with cancer, ctDNA represents a small proportion of total cfDNA, varying from less than 0.1% to over 10% depending on disease burden, stage, cellular turnover, and treatment response [38]. However, the quantification of cfDNA concentrations alone cannot be exploited for diagnostic purposes owing to the overlapping DNA concentrations that are found in healthy individuals with those in patients with malignant disease and the fact that exercise and inflammation are known to modulate cfDNA concentration levels. Some studies linked higher cfDNA concentration with shorter overall survival (OS) of patients with breast [51], ovarian [52], lung [53], gastric [54] and colorectal cancer [38], but the clinical value of this information remains to be confirmed.

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The clinical deployment of liquid biopsies requires overcoming several challenges regarding ctDNA detection and analysis. For example, routine liquid biopsy application in clinical practice is presently limited by the lack of standardization and absence of broadly accepted standard operating procedures (SOPs) for ctDNA assessments procedures [55]. Due to the low concentration of ctDNA in patient-derived biological samples, analytical specificity and sensitivity are necessary to generate reproducible results [56]. Improvement in DNA isolation technologies, detection and analysis have recently allowed reproducible detection of low ctDNA levels from a background signal of wild-type DNA released in the circulation by healthy tissues [57]. Preanalytical variables must be accurately controlled. For example, during blood collection and processing it is essential to avoid white blood cell lysis which increases dilution of tumor circulating fragments from genomic DNA derived from white blood cells. Plasma is the preferred source for ctDNA compared to serum due to greater cell lysis that occurs during the clotting process [58]. Preanalytical procedures such as blood collection, transportation, processing and storage temperature are also critical as they affect leukocytes stability and ctDNA degradation. Analytical and post-analytical variants may affect ctDNA quantitation as well, especially since ctDNA has a short half-life and there are time-dependent changes in DNA in blood collection tubes [59], due to white blood cells lysis. While a consensus on the procedures to preserve DNA integrity has not yet been achieved, most investigators agree that it is critical to isolate plasma within an hour after blood draw to avoid cfDNA degradation due to DNase activity [60]. Purposely designed blood collection tubes for cfDNA are available from multiple providers which minimize cell lysis and stabilize the total cfDNA pool by the inclusion of various additives and preservatives.

As discussed above, tumor DNA in the circulation is highly fragmented and often present in low amount. Accordingly, several protocols require as an initial step PCR-based amplification strategies, which increases the risk of artifacts due to the intrinsic error rate of the DNA polymerases and represents a limitation in many assays [11, 61]. The relevance and challenges of analytical validation of methods for liquid biopsy are not the focus of the present review and have recently been described in details elsewhere [62]. We underline that a major barrier is the current lack of comparability between existing approaches, as it was highlighted in a recent European Quality Assurance (EQA) scheme undertaken by the European Molecular Genetics Quality Network (EMQN), which assessed ctDNA detection approaches. This analysis emphasized how multiple pre-analytical and analytical techniques generate variable results [63]. International European initiatives based on consortium of several groups from different countries, such as CANCER-ID

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(https://www.cancer-id.eu/) and SPIDIA4P (http://www.spidia.eu/) are presently establishing standards for analytical and pre-analytical procedures, respectively. The analytical process to obtain ctDNA can strongly affect the standardization. A large variability in cfDNA yield and/or fragment size was reported, mainly due to the type of cfDNA extractions [64]. For example, methods based on phenol-chloroform were shown to provide higher yield, but lower DNA purity than magnetic-based techniques [65]. Multiple reports point to the silica-based membranes as the preferred approach for a high cfDNA recovery (82%–92%) [66], though this leads to loss of DNA fragments smaller than 150 bp [67].

Methodologies to genotype ctDNA

It is now generally accepted that tumors are genetically and phenotypically diverse (intra- and interpatient heterogeneity). ctDNA analyses allow longitudinal tracking of tumor dynamics during treatment [3, 12]. For example, longitudinal measurements with liquid biopsies can monitor disease progression, sub-clone evolution and patient response in real time during therapy [6, 68].

Two approaches were developed to study tumor's DNA in the blood. The first involves querying tumor-specific mutations or predefined genomic regions (targeted ctDNA strategy). This type of analysis is based on the use of very sensitive technique including PCR-based assays such as droplet digital PCR (ddPCR), Q-PCR, ARMS and BEAMing-PCR, as well as (Next Generation Sequencing) NGS-based methods such as Safe-SeqS, CAPP-Seq, and TAmSeq. PCR-based methods interrogate a limited number of known mutations, have acceptable sensitivity and high specificity. These platforms are utilized to detect *EGFR* activating mutations as well as resistance mutations in NSCLC patients who progress on *EGFR* TKIs [69]. NGS-based assays like TAM-Seq and CAPP-Seq, designed to detect multiple classes of mutations, including indels, rearrangements, and copy number alterations have also shown high sensitivity and specificity. Overall, this candidate-based strategy requires prior information on the tumor genome and allows detection of mutations at an allele frequency of 0.01% with high specificity. The second approach (untargeted strategy) aims to provide exome or genome-wide analysis of mutations and copy number aberrations (CNAs) by whole exome sequencing (WES) or whole-genome sequencing (WGS). Untargeted methods, which are based on the use of next generation sequencing (NGS) techniques, do not require prior knowledge of the genome of the primary tumors and

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allow identification of novel changes occurring during tumor treatment, but can be less sensitive (1-5%) and generally rely on high amounts of ctDNA. Among NGS approaches, deep-sequencing allows detection of variants at 0.2% low allele-frequency and identification of the mutations reported in tumor tissue with potential 100% sensitivity, although with a limited specificity (about 80%) [68, 70]. Its main advantage is the ability to investigate simultaneously multiple cancer specific variants. In the future, it is likely that increasing the number of tumor-specific variants that can be assessed simultaneously will improve both sensitivity and specificity. Importantly, NGS of ctDNA is affected by an error rate between 0.1% and 1%, which presently limits this approach. To overcome this, several protocols have been modified in order to improve the identification of those variants [71]. Several bioinformatic algorithms are now available for cfDNA analyses [26, 72, 73]. Ultimately, the quality of NGS reads is highly relevant and deeply affects downstream analysis. As sequencing costs drop, NGS-based untargeted strategies will become clinically feasible, coupled with new generations of computational strategies.

Clinical applications of liquid biopsies

ctDNA holds promise in many clinical applications including genotyping cancer at diagnosis, detection of minimal residual disease after surgery, monitoring treatment response, early detection and profiling of resistance to therapy. These aspects are discussed in separate paragraphs below and summarized in Figure 1.

Genotyping cfDNA for somatic genomic alterations found in tumors.

Many studies have shown that genetic variations in ctDNA reflect the mutational landscape of the corresponding tumor tissue. Although specificity among detection methods can approach 100%, sensitivity is generally lower and depends on the DNA alteration type [74]. A high concordance (often about 80% or more) has been reported between the mutational profile of driver oncogenes (*KRAS*, *NRAS*, *PIK3CA*, *BRAF*, and *EGFR*) in ctDNA and tumor tissue of colorectal [15, 75], lung [76] and breast [15] cancer patients. In 2014 NSCLC patients were the first to benefit from an approved liquid biopsy FDA test (Real-time PCR-based cobas® *EGFR* Mutation Test v2) to detect *EGFR* mutations associated with sensitivity to erlotinib or osimertinib. Meta-analyses assessed the performance of ctDNA compared to tissue to detect *EGFR* mutations in NSCLC, demonstrating a sensitivity of 61% to 67% and a specificity of 90% to 95.9% [77]. These data should be interpreted with caution since they

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were obtained with different platforms. The EURTAC trial [78], comparing first-line erlotinib and chemotherapy in advanced NSCLC, proposed that patients with ctDNA negative for the *EGFR* p.T790M mutation underwent a further tissue biopsy to rule out the event of false negatives, generally due to non-shedding tumors rather than a failure of the testing platforms. On the other hand, plasma positive testing in presence of tissue negativity may be due to the failure of tissue biopsy to detect tumor heterogeneity. The need for dual tissue/plasma testing in NSCLC seems to be confirmed also by recent results [79]**.**

Detecting minimal residual disease and monitoring treatment response

Two areas are emerging in which liquid biopsies are poised to radically transform clinical practice in oncology: The first is signaling the presence of an already (molecular) metastatic status in an otherwise phenotypically local disease and the second is forecasting the outcome of systemic therapy in metastatic disease. In both clinical settings, ctDNA acts as synthetic proxy of the tumor biology: in the first case by heralding an inherent resistance to therapy and in in the latter by foreshadowing metastatic spread, signaled by the persistence of minimal residual disease (MRD) after surgery, that distinguishes between indolent and aggressive, metastatic prone tumors.

Detecting the presence of minimal residual disease

The liquid biopsy applications in this area are far reaching and could potentially parallel the studies that have led to changes in the management of leukemias and other blood cancers. The rate limiting step to applying blood-based MRD tests in solid cancer is technical. To detect residual tumor DNA, highly sensitive tests are necessary. While innovative approaches to detect ctDNA with high sensitivity are being developed, proof of concept studies have been performed. Several analyses have shown that ctDNA levels can be used to monitor MRD following surgery or other curative treatment in colorectal, breast and other cancer types. In a landmark study, Diehl and colleagues were first to show that in colorectal cancer (CRC) patients who received surgery, those with MRD detected generally in cfDNA had disease relapse within 1 year of localized surgery. More recently, Tie and colleagues reported a prospective trial evaluating the relationship of postoperative ctDNA levels with tumor recurrence in patients with stage II CRC. The authors observed that recurrence rates were >10-fold higher in patients with detectable postoperative ctDNA than those in whom ctDNA was undetectable, suggesting that ctDNA analysis could be used as predictive biomarker. In parallel studies performed in breast cancers, *PIK3CA* mutations were detected in DNA from plasma samples obtained before surgery in 93% of patients with localized

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tumors and a limited tumor burden [80]. Given the short half-life of ctDNA and the possibility of minimally invasive repeat sampling, liquid biopsies can enable real-time monitoring of disease during therapy, as its assessment can be performed more often than imaging. Studies monitoring patients during treatment have shown that ctDNA dynamics correlates with treatment response, and may identify response earlier than clinical detection [38, 75, 81-86].

F*orecasting the outcome of systemic therapy in metastatic disease*

A number of studies have employed ctDNA as biomarkers of metastatic disease, to monitor disease response and to assess overall disease burden. Levels of ctDNA decrease after surgery and/or chemotherapy. In a study, an increase in ctDNA correlated with disease progression in 89% of metastatic breast cancer patients, with an average of 5 months prior to radiological progression [82], and with increased accuracy as compared to standard serum markers such as CA153 and CA15-3 (MUC1). In ovarian cancer, pre-treatment ctDNA levels and the extent of ctDNA decrease after chemotherapy initiation were significantly associated with time to progression, and were more informative than CA125 levels [87]. The same applies to NSCLC; one study reported that its levels were significantly correlated with overall tumor volume and could differentiate between residual disease and treatment-related imaging modifications [84]. Another study [88] demonstrated that in patients with *EGFR* mutation-positive ctDNA at baseline, ctDNA dynamic changes are associated with treatment outcomes. Similar studies in other cancer types, including metastatic melanoma undergoing immune checkpoint blockade, metastatic colorectal cancer (mCRC), and primary gynecological malignancies, reached similar conclusions [89-91]. Data suggest that these changes can occur rapidly, with ctDNA variations at two-weeks being predictive of subsequent radiographic results in restaging studies at two-months [91]. These studies reported that while ctDNA analysis cannot yet fully replace imaging, it can be used in combination with radiological assessments and may provide a more rapid assessment of tumor response than traditional imaging assessment.

The role of ctDNA to anticipate response to systemic therapy has been also evaluated. In 2015, 53 mCRC patients receiving 1st-line chemotherapy had their ctDNA levels monitored using SafeSeq [91]. In this prospective study, blood ctDNA samples were collected before treatment, 3 days after treatment and before cycle 2. Results indicated that 92% of patients had positive ctDNA at baseline and those with a reduction in ctDNA just prior to cycle 2 also had a radiological-confirmed response 8 to 10 weeks later. However, this study could not define whether ctDNA is able to predict disease progression as only 1 case relapsed on the

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8–10 week CT scan. More recently, the results reported in the PLACOL study confirmed Tie's data: changes in ctDNA concentrations among first, second and third chemotherapy cycles were assessed and found to discriminate responding patients [92]. The prognostic role of baseline ctDNA levels has also been confirmed in pretreated mCRC as reported by the CORRECT study [93], a large phase 3 trial whose results demonstrated the clinical benefit of regorafenib in chemo-refractory patients. An early spike in ctDNA levels may also predict response, as suggested by a recent study which assessed allele fractions of *BRAF* mutations in the first week following the initiation of immunotherapy in melanoma patients [94]. This behavior likely reflects a transient ctDNA increase due to massive cell death

In ER+, HER2- metastatic breast cancer, the ongoing PADA-1 study [95] included 803 patients receiving palbociclib combined with an aromatase inhibitor as standard of care. Within this trial, circulating levels of mutant ESR1 were detected prior to therapy in 17 cases, and were associated with prior use of aromatase inhibitor in the adjuvant setting. Of note, in most case where circulating ESR1 mutated alleles were identified prior to therapy, their levels became undetectable one month after combinatorial treatment initiation, suggesting that this combination may retain early antitumor efficacy.

Using ctDNA to understand secondary drug resistance

The utility of liquid biopsies to detect mechanisms of primary and acquired resistance to treatments has been extensively investigated alongside with ctDNA application to direct additional lines of therapies and to guide rechallenge protocols. ctDNA can be exploited to monitor clonal evolution and identify heterogeneous resistance mechanisms to drug exposure [13, 69, 75, 96-98]. In NSCLC, ctDNA has been used to identify several actionable mutations for targeted treatments such as in *EGFR*, *ALK*, *ROS1*, *RET* rearrangements, *ERBB2* and *MET* alterations, as well as monitoring acquired resistance mutations (i.e. T790M mutation, amplification of the MET receptor tyrosine kinase or ERBB2, and mutations in *PIK3CA*, *BRAF*, *STAT3*, or *AXL* amplifications) [99]. In CRC, liquid biopsy analysis in 503 cases confirmed the capability of detecting the continuous dynamic change of *KRAS*, *BRAF*, and *PIK3CA* mutations in ctDNA on regorafenib treatment [93]. Therefore, ctDNA detection can be used to nominate mechanisms of tumor resistance and guide earlier selection of appropriate follow up therapies that may improve clinical outcome. In 2012, two independent groups uncovered KRAS alterations as mechanisms of emerging resistance to anti-EGFR therapy in CRC with ctDNA analysis [13, 14]. Of note, it was revealed that ctDNA anticipated

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the emergence of KRAS resistant subclones as early as 10 months prior to radiographic progression. Subsequent data demonstrated that withdrawal of EGFR blockade drugs after progression leads to further clonal evolution which can be exploited pharmacologically [75, 100]: ctDNA analysis showed that mutated *KRAS* alleles detected at progression start declining when anti-EGFR blockade is withdrawn suggesting a potential role for anti-EGFR re-challenge. ctDNA analyses were also pivotal in showing that a subset of CRC patients acquire *EGFR* extracellular domain (ECD) mutations as mechanisms of resistance to EGFR blockade with the anti-EGFR antibodies cetuximab and panitumumab. Patients with *EGFR* ECD mutations had greater and longer responses to anti-EGFR therapy as compared to those acquiring RAS mutations [101]. In a separate, recent study [102], genetic mechanisms of primary and acquired resistance to double HER2 blockade in a cohort of *ERBB2*-amplified metastatic colorectal cancer was uncovered by ctDNA analysis. Blood samples were collected prior to initiation of treatment (baseline), every 15 days during therapy, and at radiological progression or at the end of treatment. NGS analysis was performed using a targeted NGS panel. Alterations in *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *MAP2K1*, *ERBB2*, *EGFR*, and *MET* mutations and/or copy number changes, while *ERBB2* copy number alteration was confirmed in plasma with a sensitivity of 97.9% and a positive predictive value of 100% [102]. These findings highlighted the relevance of the HER-RAS-MAPK pathway as key mediator of resistance to anti-HER2 therapies and suggested an involvement of PI3K-AKT signaling in the acquisition of resistance to dual HER2 blockade.

Ongoing ctDNA-driven clinical trials.

A large number of clinical trials have either been completed or are ongoing to evaluate application of ctDNA in cancer precision medicine. These can be broadly divided into observational trials that attempt to prospectively or retrospectively correlate ctDNA findings with clinical outcome, or interventional trials in which ctDNA results prompts therapeutic actions. Many of the observational trials have demonstrated a prognostic role for ctDNA in the MRD setting across different histology, where the detection of ctDNA typically precedes clinical or radiological relapse by a median lead-time of 3-5 months [103-105]. Observational studies in the metastatic setting have likewise shown that the presence or absence of specific ctDNA clones, or their dynamics early on in the treatment course, can prognosticate clinical outcome [93, 106]. In the immuno-oncology field, blood tumor mutational burden (bTMB) is actively being investigated for its value as a predictive biomarker for response to checkpoint inhibitors. The B-F1RST trial (A Study of Atezolizumab as First-line Monotherapy

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for Advanced or Metastatic Non-Small Cell Lung Cancer, NCT02848651) is a single-arm phase II trial with a planned enrollment of 150 patients. Its primary biomarker objective is to prospectively collect and retrospectively measure bTMB in all patients to evaluate whether it can predict for progression-free survival (PFS) in this population. Interim analysis of bTMB in 58 biomarker-evaluable patients in the B-F1RST trial, using a pre-specified cut-off score of 16 mutations per coding sequence, has demonstrated a trend to longer PFS in bTMB high versus low subgroups (9.5 months versus 2.8 months, hazard ratio = 0.51, 90% C.I. 0.24-1.08, p = 0.1315) [107]. Similarly, several non-small cell lung cancer studies with checkpoint inhibitors have retrospectively analyzed their efficacy outcome in relationship to bTMB [108] and demonstrated positive correlations, but validation is needed before these results can be extrapolated to other tumor types.

Interventional studies are emerging to assess the role of ctDNA as a predictive biomarker, building on the hypothesis that clearance of ctDNA or alteration of its dynamics with therapeutically active agents can change clinical outcome. One example of an interventional ctDNA-based trial in the MRD setting is the c-TRAK TN study in patients with moderate- and high-risk early stage triple negative breast cancer (TNBC) (*NCT03145961*). The framework of this trial is based on a “marker by treatment interaction design” (**Figure 2**). In this study, patients undergo blinded serial ctDNA screening post standard primary treatment for TNBC. Those (n = 200 patients) who have a positive ctDNA result on or before the 12-month time point will be informed and randomized in a 2:1 ratio to pembrolizumab versus observation. Primary endpoints of c-TRAK TN include proportion of patients with positive ctDNA detection at 12 and 24 months, and the absence of detectable ctDNA or disease recurrence 12 months after commencing pembrolizumab. Disease-free survival events are collected in all treatment arms to correlate with ctDNA findings. Other examples are the DYNAMIC II and III trials (A*CTRN1261500 and 0381583*), respectively, in stage II and III colon cancer. The former randomizes 450 stage II patients in a 2:1 ratio either to be treated according to ctDNA result (Arm A), or blinded to ctDNA result and managed as per standard clinical criteria at the discretion of the treating physician (Arm B). Patients in Arm A who have positive ctDNA result will receive adjuvant chemotherapy whereas those with negative ctDNA result will not receive chemotherapy. In the DYNAMIC III trial, 1000 patients will be randomized 1:1 to be treated according to ctDNA results after surgical removal of their bowel cancer or per standard of care. For the ctDNA–informed group, patients with a positive test will be treated with stronger chemotherapy than routine treatment. Patients with a negative test result will

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receive milder chemotherapy or a shorter duration of routine treatment. The framework of the DYNAMIC studies is based on “marker-based strategy design” (**Figure 2**).

In the advanced disease setting, interventional ctDNA-based clinical trials focus primarily on guidance to specific therapy to optimize outcome. One such liquid biopsy-based precision oncology trial is BFAST (A Study to Evaluate Efficacy and Safety of Multiple Targeted Therapies as Treatments for Participants with Non-Small Cell Lung Cancer, *NCT03178552*), which uses ctDNA genotyping results to match patients to specific agents. Patients with translocations in anaplastic lymphoma kinase (*ALK*) and rearranged during transfection (*RET*) are offered alectinib, those with *ROS1* fusions are matched to entrectinib, and those with positive bTMB are randomized to atezolizumab versus platinum-based chemotherapy. Additional BFAST cohorts that evaluate other molecular aberrations may be included in the future. The ongoing CHRONOS trial (Rechallenge with Panitumumab Driven by RAS Dynamic of Resistance, *NCT03227926*) is an interventional ctDNA-driven study in advanced colorectal cancer. It is based on the finding that in patients with *RAS/BRAF* wild-type metastatic colorectal cancer who have progressed on anti-epidermal growth factor receptor (anti-EGFR) therapy, acquired *RAS* mutant clones decay exponentially after drug withdrawal [75, 109], rendering these tumors potentially sensitive again to these agents. This open-label, single arm, phase II study will enroll 129 patients with *RAS/BRAF* wild-type metastatic colorectal cancer aims to evaluate the efficacy of panitumumab re-administration when *RAS* mutational load decreases by greater than 50% compared to basal mutational load after initial progression on anti-EGFR therapy. It is important to underscore that the clinical utility of ctDNA in these studies relies not only on technical aspects such as assay validity and performance, but is also contingent on the therapeutic efficacy of the intervention.

Other clinical settings where liquid biopsies could change clinical practice.

In additional to the aforementioned clinical trials, ctDNA-based investigations may help to address uncertainties in other clinical settings with the potential of altering treatment practice. A dilemma that clinicians frequently face is the optimal duration of adjuvant therapy, especially with targeted therapy or immunotherapy. In the metastatic setting, the duration of these agents is also unclear for patients who have achieved complete response, sustained partial response, or prolonged disease stabilization. For instance, many immunotherapy trials advocate treatment duration of one to two years for adjuvant therapy

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or for maintenance of objective response, without clear biological justifications for the proposed timeframe. A prospective clinical trial in such patients who have positive ctDNA result, comparing an investigational approach of treatment until confirmed ctDNA clearance versus a control arm of standard treatment duration, would be appealing. For those with negative ctDNA results, the comparison can be that of shortened treatment duration versus standard treatment duration. If ctDNA is proven useful in these scenarios, treatment-related costs and patient exposure to unnecessary drug-related toxicities can be minimized. Another scenario in which liquid biopsy may be of relevance is to rule out true progression from pseudoprogression in the clinical setting, the latter being reported at a 5-10% frequency with immuno-oncology agents in solid tumors [110, 111]. A recent report highlighted the application of ctDNA to differentiate between true progression and pseudoprogression in a cohort of 29 patients with melanoma treated with anti-PD-1 antibodies with sensitivity of 90% and specificity of 100% [112]. These data support suggest that the use of liquid biopsies in this setting could reduce prolonged treatment in patients who are not benefitting from checkpoint blockade.

Another exciting area where ctDNA analysis might be successfully exploited is screening of asymptomatic individuals for early cancer diagnostics. However, these types of screenings are at present limited by several factors [113]. First, analogously to monitoring of MRD, early cancer detection is affected by the limit of detection of current technologies. Second, minimizing false positives to achieve high specificity is of paramount importance in screening. Presence of somatic mutations in cfDNA derived from non-cancer cells can confound analysis and result in false positive results leading to unnecessary follow-ups [116]. For example, it is known that aging can lead to development of somatic mutations (clonal hematopoiesis of indeterminant potential- CHIP) [114, 115]. Recent studies [116-118] are trying to address these issues. However, well-designed prospective studies will be necessary to provide real-world evidence for the applicability of early detection.

Conclusions

Liquid biopsy-based, ctDNA analyses have rapidly emerged as an effective strategy for non-invasive cancer assessments in many stages of patients' monitoring. The next step of this exciting field of research is the execution of prospective clinical trials ultimately leading to practice changing protocols. To this end, standardization of laboratory procedures to ensure high reproducibility of the results is key. Larger studies should be performed on the utility of

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ctDNA in combination with standard blood markers and radiological imaging, to better select and stratify cancer patients and to guide therapy. The improvement of liquid biopsy technologies and their increased sensitivity will allow detection of minimal residual disease and ultimately early cancer detection in asymptomatic populations.

Figure legends

Figure 1: Clinical application of liquid biopsy with ctDNA analysis.

Figure 2: Clinical trial designs to evaluate ctDNA using predictive marker validation frameworks.

A) Marker by Treatment Interaction: in this design, it is assumed that ctDNA testing will split the population in two groups. Patients in each ctDNA status are randomly assigned to two different treatment strategies (in this case: treatment or active surveillance), and the testing plan determines whether one strategy is superior to the other within each ctDNA marker group.
B) Marker-based strategy design: after ctDNA determination, each patient is randomized to receive therapy either independently of ctDNA status (2) or assigned by ctDNA status (1).

Funding

This work was supported by European Community's Seventh Framework Programme under grant agreement no. 602901 MErCuRIC (A.B.); H2020 grant agreement no. 635342-2 MoTriColor (A.B.); IMI contract n. 115749 CANCER-ID (A.B.); AIRC IG 2015 n. 16788 (A.B.); FONDAZIONE AIRC under 5 per Mille 2018 - ID. 21091 program – P.I. Bardelli Alberto (A.B.); AIRC IG 2018 - ID. 21923 project - PI Bardelli Alberto (A.B.); AIRC-CRUK-FC AECC Accelerator Award contract 22795 (A.B.); Progetto NET-2011-02352137 Ministero della Salute (A.B.); Fondazione Piemontese per la Ricerca sul Cancro-ONLUS 5 per mille 2014 e 2015 Ministero della Salute (A.B.). G.S. was funded by Roche per la Ricerca grant 2017 and AIRC three years fellowship 2017. Asociación Española contra el Cáncer GCTRA16015SEDA (J.S.), FIS-ISCIII grant (PI16/01278) (J.S.), FERO -EDM support- and Cellex foundations (J.S.). The Commonwealth Fund (N.P.).

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Discosures

N.P. is a co-founder and board member of Thrive and co-founder of Personal Genome Diagnostics and a member of the Scientific Advisory Board of NeoPhore Ltd. These companies, as well as other companies, have licensed technologies from Johns Hopkins University, on which Nickolas Papadopoulos is an inventor. These licences and relationships are associated with equity or royalty payments to Nickolas Papadopoulos. The terms of these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies. L.S. is a consultant/advisory board member for Novartis, Bristol-Myers Squibb, Pfizer, Boerhinger-Ingelheim, Regeneron, GlaxoSmithKline, Roche/Genentech, Karyopharm, AstraZeneca/Medimmune, Merck, Celgene, Astellas, Bayer, Abbvie, Amgen, Symphogen, and Intensity Therapeutics. L.S. holds stocks in Agios. L.S. received compensation from Merck, Pfizer, Celgene, AstraZeneca/Medimmune, Morphosys, Roche, GeneSeeq, Loxo, Oncorus and Symphogen. R.B.C. is a consultant/advisory board member for Amgen, Array Biopharma, Astex Pharmaceuticals, Avidity Biosciences, BMS, Chugai, Fog Pharma, Fount Therapeutics, Guardant, Genentech, LOXO, Merrimack, N-of-one, Novartis, nRichDx, Roche, Roivant, Shire, Spectrum Pharmaceuticals, Symphogen, Taiho, and Warp Drive Bio; holds equity in Avidity Biosciences, Fount Therapeutics, and nRichDx; and has received research funding from Asana, AstraZeneca, and Sanofi. A. Bardelli is a consultant for Biocartis and Guardant. All other authors declare no disclosures.

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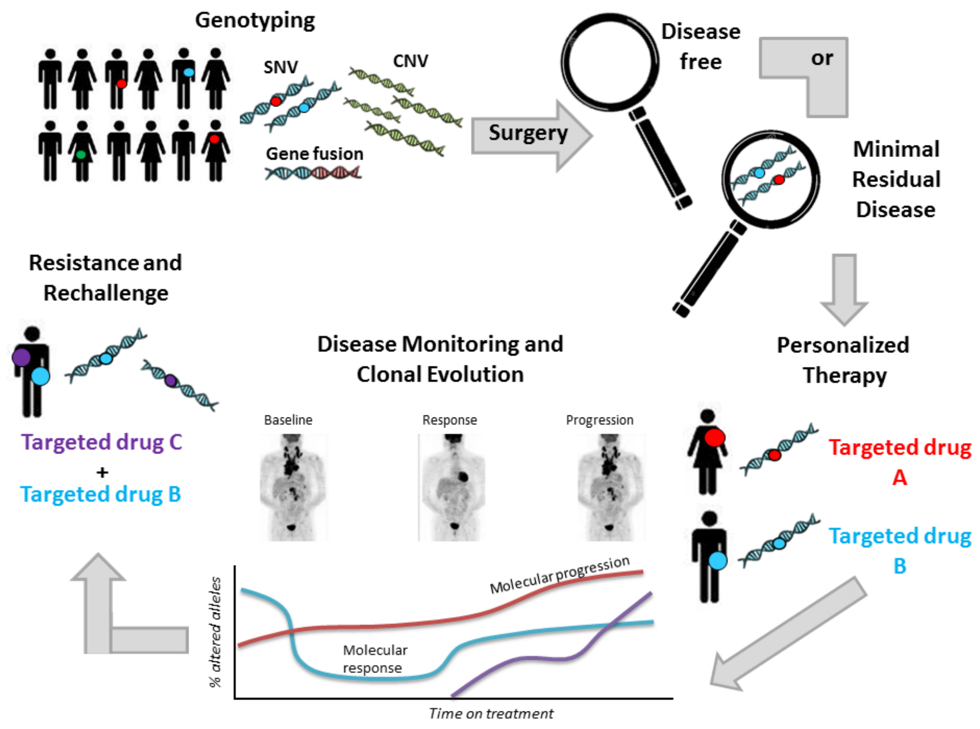

Clinical application of liquid biopsy with ctDNA analysis.

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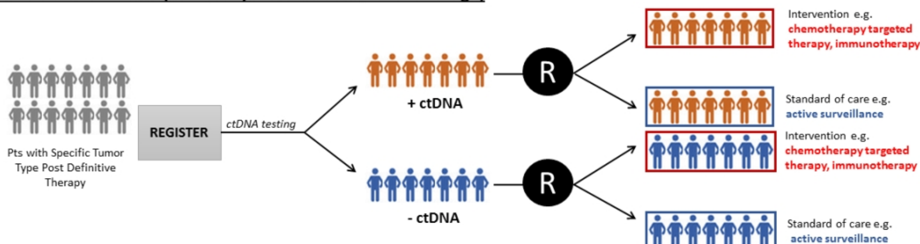

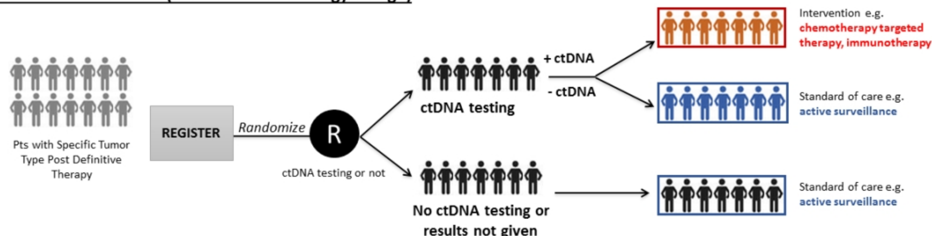

Clinical trial designs to evaluate ctDNA using predictive marker validation frameworks.
A) Marker by Treatment Interaction: in this design it is assumed that ctDNA testing will split the population in two groups. Patients in each ctDNA status are randomly assigned to two different treatment strategies (in this case: treatment or active surveillance), and the testing plan determines whether one strategy is superior to the other within each ctDNA marker group.
B) Marker-based strategy design: after ctDNA determination, each patient is randomized to receive therapy either independently of ctDNA status (2) or assigned by ctDNA status (1).

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