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Manuscript Details

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Abstract

Cancer management paradigms are shifting towards a personalized approach thanks to the advent of the -omics technologies. Liquid biopsies, consisting in the sampling of blood and other bodily fluids, are emerging as a valid alternative to circulating tumor biomarkers and tumor tissue biopsies for cancer diagnosis, routine monitoring and prognostication. The content of a liquid biopsy is referred to as the "tumor circulome". Among its components, circulating tumor DNA (ctDNA), including both cell-free and exosome-associated DNA, is the most widely characterized element. ctDNA analysis has a tremendous capability in the diagnostic arena. Its potential has been demonstrated at each level of disease staging and management and supported by a recent FDA approval for companion diagnostic, and the investments being made by pharmaceutical companies in this sector are numerous. The approaches available for ctDNA analysis allow both quantitative and qualitative studies and range from PCR and dPCR-mediated single/multiple gene mutational assessment to whole genome next generation sequencing and methylation mapping. Although the principal object of a liquid biopsy is blood, other body fluids such as urine and saliva show potential as complementary DNA sources for tumor analysis. In this review we provide a synopsis on the state of play of current ctDNA application. We discuss the clinical significance of ctDNA analysis and review the state of the art of technologies being currently developed to this aim. We also discuss the current issues limiting ctDNA application and highlight the promising approaches being developed to overcome these.

Keywords	cancer diagnosis; ctDNA; liquid biopsy; oncology; personalized medicine

Corresponding Author	Mary Bebawy
Corresponding Author's Institution	University of Technology Sydney
Order of Authors	Gabriele De Rubis, Sabna Rajeev Krishnan, Mary Bebawy
Suggested reviewers	Lesley Cheng, Richard Cristopherson, Quin Wills

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FROM:

A/Prof. Mary Bebawy

Graduate School of Health

University of Technology Sydney

PO Box 123 – Broadway

NSW 2007 Australia

mary.bebawy@uts.edu.au

TO:

Prof. Enrico Clementi

Editor-in-Chief – Pharmacological Research

Dear Prof. Clementi,

I am pleased to submit a review paper, on behalf of co-authors, entitled "**Circulating Tumor DNA - current state of play and future perspectives**" by Mr. Gabriele De Rubis, Dr. Sabna Rajeev Krishnan, Prof. Michael Wallach and A/Prof. Mary Bebawy, for consideration for publication in *Pharmacological Research*.

In this manuscript we describe the current status and the exciting future perspectives of the use of circulating tumor DNA (ctDNA) as liquid biopsy for cancer diagnosis, screening, routine monitoring, prognostication and pharmacological treatment selection. We discuss the different types of information obtainable by analyzing ctDNA and their clinical implications. We also detail the most innovative technologies developed for ctDNA analysis, and the future progresses needed to overcome the current limitations in this field.

We believe that this manuscript is appropriate for publication by *Pharmacological Research* because the liquid biopsy field is currently an extremely relevant topic in targeted cancer therapy, precision medicine and personalized therapy. Liquid biopsies overcome the limitations of current golden standards for cancer screening and molecular characterization (i.e. tissue biopsies) and, thanks to their low-to –null invasiveness and repeatability, are set to revolutionize the current paradigms of cancer management. ctDNA in particular is, as of today, the most deeply characterized among the different components of a liquid biopsy. This is demonstrated by the very recent approval, by the US FDA, of the first companion diagnostic based on the analysis of ctDNA (cobas EGFR Mutation Test v2 – Roche) and by the large number of clinical studies and

clinical trials being currently conducted and having the study of ctDNA among the primary outcome measures. Considering the high-level reputation of *Pharmacological Research* and its commitment to share the knowledge on cutting-edge topics among specialists in different disciplines, we believe that this is the journal that mostly suits our review for publication.

We state that this manuscript has not been published and is not under consideration for publication elsewhere. We also declare no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

A/Prof. Mary Bebawy

Comments from the editors and reviewers:

-Reviewer 1

-

P2 The term ‘liquid biopsies’ could be defined Done

there are multiple types of constituents. We define them in the Introduction (Page 5, lines 82-84) and in Figure 1

Why does ctDNA have the greatest potential? We don’t state that ctDNA has the greatest potential, but that it has “tremendous capability”, and that it is the most widely characterized (especially with regards to the FDA-approvals of ctDNA-based diagnostic tests, see throughout text)

P4 Replace ‘progresses’ with ‘advances’ through the text. Done

The use of a single marker is prone to error, the trend is to use patterns of markers to make diagnoses. Done (Lines 62-63)

P5 The term ‘Darwinian evolution’ seems out of place; mention selective pressure on tumours to grow. Done

Why are ctDNA the preferred markers? we recognize that stating that ctDNA is “the most promising component” of the circulome might be misleading. We changed the sentence in Line 84 from “Among these, ctDNA is the most promising and extensively studied component of the Tumor Circulome” to “Among these, ctDNA is the most extensively characterized component of the Tumor Circulome”

What is the half-time for ctDNA in the circulation, We mention it at Page 8, Line 148

provide a size distribution curve. authors do not see this necessary to include

Do the differential ctDNA encode tumour drivers? We mention driver mutations in Pag. 6 Line 114.

P7 Could rearrangement of DNA occur after cell death? Not known. Authors do not see relevant to include

P8 Some ctDNA must be more stable in the circulation? Exosome-associated DNA is more stable in circulation: we discuss this in Paragraph 2.3 (P13)

P9 Methylation sites will be more variable than ctDNA sequences. Current literature says the opposite. mutations, compared to methylations, are more variable and show lower consistency within the same tumor. Furthermore, the high diversity of mutations are often spread over several exons of the same gene. (Warton K et al. 2015, Front Mol Biosci, doi: 10.3389/fmolb.2015.00013)

P10 You could use the development of KRAS as a marker from 1994 as a an example Done (see Lines 200-201)

Note that patterns of markers are now preferred. Done (Pag. 11, Lines 214-217)

P11 Provide some examples of calculation of differential mutant allele frequencies (MAF) found in ctDNA. Not Relevant – a practical example of MAF calculation is unnecessary in this section. We define the MAF earlier in the text (Line 128)

P13 Is ctDNA protected from degradation in extra-cellular vesicles? Done Pag. 14 Lines 290-292

P15 Levels of leukocyte subtypes change in cancers, is this mixture of markers a problem?

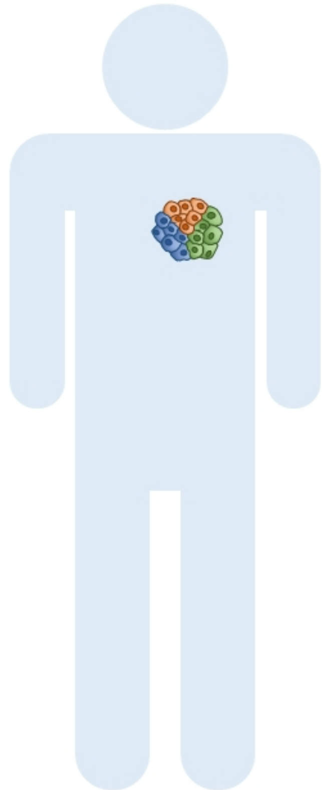
Leukocyte-derived DNA contamination is an issue because it “dilutes” ctDNA with wild-type copies of the genes of interest, further reducing the MAF (Lines 398-402). Therefore, the relative leukocyte subtype composition is irrelevant for the purpose of ctDNA mutation analysis

P16 Is bacterial DNA in stool samples a problem? We couldn’t find any literature about the possible influence of bacterial DNA in stool cancer biomarker analysis

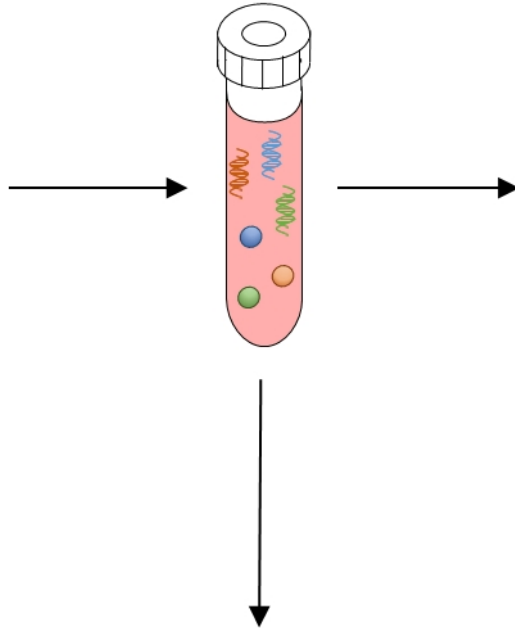
P19 Early detection is crucial for curing cancer, what is the sensitivity of the best method for analysing ctDNA? Done (Line 432)

Replace Figure 1 with a detailed workflow for the best method for analysis of multiple ctDNA from a blood sample. We don’t agree with this suggestion. The authors request Figure 1 remain as it displays the different components of the Tumor Circulome and is used to highlight the representativeness of the tumor heterogeneity held by liquid biopsies

Include reference: Shu et al., (2017) Scientific Reports 7: 583 Done (Line 245)



Blood Sample – Liquid Biopsy



Analytical technologies:
- PCR / dPCR / ddPCR
- Sequencing / NGS
- Bisulphite-dependent PCR and Sequencing

Information obtained:
- **Quantitative:** Mutant Allele Fraction
- **Qualitative:** Mutation profiles, Translocations, Deletions, Duplications, Methylation

Outcomes:
- Screening / Early detection
- Personalized Therapy decision
- Prognosis / Relapse Prediction
- Monitoring of therapeutic outcome
- Post-treatment Follow-Up

exoDNA

ctDNA

Limitations:
- Assay sensitivity
- Clonal Haematopoiesis / Predictive value of mutations
- Pre-analytical sample handling

Possible Solutions:
- Matching blood sampling with other body fluids
- Coupling ctDNA and other biological classes of biomarkers
- Compact lab-on-a-disc systems

Circulating Tumor DNA – current state of play and future perspectives

Gabriele De Rubis[a], Sabna Rajeev Krishnan[a] and Mary Bebawy[a]*

[a]: Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney Australia - PO Box 123 - Broadway - NSW 2007 Australia

Email addresses:

Mr. Gabriele De Rubis: gabriele.derubis@student.uts.edu.au

Dr. Sabna Rajeev Krishnan: Sabna.RajeevKrishnan@uts.edu.au

***Corresponding author: mary.bebawy@uts.edu.au (A/Prof. Mary Bebawy - Head, Laboratory Cancer Cell Biology and Therapeutics)**

67 Thomas St, UTS Building 7, Level 4, Ultimo NSW 2007 – Sydney - Australia

Circulating Tumor DNA – current state of play and future perspectives

Abstract:

Cancer management paradigms are shifting towards a personalized approach thanks to the advent of the -omics technologies. Liquid biopsies, consisting in the sampling of blood and other bodily fluids, are emerging as a valid alternative to circulating tumor biomarkers and tumor tissue biopsies for cancer diagnosis, routine monitoring and prognostication. The content of a liquid biopsy is referred to as the “tumor circulome”. Among its components, circulating tumor DNA (ctDNA), including both cell-free and exosome-associated DNA, is the most widely characterized element. ctDNA analysis has a tremendous capability in the diagnostic arena. Its potential has been demonstrated at each level of disease staging and management and supported by a recent FDA approval for companion diagnostic, and the investments being made by pharmaceutical companies in this sector are numerous. The approaches available for ctDNA analysis allow both quantitative and qualitative studies and range from PCR and dPCR-mediated single/multiple gene mutational assessment to whole genome next generation sequencing and methylation mapping. Although the principal object of a liquid biopsy is blood, other body fluids such as urine and saliva show potential as complementary DNA sources for tumor analysis. In this review we provide a synopsis on the state of play of current ctDNA application. We discuss the clinical significance of ctDNA analysis and review the state of the art of technologies being currently developed to this aim. We also discuss the current issues limiting ctDNA application and highlight the promising approaches being developed to overcome these.

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KEYWORDS: cancer diagnosis; ctDNA; liquid biopsy; oncology; personalized medicine

1. Liquid Biopsy – a revolutionary approach to diagnosis and disease state management

Cancer is the major cause of morbidity and mortality worldwide, as it has been estimated that 14.1 million new cancer cases have been diagnosed and that cancer caused 8.4 million deaths in 2012 [1]. During the last decades, enormous advances have been made in the study and dissection of tumors' molecular characteristic, thanks also to the advent of the –omics era, and these advances have led to the birth of precision oncology as a branch of precision medicine [2, 3], shifting from a "one size fits all" therapeutic approach towards the identification of "the right treatment, for the right patient, at the right time". The applications of tumor molecular profiling encompass all the stages of cancer management, including early detection/screening, prognosis, patient stratification for predicting response to therapy and for the selection of personalized therapies, monitoring of treatment effectiveness and response and follow-up for the early detection of relapse and metastasis occurrence [4]. The two main instruments that are still used by clinicians to assess these goals are the use of circulating tumor-derived protein markers and tumor tissue biopsies.

Protein markers have been extensively used for cancer screening (e.g. the PSA – Prostate Specific Antigen - protein for prostate cancer) [5] or for postoperative follow-up of recurrence (e.g. CA 15-3 for breast cancer) [6] and, although they allow a longitudinal monitoring of cancer patients, their real clinical value is still highly debated. As single biomarkers generally have low sensitivity and specificity, the use of panels of markers is a current trend [7]. In particular, with regards to routine PSA screening, its net benefit has proven to be marginal in terms of mortality reduction, while the harms associated to overdiagnosis and to the side effects of standard therapies are considerable [5]. Furthermore, the use of CA 15-3 for post-operative follow up lacks

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240 evidence of benefit in terms of patient outcome or quality of life. Further, it is only
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242 recommended for supportive application in the evaluation of chemotherapeutic
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244 response, particularly in poorly accessible disease [6]. Tissue biopsies, although
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246 currently representing the standard procedure for tumor diagnosis, have two main
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248 disadvantages: first of all, they are invasive procedures, which very often cause
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250 complications [8] and in most cases don't allow longitudinal monitoring of patients [9];
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252 secondly, they are virtually never representative of the tumor heterogeneity and
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254 multiclonality generated by the selective pressure to which the tumor is subjected
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256 during its growth [9, 10].
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259 As a favourable alternative to tissue biopsies, during the last years the concept of
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261 "liquid biopsy", consisting in the sampling of non-solid biological tissues, blood *in*
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263 *primis*, gained an exceptional momentum and is becoming a topical concept in
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265 precision oncology [9]. The content of a liquid biopsy is an instantaneous snapshot of
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267 what we define "the Tumor Circulome". It comprises a subset of tumor-derived factors,
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269 circulating in the bloodstream, which can be directly or indirectly used as a source of
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271 tumor biomarkers [11]. These include circulating proteins, cell-free circulating tumor
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273 DNA (ctDNA) and RNAs, circulating tumor cells (CTCs), tumor extracellular vesicles,
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275 and tumor-educated platelets (TEPs) **(Figure 1)**. Among these, ctDNA is the most
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277 extensively characterized component of the Tumor Circulome as of today, and it is the
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279 only component on which an FDA (Food and Drug Administration)-approved
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281 companion diagnostic test is based [12]. The potential applications of ctDNA in cancer
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283 management have just started to be explored, and we retain very likely that ctDNA will
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285 become the protagonist in the development of novel companion and complementary
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287 diagnostics. This review provides an up-to-date synopsis of the current state of the
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289 use of ctDNA as liquid biopsy in cancer management. We discuss the current clinical
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applications of ctDNA in cancer diagnostics and disease state management and the exciting future perspectives, but also the current limitations, on the use of this component of the Tumor Circulome in liquid biopsies. We also detail the analytical technologies required and advances made in purification and analysis of ctDNA.

2. Circulating Tumor DNA - a key player in the liquid biopsy arena.

ctDNA is the fraction of circulating cell-free DNA (cfDNA) that originates from tumor cells. It consists of fragmented DNA, between 80 and 200 bp (base pairs) in length, with peaks of length corresponding to multiples of the canonical nucleosome-associated DNA length (147 + 20-90 bp) [13]. The presence of circulating cfDNA was first reported 70 years ago [14] and, in healthy individuals, hematopoietic cells represent the major source of cfDNA [15].

The early association between malignancy and cfDNA levels came about from clinical reports of patients with malignant disease having higher levels of cfDNA compared to patients with benign diseases [16]. This was followed by work conducted by Stroun and colleagues in 1989 which traced the neoplastic origin of such DNA present in the plasma of oncologic patients [17]. The mechanism by which ctDNA is released in circulation is not entirely known. Apoptosis and necrosis of cancer cells are thought to be the major contributors [18, 19]. Other possibilities include active secretion of DNA by tumor cells [20] as well as potential if not minor contributions from CTCs [21].

The potential for using ctDNA as cancer biomarkers for disease screening were first published in the mid-nineties with the identification of mutations in the driver gene *KRAS* in the ctDNA isolated from colorectal and pancreatic cancer patient blood samples [22-25]. The clinical utility in identifying mutations associated with the onset

or recurrence of cancer in ctDNA became immediately obvious. Since these early studies, during the last two decades numerous tumor-associated mutations have been identified in ctDNA for different types of cancers, including colorectal, breast, ovarian, pancreatic and lung cancer [21].

2.1. What does ctDNA analysis tell us?

By analyzing ctDNA it is possible to obtain two broad classes of information: quantitative and qualitative or genomic information, on the patients' disease state [26]. Quantitative information in the context of gauging tumor burden has application in disease staging, treatment response and relapse monitoring, minimal residual disease (MRD) detection and prognostication. This type of information derives from the measurement of ctDNA concentration, expressed as mutant allele concentration (copies / volume of plasma) or mutant allele fraction (MAF, proportion of mutant allele in a given locus). This quantitative capacity of ctDNA has been applied across many different cancers and has been shown to correlate with tumor stage [27], tumor volume and, indirectly, with time to progression after chemotherapy [28]. Expanding on this, an interesting prospective study published by Tie et al. in 2016 demonstrated, in patients with colorectal cancer, that the detection of ctDNA after surgical resection of tumor strongly correlated with recurrence of disease within three years [29].

Genomic or qualitative information consists of profiling of mutations, amplifications, deletions and translocations in ctDNA, in selected loci or across the whole genome. This type of information may provide identification of resistance-related mutations, thus supporting treatment selection. The identification of genetic alterations associated with drug resistance or drug sensitivity through ctDNA is of crucial

importance in cancer management, especially considering that the emergence of acquired drug resistance is believed to be the cause of treatment failure in 90% of patients with metastatic disease [30]. It also has application in monitoring the clonal evolution of the tumor over time and supporting decision making for "adaptive or reactive" strategies. This approach entails continuous patient follow-up after surgery and/or treatment, monitoring for new resistance mutations arising from the adaptation of tumor subclones in response to treatment [31], and finally, in the adjustment of the therapeutic regimen accordingly [26]. The relevance of this process is supported by the short half-life of ctDNA (approximately 1.5h [32]), which provides an "instantaneous snapshot" of tumor mutational state at the time of detection. An excellent example of this strategy is the study published by Siravegna and colleagues in 2015 [33], in which they continuously monitored the clonal evolution of disease in metastatic colorectal cancer (mCRC) patients through ctDNA mutational profiling and demonstrated that levels of mutant *RAS* clones, which rise in blood during EGFR blockade and are at the root of anti-EGFR therapy resistance, drop after anti-EGFR therapy interruption thus restoring, at least partially, drug sensitivity. This study provided a rationale for the efficacy of re-challenge anti-EGFR therapy [33].

The first ctDNA companion diagnostic test for the identification of *EGFR* mutations in non-small-cell lung cancer (NSCLC) patients (cobas® EGFR Mutation Test v2 – Roche Diagnostics) has been recently approved by FDA [12]. The aim of this test is to guide the use of EGFR-Tyrosine Kinase Inhibitors (EGFR-TKI) based on the presence of specific mutations, in the event that a solid biopsy of the tumor is not available. A newly emerging application of this test is currently the detection of the T790M mutation for the use of the third generation EGFR-TKI Osimertinib, and the feasibility of this approach has been recently validated by different studies, including a phase III clinical

trial [34, 35]. A similar diagnostic test, therascreen EGFR Plasma RGQ PCR Kit (Qiagen), has been approved by the CE as In Vitro Diagnostic test in 2015 [36]. At the moment, several clinical studies are ongoing to evaluate the clinical utility of ctDNA analysis across different types of cancer and the investments being made by pharmaceutical companies in this sector are numerous. Relevant examples of these studies are summarized in **Table 1** and **Table 2.**

Other qualitative information obtainable by ctDNA analysis is the identification of tumor-specific epigenetic/epigenomic variations, which include ctDNA methylation and histone post-translational modifications (PTMs) in circulating cell-free nucleosomes [37]. The first report detailing the feasibility of detecting tumor-associated aberrant ctDNA methylation was by Wong et colleagues in 1999 [38]. The team compared the methylation status of the *p16* gene in ctDNA and in tumor-derived DNA, finding a concordance of 81%. Since then, numerous studies have been conducted with the aim of characterizing the potential use of ctDNA methylation as biomarkers for the early diagnosis and prognosis of cancer, for cancer screening, and for real-time follow up of tumor dynamics [39-41]. One potential advantage of the analysis of ctDNA methylation compared to the analysis of mutations and rearrangements is that changes in DNA methylation in cancer have greater consistency than mutations, and are usually concentrated in narrower regions of each gene (for example, CpG islands). As a consequence, potentially, smaller proportions of the genome could be analysed for methylation to provide a test of adequate sensitivity [41]. An important milestone in the area of ctDNA methylation analysis has been the approval by the FDA in 2016 of Epi proColon®, a screening test for colorectal cancer, based on the analysis of the methylation of the *SEPT9* promoter region [42]. Another important potential application

of ctDNA methylation analysis is the identification of the tissue of origin in cancers of unknown primary [43].

2.2. Technological approaches to ctDNA analysis

The technologies available for ctDNA analysis are numerous and encompass a scale which ranges from single mutation detection to genome-wide analysis. They are schematically represented in **Figure 2**.

With regard to the identification of sequence alterations (mutations, copy number variations and chromosome rearrangements), the analytical approaches can be divided in PCR-based and Next-Generation Sequencing (NGS)-based techniques.

PCR-based techniques are used for single-locus or in multiplexed assays and were historically the first to be used, with allele-specific PCR being used in 1994 for the identification of mutant *KRAS* in pancreatic carcinoma [25]. The cobas® EGFR Mutation Test v2 uses this approach in a real-time PCR context, while the therascreen EGFR Plasma RGQ PCR Kit is based on a variation of this technique called Amplification-Refractory mutation system PCR (ARMS-PCR). Although useful, allele-specific PCR has very limited analytical sensitivity [26] and it is currently being substituted by more sensitive approaches. Among those, digital PCR (dPCR) [44-46] and its variants droplet digital PCR (ddPCR) [47, 48] and BEAMing (Beads, Emulsion, Amplification, Magnetics) [49] are being successfully used for ctDNA analysis. These techniques approach sensitivities ranging between 0,001% and 0,01% MAF [50] and have the advantage, compared to traditional qPCR, to offer an absolute quantification of the number of mutant alleles in a sample [51]. Although dPCR-based approaches have high sensitivity and absolute quantification as important points of strength, an

important limitation of these approaches relies in the fact that they are applicable for the analysis of a limited number of mutation hotspots at the same time. This is an important issue: as single mutations or small panels may not be informative, the study of patterns of mutations has a higher predictive value and it is a preferable approach [26].

NGS-based techniques have the important feature that they can also be used to identify *de novo* mutations, and they can be divided into two subcategories basing on the extent of genomic coverage, namely targeted sequencing and genome-wide sequencing techniques. Targeted sequencing involves the sequencing of specific loci (from individual exons to the whole exome) after selective amplification by PCR (amplicon-based) or hybrid capture-based enrichment. It has the advantage of interrogating a larger number of loci compared to dPCR-based approaches, although with higher costs, longer times and generally lower sensitivity [4].

Among the amplicon-based technologies, Enhanced Tagged Amplicon-Sequencing (eTAm-Seq™ - Inivata Ltd) is worth noting in that it has been recently used to develop and validate the InVision™ liquid biopsy analytical platform, which is capable of detecting mutant alleles down to a MAF of 0.02% with high reproducibility [52]. A similar technology is the Ion AmpliSeq™ platform (ThermoFisher Inc.), which has recently been used to assess mutations in a large panel of genes in lung cancer patients. The results obtained were then validated using ddPCR [53].

Hybrid-capture based technologies allow the selection and sequencing of a number of target sequences ranging from panels of genes to the whole exome (WES, Whole Exome Sequencing). WES has been recently used, paired with deep coverage targeted sequencing, to study clonal heterogeneity in neuroblastoma, in an elegant

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653 study in which somatic mutations and copy number alterations were compared
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655 between solid biopsy and cfDNA [54]. In another study, Manier and colleagues used
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657 WES to compare the mutational landscape and the copy number alteration profile
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659 between CTCs, cfDNA and matched tumor biopsies, obtaining high concordance
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661 between the different classes of biopsy [55]. Usually, for the same cost, the sensitivity
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663 of hybrid capture-based approaches is inversely proportional to the number of loci
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665 analysed [4] and, generally, WES limit of detection is around 5% MAF [26]. In order to
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667 enhance the sensitivity, patient-specific or cancer-specific assays can be designed
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669 including only genes of interest with an important prognostic or diagnostic value [56].
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671 An example of this approach is the very recent CAPP-Seq (Cancer Personalized
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673 Profiling by deep Sequencing) [57] technology, which has been used for the early
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675 detection of MRD [58] and the investigation of tumor heterogeneity in lung cancer [59],
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677 as well as in diffuse large B cell lymphoma [60] and leiomyosarcoma [61].
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681 In order to improve the sensitivity of sequencing techniques, recently the use of Unique
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683 Molecular Identifiers (UMIs) has been adopted: this approach consists in tagging each
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685 template molecule with a “molecular barcode”, a unique sequence, during the library
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687 preparation phase. In this way, for each template molecule, a consensus sequence is
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689 obtained, thus drastically reducing the background noise generated by the random
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691 errors occurring during the PCR amplification step [62]. This approach is used in both
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693 the above-described eTAm-Seq™ and Ion AmpliSeq™ technologies. Another
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695 example of the use of UMIs is the Simple, multiplexed, PCR-based barcoding of DNA
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697 for sensitive mutation detection using sequencing (SiMSen-Seq) technology [63].
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699 Moreover, considering that ctDNA is slightly shorter than non-tumor cfDNA, this
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701 difference can be exploited for the *in vitro* or *in silico* concentration of ctDNA, thus
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703 further increasing the sensitivity of the following tests [26, 64, 65]. Another recently
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developed methodology to concentrate mutant DNA is the Nuclease-assisted Minor-allele Enrichment with Probe-Overlap (NaME-PrO), which is based on the selective digestion of wild-type alleles through the use of a double stranded DNA-specific nuclease guided on the target sequence by selective oligonucleotide probes that pair with wild-type alleles of the genes of interest. This approach enables mutation detection at 0.01 - 0.00003% MAF [66].

Approaches for t he identification of copy number alterations from ctDNA are usually genome-wide, and they include Array-Comparative Genome Hybridization (Array-CGH) [67] and low coverage whole genome sequencing, such as shallow whole genome sequencing (sWGS) [68] and Plasma-Seq [69]. Moreover, a suitable technique for the identification and quantification of chromosomal translocations is the Personalized Analysis of Rearranged Ends (PARE), a PCR-based approach consisting in the use of primers spanning translocation breakpoints previously identified via NGS [70].

Finally, with regards to the analysis of ctDNA methylation, the methodologies used range from single-locus to genome-wide and are mostly based on bisulphite conversion of non-methylated cytosines. They involve methylation-specific PCR, the use of methylation-sensitive restriction enzymes, array based hybridization, and bisulphite sequencing [39].

2.3. Exosomal DNA (exoDNA)

Another important source of circulating tumor-derived DNA is the fraction contained within circulating extracellular vesicles (EVs). EVs are membranous particles released

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771 from all cell types in physiological and pathological conditions, as well as following
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773 different types of stimuli. They can be found in almost every body fluid, especially in
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775 blood [71, 72]. Once considered inert elements of “cell debris”, or as a simple means
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777 to eliminate unneeded components from the cytoplasm of cells [73, 74], during the last
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779 decade EVs have been recognized as fundamental mediators of intercellular
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781 communication [75]. The fact that EVs shield their molecular cargo from degradation,
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783 increasing its stability, makes EV-associated DNA a potentially favourable alternative
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785 to cell-free DNA [76]. Although EVs, exosomes in particular, are well recognized as an
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787 important novel component of the tumor circulome suitable for biomarker analysis,
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789 most of the efforts until now have been made in exploring their protein and RNA
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791 (mRNA and miRNA) content [77-79] and very few studies have been focused on the
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793 analysis of their DNA cargo. The first paper describing the feasibility of the analysis of
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795 exosomal DNA as liquid biopsy was published in 2014 [80]. In this study, the authors
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797 demonstrated that exosomes derived from both cancer cell lines and the serum of
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799 pancreatic cancer patients contain long DNA fragments (> 10 kb) harbouring tumor-
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801 characteristic mutations, and that these DNA fragments where uniformly
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803 representative of nuclear genomic DNA, with reads spanning across all chromosomes
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805 [80]. Similar results were obtained independently the same year on exosomes derived
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807 from different cancer cell lines [81]. In a successive study, San Lucas and colleagues
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809 performed a comprehensive NGS profiling of exoDNA and exoRNA isolated from
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811 pleural effusion- or plasma- derived exosomes of three patients with pancreaticobiliary
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813 cancers [76]. The results obtained demonstrated that a wide number of biomarkers
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815 could be detected by profiling exosomal nucleic acids, including point mutations, copy
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817 number variations and gene fusions. Interestingly, in this study an unexpected
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819 mutation in the *BRCA2* gene (V3091I) was identified in a patient with ampullary
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830 carcinoma, which conferred an exceptional response to platinum-containing adjuvant
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832 regimen [76]. Finally, more recently the same group published a larger scale study in
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834 which they compared the potential of cfDNA and exoDNA analysis for the
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836 ultrasensitive identification of mutations of the *KRAS* gene as biomarker of pancreatic
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838 ductal adenocarcinoma (PDAC) [82]. In this study, exoDNA has proven better than
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840 cfDNA for the detection of mutant *KRAS* (especially in early stages of disease), and
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842 better than CA 19-9 (which is currently the only guideline-recommended protein
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844 biomarker for PDAC[83]) for prognostic stratification [82]. However, mutations in *KRAS*
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846 were identified in a significant proportion of healthy donors too, thus indicating that the
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848 identification of a single genetic mutation as a predictor of PDAC may have low
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851 predictive value [82].
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856 **3. *Proximal Samplings: an alternative source of tumor DNA***
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859 Proximal sampling consists in the sampling of body fluids different from blood, such
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861 as urine, saliva, sputum, cerebrospinal fluid (CSF), pleural effusions and stool. These
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863 fluids have great potential as an alternative or complementary source of tumor DNA
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865 for liquid biopsy. Their analysis is generally limited to detection of local cancers, with
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867 the major exception of urine. In these cases the close proximity, if not the direct
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869 contact, of the diseased organ with the body fluid may increase the yield of tumor DNA
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871 compared to systemic sources [84]. Another advantage of proximal samplings is that,
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873 except in the case of inflammation and late stage organ damage, they don’t contain
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875 immune cells, thus having lower interference by non-tumor DNA [85]. Generally, every
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877 body fluid has two populations of cell-free DNA: a low molecular weight (MW)
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880 population derived from circulation and a high MW population derived from exfoliating,
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889 apoptotic and necrotic cells [84]. These fluids can be divided into two groups, “non-
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891 invasive” and “invasive”, based on whether their collection is more or less invasive
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893 relative to blood sampling.
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896 The “non-invasive” group includes urine, saliva, sputum and stool. Their ease of
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898 collection, which requires little or no participation from healthcare professionals, is an
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900 important point of strength [84]. Sputum-derived DNA is gaining attention in the context
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902 of lung cancer diagnosis and patient stratification [86]. The analysis of stool-derived
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904 DNA has been recently validated as a powerful diagnostic tool of colorectal cancer
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906 [87] and showed potential in the diagnosis of pancreatic cancer [88]. Urinary DNA has
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908 great potential as liquid biopsy for several types of cancers including prostate, bladder,
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910 cervical but also non-urogenital malignancies like NSCLC, colorectal and gastric
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912 cancer [89]. Finally, saliva is an important source of biomarkers for head and neck
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914 squamous cell carcinomas (HNSCC) [90]. Interestingly, salivary DNA has also been
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916 used for the detection of EGFR mutations in NSCLC [91].
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919 The “invasive” group of body fluids mainly includes pleural effusions and cerebrospinal
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921 fluid (CSF). Malignant pleural effusions are caused by malignant pleural mesothelioma
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923 (MPM) and lung cancer and the deriving DNA has been used for the detection of these
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925 pathologies [92, 93]. CSF is set to become an important source of ctDNA for central
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927 nervous system (CNS)-restricted tumors, overcoming the limitations caused by
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929 scarcity of ctDNA in the blood of these patients [84]. In a ground-breaking study, De
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931 Mattos-Arruda et al. demonstrated that CFS ctDNA better represented the genomic
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933 alterations of brain tumors, including primary tumors and brain metastatic lesions,
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935 compared to plasma ctDNA [94].
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A further class of body fluids showing potential for liquid biopsy is represented by the fluids derived from medical procedures. For example, bronchoalveolar lavage-derived DNA has potential for NSCLC diagnosis [95], while uterine lavage has been used to identify cancer-associated mutations in patients with early-stage endometrial cancer. In this study, high MAFs of cancer driver mutations were observed in patients without a cancer diagnosis, suggesting the presence of a premalignant landscape of mutations [96].

4. Current limitations of ctDNA analysis

ctDNA analyses possess enormous potential in the early diagnosis of cancer, however at this early stage, their capacity to achieve this is limited by detection sensitivity. Early-stage cancers very often are characterized by a very low amount of ctDNA, resulting in a MAF that may be undetected using existing techniques [26]. Approaches to improve sensitivity consider proximal samplings, i.e., the use of other body fluids, as an alternative or in combination with plasma: for example, urine samples for bladder cancer. Other approaches could be the use of *in vivo* implanted devices containing cfDNA-binding materials, with the aim of increasing the yield of cfDNA without the need of extensive blood draws. This approach has already been successfully tested for CTCs [97].

Another challenge for early diagnosis of cancer relies in the fact that the identified mutations need to have a high predictive value. Single or small panels of mutations may not be predictive of cancer, as it has been shown that cancer-associated mutations can be present in plasma of healthy individuals [26, 82]. In order to improve predictive capacity, other biomarkers should also be considered as part of the

analysis. The CancerSEEK platform for instance is a multi-analyte blood test recently described by Cohen et al. capable of detecting eight different types of cancer through a combined analysis of 8 proteins and genetic mutations in ctDNA, achieving a sensitivity between 69 - 98% basing on the type of cancer and, most importantly, a specificity of >99% [98]. A different approach for early detection being trialled is the "The Circulating Cell-free Genome Atlas Study", a large prospective, multi-center, observational study which aims at using deep genome-wide sequencing of circulating nucleic acids in order to develop computational models for distinguishing cancer from non-cancer specimens, thus enabling early diagnosis. The first results of this trial showed encouraging results for the early diagnosis of lung cancer [99]. This approach could also benefit, in the future, of machine learning as a strategy to detect tumor biomarker "signatures" [100].

A last important factor to be considered in improving the quality of ctDNA analysis is the pre-analytical handling of blood samples [101], especially with regards to the purification of ctDNA from whole blood. Current ctDNA isolation procedures involve the shipment of the blood sample to central laboratories, separation of plasma by centrifugation and ctDNA purification from plasma. These procedures are complex and time consuming and require intense handling of the samples, which can result in ctDNA degradation (resulting in a drop in mutant allele concentration) or in the release of genomic wild-type DNA caused by the lysis of blood cells (resulting in a drop of mutant allele fraction) [26]. Although blood samples could be stabilized by different types of specialized blood collection tubes [102], it would be advantageous to perform ctDNA purification on-site, right after the blood draw, minimizing the number of steps requiring handling of the sample and the overall time needed for purification. In order to achieve this goal, Kim and colleagues have developed a fully automated lab-on-a-

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1066 disc system, which integrates all the steps of ctDNA purification starting from whole
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1068 blood and performs the extraction in 30 minutes. Furthermore, as a proof of its
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1070 applicability, they used this system to isolate ctDNA from NSCLC patients and
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1072 successfully detected EGFR mutations arising after drug therapy [103].
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5. Concluding Remarks and Future Directions

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1081 The birth of precision oncology, which has been made possible by recent
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1083 advancements in molecular profiling of cancer, has shifted the cancer management
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1085 strategies towards a more and more personalized approach, overcoming the old "one
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1087 size fits all" therapeutic paradigm. Genetic analyses of tumor samples obtained by
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1089 conventional solid tissue biopsies and evaluation of circulating levels of biomarker
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1091 proteins are currently routine for treatment decision and patient stratification and
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1093 prognosis, although both approaches have important limitations. Conventional tissue
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1095 biopsies, in particular, are invasive procedures that don't allow longitudinal monitoring
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1097 and are not representative of tumor spatial and temporal clonal heterogeneity. As a
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1099 more favourable alternative to tissue biopsies, the concept of "liquid biopsy", consisting
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1101 in the sampling and analysis of the tumor circulome, is now gaining an exceptional
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1103 momentum. At present ctDNA, including exosome-associated ctDNA, is a promising
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1105 constituent within the tumor circulome for use in liquid biopsies, and this is supported
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1107 by recent FDA approvals. The technologies available for ctDNA characterization allow
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1109 a range of analyses spanning from single gene mutational assessment to next-
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1111 generation deep genome sequencing and methylation analysis, and their applicability
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1113 encompass all the stages of cancer management. Despite this, ctDNA use as liquid
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1115 biopsy has still some limitations on which further research should be focused. Its
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1125 capacity to achieve early detection is limited by detection sensitivity of low blood
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1127 ctDNA concentrations correlating to low tumor burden. The sensitivity of the most
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1129 recent analytical platforms is around 0.02% MAF. Approaches to improve sensitivity
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1131 may involve association between blood and proximal samplings and the use of *in vivo*
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1133 DNA capture devices. Furthermore, technological advancements such as the use of
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1135 UMIs in the amplification step preceding sequencing and the *in vitro* / *in silico*
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1137 enrichment of ctDNA fragments by virtue of their smaller size are also valid
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1139 approaches to enhance the sensitivity.
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1142 Another challenge in early diagnosis of cancer relies in the low predictive value of
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1144 single mutations. To improve predictive capacity, other strategies should also be
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1146 considered including multi-analyte systems, such as the CancerSeek Platform, or the
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1148 use of computational models and machine learning.
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1150
1151 Improving of the quality of ctDNA analysis is central to successful application. The pre-
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1153 analytical handling of blood samples, including streamlining workflows and protocols
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1155 for optimal purification of ctDNA from biological samples that minimize analyte
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1157 degradation, should be prioritized. Technologies being developed for this purpose
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1159 include fully automated lab-on-a-disc systems, which integrate all the steps of
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1161 purification quickly and effectively.
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1164 Taken together, the studies reviewed here highlight the prominent role played by
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1166 ctDNA in the liquid biopsy field and its enormous potential to change current strategies
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1168 in personalized cancer management. We have also shed light on current limitations in
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1170 this field, giving a hint on where future research should be focused. Numerous clinical
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1172 studies are ongoing, and further studies will be needed, to comprehensively assess
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the clinical value and applicability of ctDNA, but this exciting new field is already revolutionizing the way we diagnose and treat cancer.

Although it is unlikely that ctDNA would replace the golden standards of disease diagnosis, prognosis and monitoring in a short timeframe, its analysis already provides a valuable complementary approach. This is especially true in cases where available disease monitoring methodologies are inadequate, such as when the patient's health status is not compatible with an invasive biopsy.

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ABBREVIATIONS:

BEAMing: Beads, Emultion, Amplification, Magnetics

bp: Base Pairs

cfDNA: Circulating Cell-free DNA

CNS: Central Nervous System

CRC: Colorectal Cancer

CSF: Cerebrospinal Fluid

CTC: Circulating Tumor Cell

ctDNA: Circulating Tumor DNA

ddPCR: Droplet Digital PCR

dPCR: Digital PCR

EV: Extracellular Vesicle

HNSCC: Head and Neck Squamous Cell Carcinoma

MAF: Mutant Allele Fraction

MW: Molecular Weight

NGS: Next-Generation Sequencing

NSCLC: Non-small Cells Lung Cancer

PCR: Polymerase Chain Reaction

PDAC: Pancreatic Ductal Adenocarcinoma

PTM: Post-Translational Modification

qPCR:Qquantitative PCR

TEP: Tumor Educated Platelet

TKI: Tyrosine Kinase Inhibitor

UMI: Unique Molecular Identifier

WES: Whole Exome Sequencing

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FIGURE CAPTIONS

Figure 1 – The Tumor Circulome

The tumor circulome is defined as the set of tumor-derived elements, circulating into the bloodstream, which can be used as a direct or indirect source of tumor biomarkers and can be obtained with a simple blood draw. It includes nucleic acids (ctDNA and RNA), proteins, extracellular vesicles, circulating tumor cells and tumor-educated platelets. The tumor clonal heterogeneity is indicated in the figure by the use of different colours. The advantages of liquid biopsies over conventional tissue biopsies are many. First of all, liquid biopsies are non-invasive and therefore allow a longitudinal follow-up of the patient. Most importantly, as shown in the figure, the content of a liquid biopsy is usually representative of all the different clones contributing to the primary tumor and the metastatic lesions, thus allowing for a more accurate characterization of the disease. The same representativeness is not achievable by conventional tissue biopsy as only a small portion of the tumor is sampled.

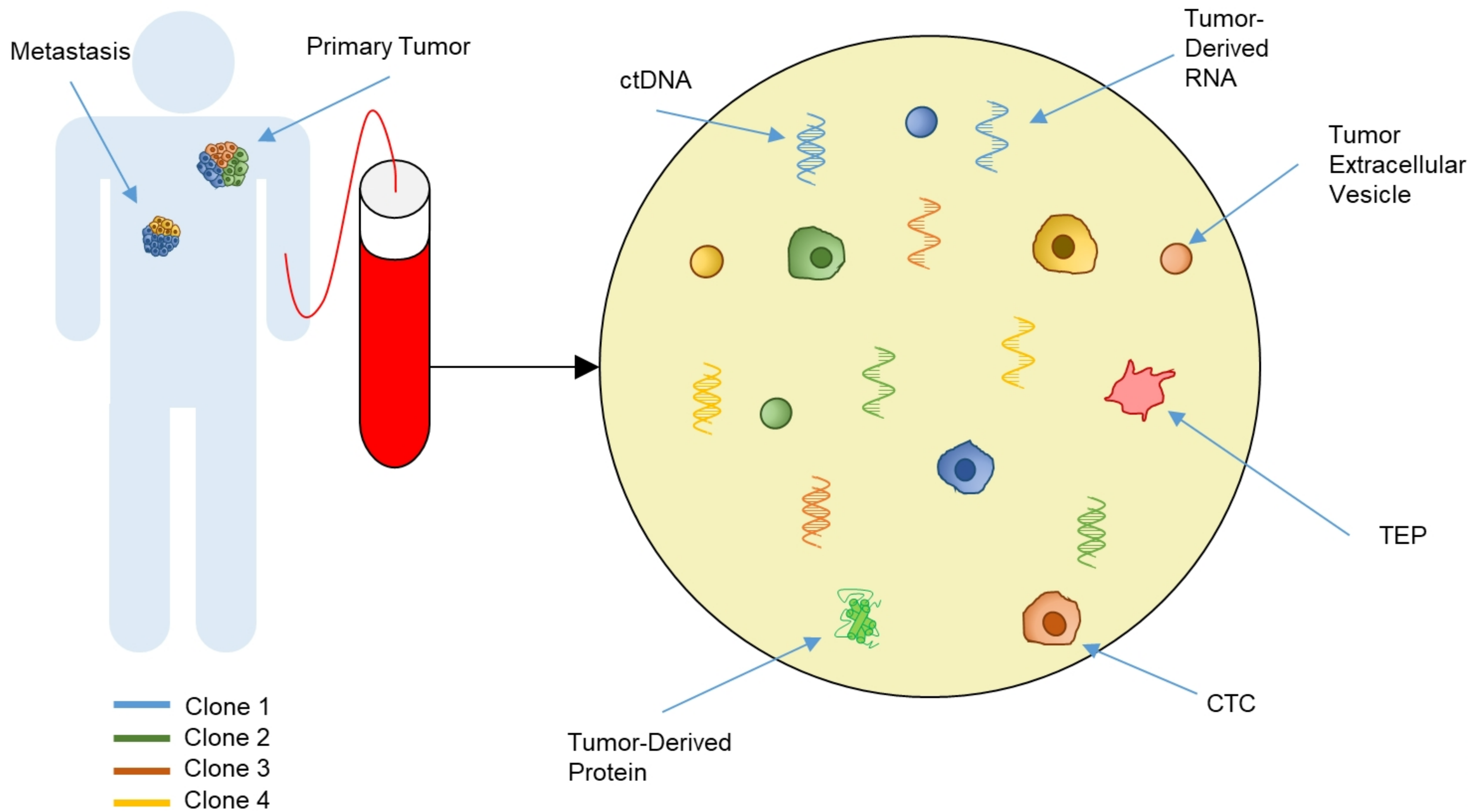
CTC: Circulating tumor cell; ctDNA: circulating tumor DNA; TEP: Tumor-educated platelet

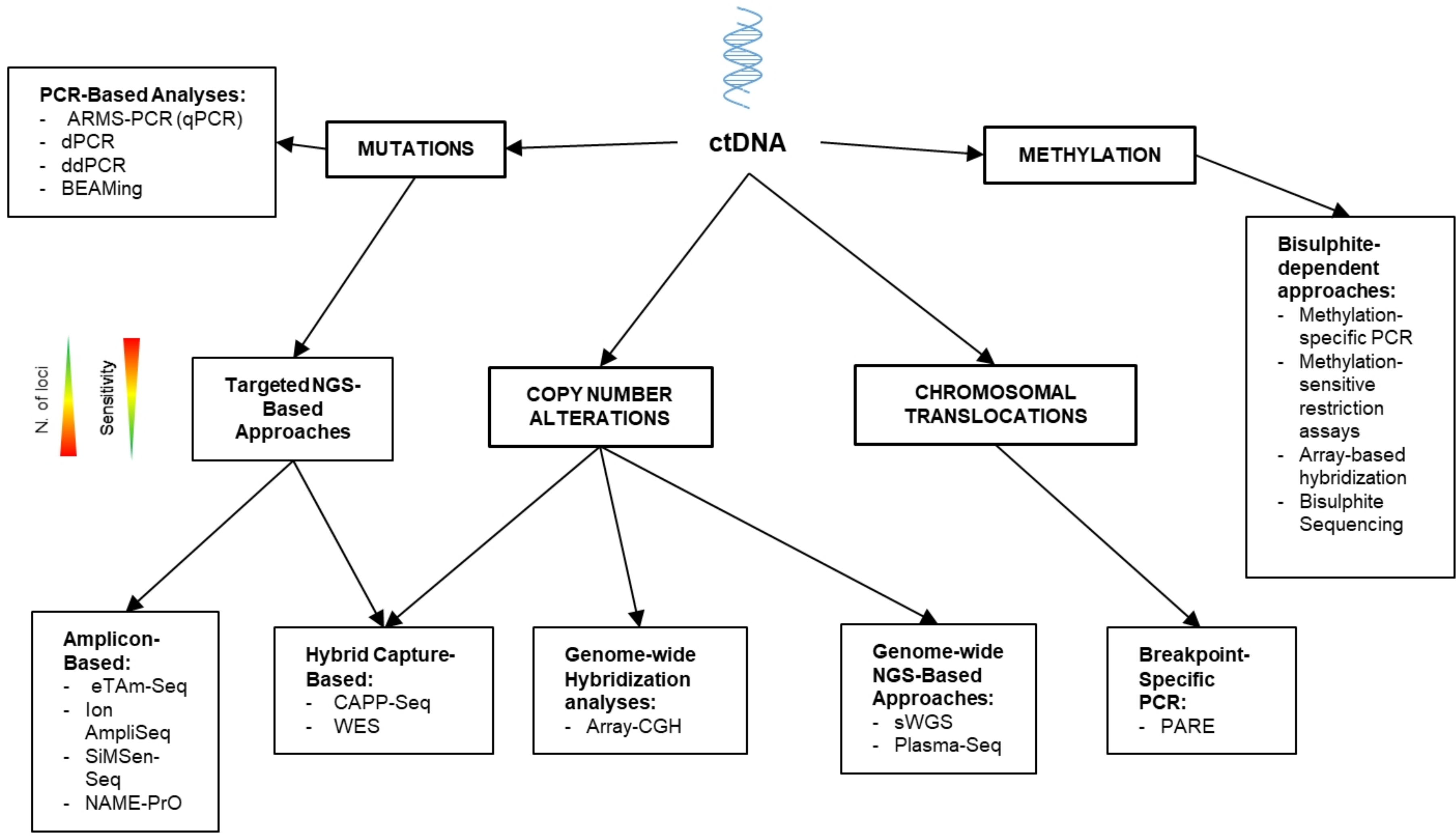
Figure 2 – Technologies used for ctDNA analysis

The technological approaches available for ctDNA analysis range from single locus to genome-wide. They allow the identification and quantification of mutations, copy number alterations (duplications and deletions), chromosomal translocations and the assessment of CpG island methylation patterns. Most of these approaches are based either on NGS or PCR and PCR-derivated technologies or on associations

between them. With regards to NGS-based approaches, usually the number of loci analyzed is inversely proportional to the sensitivity of mutant detection. The technologies used for methylation assessment are based on the bisulphite-mediated conversion of unmethylated cytosines into uracil and subsequent analysis through PCR, NGS, arrays or restriction enzymes.

ARMS-PCR: Amplification-Refractory Mutation System PCR; Array-CGH: Array-Comparative Genome Hybridization; BEAM: Beads, Emulsion, Amplification, Magnetics; CAPP-Seq: CAncer Personalized Profiling by deep Sequencing; ddPCR: Droplet digital PCR; dPCR: Digital PCR; eTAm-Seq: Enhanced Tagged Amplicon Sequencing; NAME-PrO: Nuclease-assisted Minor-allele Enrichment with Probe-Overlap; NGS: Next Generation Sequencing; PARE: Personalized Analysis of Rearra nged Ends; PCR: Polymerase Chain Reaction; SiMSen-Seq: Simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; sWGS: Shallow Whole Genome Sequencing; UMI: Unique Molecular Identifier; WES: Whole Exome Sequencing.


Metastasis
Primary Tumor
ctDNA
Tumor-
Derived
RNA
Tumor
Extracellular
Vesicle
TEP
CTC
Tumor-Derived
Protein
Clone 1
Clone 2
Clone 3
Clone 4



ctDNA
MUTATIONS
PCR-Based Analyses:
- ARMS-PCR (qPCR)
- dPCR
- ddPCR
- BEAMing
METHYLATION
Bisulphite-dependent approaches:
- Methylation-specific PCR
- Methylation-sensitive restriction assays
- Array-based hybridization
- Bisulphite Sequencing
N. of loci
Sensitivity
Targeted NGS-Based Approaches
COPY NUMBER ALTERATIONS
CHROMOSOMAL TRANSLOCATIONS
Amplicon-Based:
- eTAm-Seq
- Ion AmpliSeq
- SiMSen-Seq
- NAME-PrO
Hybrid Capture-Based:
- CAPP-Seq
- WES
Genome-wide Hybridization analyses:
- Array-CGH
Genome-wide NGS-Based Approaches:
- sWGS
- Plasma-Seq
Breakpoint-Specific PCR:
- PARE

Table 1 – Ongoing Interventional Industry-funded clinical studies on ctDNA

Study type / Phase	NCT Number	Title (Acronym)	Description	Cancer Type(s)	Sponsors / Collaborators	Explored parameters	Techniques / Platforms	Enrollment N.	Status
Interventional / N/A	NCT03302884	Circulating Tumor DNA as an Early Marker of Recurrence and Treatment Efficacy in Ovarian Carcinoma (CIDOC)	Evaluation of the prognostic value of ctDNA levels measurement for recurrence prediction (3 years)	Ovarian Carcinoma	Institut Paoli-Calmettes / AstraZeneca	Mutation analysis	N/A	150	Not yet recruiting
	NCT03017183	Detection of Heterogeneity in Central Lung Cancer - EBUS-TBNA and ctDNA Analysis vs. Endobronchial Forceps Biopsy	Evaluation of the Diagnostic Value of EBUS-TBNA and ctDNA Analysis compared to conventional forceps sampling	Lung Neoplasms	Wissenschaftliches Institut Bethanien / AstraZeneca / Institute of Pathology, University Clinic Düsseldorf	Mutation analysis (multiple genes)	N/A	30	Recruiting
Interventional / Phase 1	NCT02808884	Cancer DNA Screening Pilot Study (CANDACE)	Evaluation of positive and negative predicting values (1-2 years) of a NGS-based ctDNA assay for the early detection of cancer	Pan-Cancer	British Columbia Cancer Agency / University of British Columbia / University of Utah / Pathway Genomics / Boreal Genomics	Mutation analysis (multiple genes)	NGS	1000	Active, not recruiting
	NCT02510001	MEK and MET Inhibition in Colorectal Cancer (MErCuRIC1)	Includes, among secondary outcomes, gene sequencing from ctDNA in serially collected plasma samples from mCRC patients (1 year) to develop a liquid biopsy platform for routine assessment of therapeutic efficacy.	Colorectal Cancer	University of Oxford / Pfizer / European Commission / Array Biopharma and 11 others	Mutation analysis (multiple genes)	NGS	92	Recruiting

Interventional / Phase 2	NCT02955758	Pembrolizumab in Patients With Metastatic Non-squamous Non-small Cell Lung Cancer	Correlation between ctDNA levels and radiographic tumor assessment (RECIST v1.1) to investigate the effect of Pembrolizumab on patients with NSCLS (2 years)	Metastatic Non-Squamous Non-Small Cell Lung Carcinoma	Joel Neal / Merck Sharp & Dohme Corp. / Stanford University	Mutation analysis (multiple genes) and ctDNA concentration	CAPP-Seq	25	Recruiting
	NCT02892734	Ipilimumab and Nivolumab in Treating Patients With Recurrent Stage IV HER2 Negative Inflammatory Breast Cancer	Includes, among the tertiary outcomes, the assessment of the predictive value of exosome-associated ctDNA analysis for the evaluation of treatment response using blood samples at baseline	Metastatic recurrent HER2-negative inflammatory breast cancer	Northwestern University / Bristol-Myers Squibb / National Cancer Institute (NCI)	N/A	N/A	29	Recruiting
Interventional / Phase 3	NCT02997501	T790M Plasma Testing Methodology Comparison and Clinical Validation (ADELOS)	Evaluation of the concordance between the Cobas test and three other platforms - Evaluation of sensitivity and specificity of the three platforms in comparison with the cobas test	Non-Small Cell Lung Carcinoma	AstraZeneca / TigerMed	Mutation analysis (single gene)	Cobas v2 EGFR test Super-ARMS dPCR NGS	167	Active, not recruiting
	NCT03038217	Investigation of the Value of ctDNA in Diagnosis, Treatment, and Surveillance of Surgically Resectable Colorectal Cancer	Determination of the value of circulating tumor DNA (ctDNA) analysis in predicting the therapeutic effects of combined and surgical treatments and the long-term (5 years) prognosis.	Rectal Cancer, Adenocarcinoma	gwcmc / Geneplus-Beijing Co. Ltd.	Mutation analysis	NGS	300	Not yet recruiting

	NCT03439046	Study of the Molecular Features of Postmenopausal Women With HR+ HER2-negative aBC on First-line Treatment With Ribociclib and Letrozole (BioItaLEE)	Evaluation of the molecular features of postmenopausal women with hormone receptor-positive, HER2-negative advanced breast cancer on first-line treatment with ribociclib and letrozole. Includes associations of the identified mutational landscape with treatment response	Advanced Breast Cancer	Novartis Pharmaceuticals	Mutation analysis (multiple gene) ctDNA concentration	N/A	350	Not yet recruiting
	NCT03079011	PAlbociclib and Circulating Tumor DNA for ESR1 Mutation Detection (PADA-1)	Evaluation of the efficacy of a change of the hormone therapy (from aromatase inhibitor to fulvestrant) associated with palbociclib driven by the identification of ESR1 mutants in ctDNA	Metastatic Breast Cancer	UNICANCER / Pfizer	Mutation analysis (single gene)	N/A	800	Recruiting

The indicated details about the clinical studies have been obtained from the NIH clinical trials server (https://clinicaltrials.gov/) – Last visit: 06/07/2018

EBUS-TBNA: Endobronchial Ultrasound-guided Transbronchial Needle Aspirate; N/A: Information not available on the study's corresponding page; RECIST: Response Evaluation Criteria in Solid Tumors – International guidelines for tumor response assessment published by the World Health Organization.

Table 2 – Ongoing Observational Industry-funded clinical studies on ctDNA

Study type	NCT Number	Title (Acronym)	Description	Cancer Type(s)	Sponsor / Collaborators	Explored parameter(s)	Technique(s) / Platform(s)	Enrollment N.	Status
Observational	NCT03085888	The STRIVE Study: Breast Cancer Screening Cohort	Training and validation of an assay to detect early breast cancer: 120,000 women undergoing screening mammography will be subjected to blood draw and extensive sequencing of ctDNA and other circulating tumor derived nucleic acids.	Breast Cancer	GRAIL, Inc.	Mutation analysis (multiple genes)	High-Intensity whole genomic sequencing (NGS) Machine Learning	120000	Enrolling by invitation
	NCT03483922	HCC Screening Using DNA Methylation Changes in ctDNA	Development of a test based on the assessment of methylation profiles on ctDNA and PBMC and T Cell-derived DNA for the early detection of the transition from Chronic Hepatitis B to Hepatocellular Carcinoma	Hepatocellular Carcinoma	HKGepitherapeutics / International Centre for Diarrhoeal Disease Research, Bangladesh	ctDNA methylation (multiple genes)	Bisulphite Conversion + NGS	400	Recruiting
	NCT03519958	Epidermal Growth Factor Receptor (EGFR) T790M Mutation Testing Practices in Hong Kong	Study of the EGFR T790M mutation prevalence in ctDNA and urinary ctDNA of NSCLC patients who progressed after previous TKI treatment.	Non-small Cell Lung Cancer	AstraZeneca	Mutation analysis (single gene)	ddPCR	100	Not yet recruiting

	NCT03517332	Circulating Tumor DNA Exposure in Peripheral Blood	Test of the feasibility of using an innovative approach for ctDNA detection for the diagnosis of different types of tumors. Patients recruited must have a cancer diagnosis and must not have been treated yet.	Different Types	Quantgene Inc.	Mutation analysis (multiple genes)	N/A	10000	Recruiting
	NCT02994511	Cambridge Liquid Biopsy and Tumor Profiling Study for Patients on Experimental Therapeutics Trials (CALIBRATE)	Investigation of the potential of ctDNA as a predictive factor of resistance or response to anticancer treatment.	Different Types	CCTU- Cancer Theme / AstraZeneca / Cambridge University Hospitals NHS Foundation Trust	ctDNA concentration Mutation analysis (multiple genes), genomic profile	NGS	100	Recruiting
	NCT02889978	The Circulating Cell-free Genome Atlas Study	Development of computational models for distinguishing cancer from non-cancer specimens, thus enabling early diagnosis	Pan-Cancer	GRAIL, Inc.	Mutation analysis (multiple genes, whole genome) Methylation analysis	High-Intensity Sequencing (NGS) Whole Genome Bisulphite Sequencing Machine Learning	15000	Recruiting
	NCT02284633	Blood Sample Monitoring of Patients With EGFR Mutated Lung Cancer	Study of the evolution of EGFR mutational landscape and correlation with resistance to first line treatment and Progression Free Survival. Early identification of disease progression.	Non-Small cells Lung Cancer	Aarhus University Hospital / Odense University Hospital / Aalborg Universitetshospital / Herning Hospital / Roche Pharma AG	Mutation analysis (single gene)	qPCR (cobas)	200	Recruiting

The indicated details about the clinical studies have been obtained from the NIH clinical trials server (https://clinicaltrials.gov/) – Last visit: 06/07/2018

HCC: Hepatocellular Carcinoma; N/A: Information not available on the study’s corresponding page; PBMC: Peripheral Blood Mononuclear Cell.

CONFLICT OF INTEREST STATEMENT:

The authors of the review manuscript entitled "**Circulating Tumor DNA – current state of play and future perspectives**" (Mr. Gabriele De Rubis, Dr. Sabna Rajeev Krishnan, Prof. Michael Wallach, A/Prof. Mary Bebawy) certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.