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Liquid Biopsies in Cancer Diagnosis, Monitoring and Prognosis

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ABSTRACT

Liquid biopsy, consisting in the non-invasive analysis of circulating tumor-derived material (the Tumor Circulome), represents an innovative tool in precision oncology to overcome current limitations associated with tissue biopsies. Within the tumor circulome, ctDNA and CTCs are the only components whose clinical application is FDA-cleared. Extracellular vesicles, ctRNA and tumor-educated platelets are relatively novel tumor circulome constituents with promising potential at each stage of cancer management. Here, we discuss the clinical applications of each element of the tumor circulome and the prevailing factors that currently limit implementation in clinical practice. We also detail the most recent technological developments in the field, which demonstrate potential in improving the clinical value of liquid biopsies.

Liquid Biopsies – Investigating the "Tumor Circulome"

Cancer is one of the leading causes of death worldwide, with 8.8 million deaths estimated in 2015[i]. In the USA, more than 1,735,350 cases of cancer are foreseen to be diagnosed in 2018, causing more than 609,640 deaths[ii]. The development of "omics" technologies has led to the field of precision oncology that consists of tailoring treatment regimens to an individual's tumor molecular characteristics [1]. The current golden standard for genetic profiling of tumors typically involves the use of tissue biopsies. Because of their invasive nature, tissue biopsies are associated with many limitations including patient risk, sample preparation, **sensitivity** (see Glossary) and accuracy, procedural costs and invasive testing. This makes the procedure incompatible for clinical **longitudinal monitoring** [2]. Furthermore, a significant limitation of tissue biopsies is that they fail to capture intratumoral and intermetastatic **genetic heterogeneity**, impacting the accuracy of the test [3].

Liquid biopsies present great potential in overcoming these existing sampling limitations. They consist of the sampling and analysis of liquid biological sources, typically blood, for cancer diagnosis, screening and prognosis. The "Tumor Circulome" , defined as the subset of circulating components is derived from cancer tissue andcan be directly or indirectly used as a source of cancer biomarkers in liquid biopsy [4]. These include: circulating tumor proteins, circulating tumor nucleic acids (ctDNA, ctRNA), circulating tumor cells (CTCs), tumor-derived extracellular vesicles (EV) ~~, circulating tumor proteins~~ and tumor-educated platelets (TEPs) (**Figure 1, Key Figure**). Liquid biopsies present several advantages over conventional tissue biopsies (see **Table 1**), and technological advancements in sample isolation (such as the development of nucleic acids extraction chips to minimize the manipulation of samples [5]) and detection platforms (such as the development of high resolution flow cytometers [6] or

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single-cell Western Blot platforms [7]) are evolving to support this approach. The first important milestone in this field was reached in 2016 with the Food And Drug Administration (FDA) approval of the first **companion diagnostic** test for lung cancer based on the ctDNA content of a liquid biopsy [8].

In this review, we detail the clinical significance and potential of liquid biopsies and provide an overview of recent reports supporting elements of the Tumor Circulome as biomarkers for the diagnosis and monitoring of cancer, with a particular focus on ctDNA, CTCs, tumor-derived EVs and ctRNAs. We also discuss factors limiting implementation in clinical practice and outline significant technological advances that may overcome these. Our focus will be on blood as the biological matrix for the liquid biopsy, however other biological fluids comprise a source of promising tumor-derived biomarkers too (see **Box 1**).

Circulating Tumor-derived Proteins

The measurement of circulating protein markers has historically been the gold standard approach used for the non-invasive diagnosis, screening and postoperative follow-up in cancer management. Notable examples of circulating tumor-derived protein markers include the –**Prostate Specific Antigen (PSA)** for prostate cancer screening [9] and CA 15-3 for postoperative follow-up of breast cancer recurrence [10]. These are compromised by high false positive rates, which can lead to overdiagnosis and in some cases unnecessary anticancer treatment [9]. In the case of CA 15-3, there are questions around its application in improvements in patient outcomes [10]. The use of panels or biosignatures comprised of more than one protein is a more promising approach, as the combination of multiple

biomarkers increases the diagnostic/prognostic capability of the assay by reducing the number of false positives and false negatives [11, 12].

Circulating Tumor DNA (ctDNA)

Circulating Tumor DNA comprises the fraction of circulating cell-free DNA (cfDNA) originating from cancer cells. This includes short nucleosome-associated fragments (80-200 bp) [13] and longer fragments (> 10 kb) encapsulated within EVs [14]. The mechanisms of ctDNA release into circulation include **apoptosis**, **necrosis**, lysis of CTCs and active secretion from the tumor [15]. The proof of the suitability of ctDNA as cancer biomarker came with the identification of *KRAS* gene mutations in ctDNA from the blood of pancreatic cancer patients [16].

Clinical significance of ctDNA analysis

Both qualitative and quantitative information can be obtained from ctDNA analysis [13].Quantitative information can be obtained from the measurement of the mutant allele fraction (MAF, the percentage of mutant allele in a given locus) and is a reflection of tumor burden [13]. It finds application in the detection of **minimal residual disease** (MRD) and **occult metastases** [17] and in the monitoring of treatment response and therapeutic effectiveness [18]. CtDNA levels provide a "real time" snapshot of tumor bulk because of its short **half-life** (around 2.5 hours) [13]. The detection of ctDNA after treatment is a high sensitivity and **specificity** predictor of relapse [19].

Qualitative information can be sourced through the profiling of mutations, ~~duplications,~~amplifications, deletions and translocations in ctDNA (Figure 1), allowing the identification of genetic alterations associated with response, hence supporting decision-

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making for personalized management [13]. For example, the first ctDNA-based companion diagnostic test (cobas® EGFR Mutation Test v2 – Roche Diagnostics), recently approved by the FDA [8], is used to guide the use of Epidermal Growth Factor Receptor (EGFR)-**Tyrosine Kinase** Inhibitors on the basis of specific EGFR-sensitizing mutations in patients with non-small cell lung cancer (NSCLC). Other qualitative information obtainable through ctDNA analysis include~~s~~ assessment of methylation status. For example, a screening test for colorectal cancer, Epi proColon®, has been recently approved by FDA and analyzes the methylation pattern of the promoter of the *SEPT9* gene, a region known to be hypermethylated in colorectal cancer when compared to ~~normal~~non-malignant samples [20].

Technological approaches and current limitations

The currently available technologies for ctDNA analysis are based on polymerase chain reaction (PCR) or next-generation sequencing (NGS). Allele-specific PCR was the first approach used in ctDNA detection [16] and a quantitative PCR (qPCR) variation of this technique is currently adopted by the cobas® EGFR test [8]. Considering that the fraction of ctDNA in total cfDNA is usually very low, often less than 0.01% [21], more sensitive technologies have been developed and successfully used for ctDNA analysis, such as **digital PCR (dPCR)** [22], **droplet digital PCR (ddPCR)** [23] and Beads, Emulsion, Amplification, Magnetics (**BEAMing**) [24]. Although very sensitive, quick and relatively inexpensive, PCR-based assays are limited by low multiplexing capacity, allowing for analysis of a restricted number of loci in parallel [13].

The sensitivity of NGS-based technologies is lower than that of PCR-based technologies and inversely proportional to the number of loci analyzed, with **Whole Exome Sequencing (WES)** having the lowest sensitivity (≥5% MAF) [13]. Approaches to enhance the sensitivity of NGS

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include considering patient- or cancer-specific gene panels, such as in the **Cancer Personalized Profiling by deep Sequencing** (CAPP-Seq) technology [25], or strategies to suppress the background noise generated by random errors occurring during library preparation. These strategies involve tagging each template molecule with Unique Molecular Identifiers (UMIs). These are used by different NGS platforms, such as eTAm-Seq™ (Enhanced Tagged Amplicon Sequencing) [26]. Another approach to enhance sensitivity includes the selective nuclease digestion of non-mutated DNA, which results in an increase in MAF and has enabled mutation detection down to 0.00003% MAF [27].

Despite its potential, the use of ctDNA as a liquid biopsy has many limitations. Detection sensitivity is a serious concern, especially in early cancer detection where the low amount of ctDNA may result in a MAF lower than the limit of detection of existing techniques [13]. The sampling of other body fluids, proximal to the putative site of the tumor, can increase the detection rate, at least in individuals at risk due to, for example, hereditary predisposition. This is mainly because, especially at early stages, a proximal body fluid may have a higher concentration of tumor-derived DNA than blood [28]. Another concern in early detection is the predictive value of single or small sets of mutations, as cancer-associated mutations can be found in plasma of healthy individuals as a result of **clonal hematopoiesis** [13]. One approach to overcome this challenge is ~~to use~~ the CancerSEEK platform, which associates the analysis of 8 tumor-derived proteins to ctDNA mutation profiling and has a specificity of >99% [29].

Another limitation impeding the implementation of ctDNA analysis into clinical practice is the lack of standardized protocols for pre-analytical sample preparation and ctDNA purification. Current procedures are complex and may cause ctDNA degradation and blood cell lysis [30].

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A platform allowing a quick, single-step purification of ctDNA from blood is desirable, and **lab-on-a-chip** systems have potential to address this need [5].

Circulating Tumor Cells (CTCs)

CTCs are a population of tumor cells that have detached from the primary tumor and can be found in the peripheral blood of patients. Their presence is thought to be fundamental to the development of metastasis [31]. CTCs present systemically through active **intravasation**, with **epithelial-to-mesenchymal** (**EMT**) transition as a fundamental step [32], or through passive shedding from the primary tumor~~,~~. This latter ~~a~~ mechanism is supported by the presence of CTC aggregates or Circulating Tumor Microemboli (CTMs) in the blood [33].

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Clinical significance of CTCs and analytical technologies

The information that can be obtained from CTCs are quantitative as well as phenotypic (qualitative) through single cell genomic/transcriptomic/proteomic profiling (Figure 1). They have great potential as tools for diagnosis, monitoring, prognosis and prediction of response to therapy, and also for the discovery of novel drug targets [34]. Furthermore, the *ex vivo* culture of CTCs has an important translational value, because it allows to perform personalized drug sensitivity tests with the aim of basing treatment decision-making on evolving tumor mutational profiles and drug sensitivity patterns found in individual patients [35].

The simplest information obtainable from CTCs is their number, which is a prognostic predictor for many cancers including metastatic breast, colon and prostate cancers [36]. Currently the only FDA-cleared clinical application of CTCs is the CellSearch® platform used

for enumeration of epithelial CTCs [36]. The power of CTC counts as a criterion for the selection of the first-line treatment in metastatic breast cancer is currently being investigated in the METABREAST trial (Clinical Trial Number: NCT01710605).

With regards to the genetic and genomic information obtainable from CTCs, the technologies that can be used are similar to those of ctDNA analysis, and range from qPCR and dPCR-based mutational profiling to targeted NGS and whole genome sequencing [37]. Additionally, CTCs can be analyzed by cytogenetic analyses such as Fluorescence In Situ Hybridization (FISH) for the identification of chromosomal rearrangements [38]. NGS technologies have fundamental importance for single-CTC genomic and transcriptomic characterization, in the study of tumor heterogeneity and in comparative analysis with tissue biopsies [39]. Although the fields of single-cell genomics and transcriptomics have experienced significant developments, single-CTC protein analysis is somewhat premature in comparison, with immunocytochemistry and flow cytometry being primarily used, both of which have poor multiplexing capacity. New technologies are however emerging and Sinkala and colleagues recently developed a microfluidic-based single-cell Western Blot assay (scWB) which was used to assess the levels of 8 proteins in three metastatic cancer patient-derived single CTCs [7].

Despite numerous analytical platforms and technologies available for CTC analysis, their translation into clinical practice is limited by their isolation from blood. Challenges include their extreme rarity, fragility and physical and phenotypic heterogeneity [34]. Currently available strategies for CTC enrichment and isolation exploit their biological and physical properties, while functionality assays allow CTCs identification (**Box 2** and **Table 2**). Each of these alternatives has advantages and drawbacks, and only their combination can support a comprehensive characterization.

Extracellular Vesicles (EVs)

EVs are membranous particles released from all cell types in physiological and pathological conditions, as well as following different types of stimuli including proteases, ADP, thrombin, inflammatory cytokines, growth factors, biomechanical shear and stress inducers and apoptotic signals [40]. They can be found in almost every body fluid, especially in blood [41]. Once considered a simple means to eliminate unneeded cellular components from the cytoplasm of cells, during the last decade EVs have been recognized as fundamental mediators of intercellular communication, regulating and participating to a plethora of physiological and pathological processes including cancer [41]. Based on their biogenesis, content and secretory pathways, EVs can be divided into two broad categories: exosomes and microvesicles [41].

Clinical significance of EVs as cancer biomarkers

The suitability of EVs as cancer biomarkers lies in the fact that the molecular cargo they carry can be considered a molecular fingerprint of the cell of origin [42]. Compared to ctDNA and CTCs, whose implementation in clinical cancer diagnostics is hampered by challenges in their isolation, analytical sensitivity and by stability concerns, the potential advantages of EVs are many. EVs are typically produced and released in abundant quantities and in greater amounts compared to CTCs [43]. Likewise, the stability of the vesicular cargo is maintained through a protecting outer lipid membrane [44].

Similarly to ctDNA and CTCs, EVs can be source of quantitative and qualitative information. Quantitative information comprising EVs numbers can inform the presence of malignant

disease and tumor burden. For example, circulating exosome levels are increased in breast and pancreatic cancer [45] and the number of circulating microparticles (MPs) is higher in multiple myeloma (MM) patients compared to healthy individuals [46]. Furthermore, circulating MP levels demonstrated potential for the diagnosis and prognosis of advanced NSCLC [47].

Qualitative information through the molecular characterization of EV constituents, including nucleic acids and proteins (Figure 1), are the most readily obtained ~~(Figure 1)~~ [42]. The RNA content of EVs, including both coding and non-coding RNAs, has been widely studied [43] . The DNA content of exosomes has recently gained attention as a biomarkers source in a study in which mutations in *KRAS* and *TP53* have been detected in serum exosomes from pancreatic cancer patients [14]. In another study, the identification of exosomal *KRAS* mutations proved better than **CA 19-9** for prognostic stratification of patients with pancreatic ductal adenocarcinoma (PDAC) [48].

EVs carry proteins in their lumen and in their membrane, and numerous reports have been published demonstrating the important role of EV proteins as possible cancer biomarkers [42]. Melo et al. demonstrated the ability of circulating exosomal Glypican-1 (GPC1) to distinguish PDAC from healthy donor samples with a reported accuracy of 100% [45]. More recently, Moon et al. demonstrated the suitability of EV Del-1 [49] and Fibronectin [50] as biomarkers for early breast cancer diagnosis. Furthermore, our group demonstrated that the levels of circulating $CD138^+$ MPs increase in MM and observed a significant prognostic potential for $CD138^+$ MPs in predicting risk of relapse and therapeutic response in individual patients [46]. Finally, the levels of $AnnexinV^+$ $EpCAM^+$ Asialoglycoprotein Receptor-1 (ASGPR1)$^+$ circulating MPs are diagnostic of hepatocellular carcinoma and

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cholangiocarcinoma [51], and the levels of $CD147^{+}$ $EpCAM^{+}$ MPs are predictors of colorectal carcinoma [52].

Technologies for EVs isolation and analysis and recent advances

One important limitation to the clinical application of EVs as liquid biopsy is the lack of standardized protocols for sample handling and EV isolation and analysis, which could impact reproducibility in the clinical setting [53]. Currently used EV isolation procedures often consist of many biospecimen handling steps and can subject EVs to different types of physical and chemical insults, which may damage EVs and/or modify their biological and physical properties. Another factor influencing the reproducibility of EV studies is the lack of standardized guidelines defining EVs nomenclature and definition, and the control experiments needed for validation. In order to overcome these limitations, a comprehensive collection of guidelines and recommendations has been very recently updated by the International Society for Extracellular Vesicles [54].

Similarly to CTCs, conventional EVs isolation strategies exploit physical (density, size) and biological (expression of surface markers) properties [55]. Density-based approaches, such as differential centrifugation/ultracentrifugation and density gradient centrifugation, are the most commonly used methods for EV isolation. Among these, differential ultracentrifugation is considered the gold-standard technique, especially for exosome purification. Although widely used, these techniques rely on expensive equipments, are time-consuming and don't guarantee pure yields, often resulting in a compromise between purity and recovery [55].

Size-based techniques include filtration and size exclusion chromatography (SEC). Filtration can result in high yields and purity, but again is limited in terms of EVs adherence to the filters

and vesicle damage due to high pressure [56]. SEC allows a superior recovery of EVs compared to ultracentrifugation [56].

Immunoaffinity capture methods use magnetic beads conjugated to antibodies recognizing EV surface markers. An advantage of these methods is that they produce EV fractions with high purity, allowing the isolation of specific subsets of EVs based on the surface marker used. This feature, on the other hand, could be a limitation because it may overlook potentially important EV subpopulations lacking the expression of the selected marker [55]. A novel development of immunoaffinity capture is its integration into microchips allowing *in-situ* immunoassay analysis [57].

Another commonly used isolation method, especially for exosomes, is polymer precipitation. This method involves the use of polymers (such as polyethylene glycol (PEG)) to reduce the solubility of EVs, in order to precipitate them with a rapid low-speed centrifugation. Although producing high recovery rates, this methodology has low purity [58].

Recently emerging methodologies for EV isolation use electric fields. Lewis et al. developed an Alternating Current Electrokinetic (ACE) Chip capable of performing exosome capture from whole blood and *in-situ* immunofluorescent analysis in 30 minutes. They validated this chip by assessing the suitability of GPC-1 and CD63 levels as diagnostic markers of PDAC [59].

Finally, another promising category of potential novel approaches to EV isolation relies on microfluidics [58]. The available microfluidic approaches are based on different EV properties, eg: nanoscale size-based filtration [60], antibody-functionalized microfluidic channels [61] and spiral inertial microfluidic devices [62]. In a recent report, Ko et al. developed a magnetic nanopore sorting platform that has been used to isolate specific cancer-derived EVs. They used this system to identify, in a mouse model of PDAC, a miRNA signature to train a machine

learning algorithm for the classification of distinct cancer states [63]. Microfluidics technologies are set to boost the development of lab-on-a-chip systems for fast, cost-effective, integrated isolation and analysis of EVs, towards the development of EV-based point-of-care diagnostics.

Together with the isolation methods, EV detection methods are also experiencing advances in development, especially with regards to the analysis of protein cargo. Common analytical technologies include Western blot (WB), Enzyme-linked immunosorbent assay (ELISA), mass spectrometry (MS) and flow cytometry (FCM) [64]. With the exception of FCM, these techniques focus primarily on bulk EV analysis, without assessing their individual variability [58]. FCM is currently used for single-MPs characterization [46, 51, 52], but fails to analyze single exosomes due to their small size. Exosome FCM analysis currently involves the binding of multiple exosomes to larger beads [45]. Recently, Kibria et al. developed a microFCM platform which was capable of assessing the expression of CD47 in single circulating exosomes from breast cancer patients [6]. Another technique capable of allowing single-EV protein phenotyping, at a higher size resolution than current flow cytometers, is a variation of Nanoparticle Tracking Analysis (NTA) in which fluorescent antibodies are used to identify EVs expressing a given marker [65]. Despite better size resolution of NTA, a great advantage of FCM is its higher multiplexing capacity.

Circulating Tumor RNA (ctRNA)

The fraction of circulating cell-free RNA originating from cancer cells is referred to as circulating tumor RNA (ctRNA). The existence of extracellular RNA was first documented in 1978 [66] and the first report about its potential as cancer biomarkers was shown years later

[67]. Compared to DNA, RNA is a relatively unstable molecule, whose naked half-life in plasma is approximately 15 seconds [68]. Its stability is enhanced by its association with proteins [69], proteolipid complexes [67] and extracellular vesicles [44].

Clinical significance of ctRNA as cancer biomarker and current limitations

Nearly all known classes of RNA have been found in systemic circulation and, to a certain extent, each one of them has potential to serve as cancer biomarkers [70]. Similar to other components of the tumor circulome, ctRNA is source of quantitative and qualitative information. In fact, although expression profiles of coding and non-coding RNAs (ncRNAs) represent the most important source of information, the identification of tumor-specific fusion transcripts or alternative splice events is also possible [71]. The most important classes of ctRNA potentially suitable as biomarkers are mRNAs, miRNAs and long non-coding RNAs (lncRNAs) (Figure 1). Their analysis is performed with techniques ranging from qRT-PCR or dPCR-based assessment of single or small panels of RNAs to the comprehensive characterization of RNAs (especially miRNAs) signatures via RNA-Seq [70].

Circulating exosomal mRNA has been used to investigate the mutational status of *KRAS* and *BRAF* in patients with colorectal cancer (CRC) [72], and exosomal EGFRvIII mRNA has potential for the diagnosis of EGFRvIII-positive high-grade gliomas [73]. In another report, the detection of androgen receptor splice variant 7 (AR-V7) in plasmatic exosomes by ddPCR has been shown to be a good predictor of resistance to hormonal therapy in prostate cancer [74]. Numerous lung cancer-related gene fusions are also readily identified in both vesicular and non-vesicular mRNA and have value as biomarkers [75]. Among the non-vesicular fraction of ctRNAs, circulating human Telomerase Reverse Transcriptase (hTERT, catalytic subunit of the

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Telomerase Complex) mRNA has demonstrated a greater diagnostic and prognostic accuracy than PSA for prostate cancer [76].

With regards to miRNAs, plasma exosomal miR-196a and miR-1246 levels have potential for the early diagnosis of pancreatic cancers [77], and panels of miRNAs have been shown to be reliable biomarkers for diagnosis [78] or prognosis [79] of lung cancer. More recently, a serum exosomal miRNA signature has proven to be an innovative tool for the differential diagnosis of gliomas [80].

A novel promising source of RNA biomarkers are long-noncoding RNAs (lncRNAs). For example, plasma exosome LINC00152 levels have been linked to gastric cancer [81], and the combination between two mRNAs and one lncRNA in serum exosomes has diagnostic potential for colorectal cancer [82]. Furthermore, serum exosomal HOTAIR lncRNA has applicability in diagnosis and prognosis of glioblastoma multiforme [83]. More recently, a panel of five circulating lncRNAs has been studied as a promising diagnostic biomarker for gastric cancer [84].

To date, the most important limitations for the implementation of ctRNAs in the clinical setting involve the pre-analytical and analytical steps. Although circulating RNAs are protected by the association with different molecules and structures, they are unstable in plasma if stored at 4°C, and limited by the speed of extraction [85]. Furthermore, different extraction protocols have different recovery rates, and there is currently no consensus on an optimal extraction protocol [85]. Again, lab-on-a-chip devices seem to offer a potential solution to this issue, allowing rapid and integrated purification and analysis of samples while minimizing their handling. A proof-of-concept of this is the microdevice developed by Potrich et al., which is capable of selectively extracting miRNAs from cell culture supernatant and

allows *in situ* reverse transcription and qPCR analysis [86]. Another example is the Integrated Comprehensive Droplet Digital Detection (IC 3D) system, a microfluidic platform capable of quantifying extremely low concentrations of miRNAs directly from plasma in 3 hours [87].

Tumor-Educated Platelets (TEPs)

TEPs are perhaps the latest components of the tumor circulome to be considered for biomarker analysis. The concept of "platelet education" by cancer refers to the presence of specific RNA signatures in platelets of cancer patients. This was first reported in 2010 and 2011 with the observations that: in metastatic lung cancer patients, 197 platelet genes were downregulated and several genes were differentially spliced compared to non-cancer controls [88]; in glioma, cancer-derived microvesicles are actively taken up by platelets and transfer their RNA content, harboring a cancer-characteristic RNA signature revealed by microarray [89]. Best et al. in 2015 characterized TEPs extracted from a patient cohort across 6 cancer types via RNA-Seq, distinguishing patients with localized or metastatic tumor from healthy individuals with 96% accuracy and locating the anatomical position of the tumor with 71% accuracy. This paved the way for "pan-cancer and multiclass cancer diagnostics" [90]. In more recent work, the same group applied **Particle-Swarm Optimization (PSO)**-enhanced algorithms ~~swarm intelligence-enhanced algorithms~~ to platelet RNA-Seq libraries to generate a panel of biomarkers capable of distinguishing lung cancer patients from healthy individuals and from those with lung inflammatory conditions [91].

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With regards to tumor-derived proteins, our idea was to not include them because, although technically part of the "Tumor Circulome", they don't represent a novel approach for cancer management if compared to ctDNA, CTCs, EVs and ctRNAs (which are actually the "hot topics" in liquid biopsy). However, we do agree with the editor that it doesn't make any sense to show the proteins in Figure 1 if they are not described in the text. To this aim, we added a very short paragraph on circulating tumor-derived proteins just before the ctDNA chapter, in which both the use of single and multiple proteins are briefly discussed. We decided to discuss them at the beginning of the paper in order to maintain their position in the Figure (the smallest spaces in the figure, which are the ones at the top right and top left, are perfect to put components of the circulome which don't "contain" many elements, such as proteins and TEPs)

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Controversies on the use of Liquid Biopsies in cancer management

Despite reports demonstrating potential of liquid biopsies in addressing the current needs in cancer management, numerous controversies remain on their utility. This is particularly true for ctDNA and CTCs, which have already found application in clinical management. In a recent report Torga et al. [92] compared the performance of two commercially available NGS-based ctDNA tests for metastatic prostate cancer, finding an astonishingly low concordance (7.5% patients studied) between the two tests. The discordance was attributed to issues in study design and sample analysis, raising current limitations of preanalytical and analytical standardization in the field. Furthermore, with very few exceptions, most of the ctDNA assays available have limited evidence of clinical validity and utility in advanced cancer. Likewise, their utility in early-stage cancers, treatment monitoring or MRD detection remains to be established [93]. Additionally, with regards to CTCs, although their clinical validity has been demonstrated, particularly for their prognostic capacity in metastatic disease, evidence of their clinical utility is still missing, preventing their implementation into standard clinical practice [94]. Numerous large-scale clinical trials are clearly needed, and some are currently in progress to address this unmet need.

Concluding Remarks and Future Directions

Current standards for patient stratification and treatment selection include the analysis of tumor genetic alterations from tissue biopsies. Despite their undoubted value, tissue biopsies have important limitations, being highly invasive procedures that fail to capture tumor clonal heterogeneity. Liquid biopsies, consisting in the analysis of circulating tumor-derived factors (the Tumor Circulome), are gaining exceptional attention as a valuable alternative. The tumor circulome is source of different classes of tumor-derived biological components. Novel technologies are being developed to further improve the analysis of the tumor circulome, with the aim of fully exploring the complexity of the information obtainable from a simple blood draw.

The studies reviewed here underline the tremendous potential of liquid biopsies, and the development of novel technologies allows researchers to characterize each single component of the tumor circulome with increasing precision. Liquid Biopsies are being positioned as a game-changing tool in personalized cancer management. However, their clinical application has been comparatively slow, hampered by multiple technical challenges (listed in **Table 3**). As a consequence, several problems still need to be resolved to firmly establish the role of liquid biopsies in the clinical setting (see **Outstanding Questions**). The lack of standardization of pre-analytical and analytical variables is a significant limitation in the field. A liquid biopsy ideally should be cost effective, fast, reproducible and ensure sample integrity. One approach via which this can be achieved is through automated chip-based devices allowing for the analysis of biomarkers from whole blood without the need for lengthy and costly purification steps. While complex chip systems such as the ACE chip remain costly, alternative polymeric microfluidic devices such as the spiral microfluidic chip used for CTC isolation are, in comparison, cost effective [95]. Although much work is still needed to comprehensively define

412 the future role of liquid biopsies in cancer diagnosis, monitoring and prognosis, the promising
413 results reported so far testify to the potential of this approach in changing the current
414 paradigms of cancer management.

415

TEXT BOXES

Box 1 – Liquid biopsy of ~~other~~non-blood ~~biological specimens body fluids~~ biological specimens ~~different from~~ other than blood

Although the "standard" concept of liquid biopsy consists in the sampling of blood, virtually all body fluids are suitable as liquid biopsy. The anatomic localization of primary or metastatic tumors influences the presence of tumor-derived material in the corresponding body fluids. Common body fluids include urine, saliva, sputum, stool, cerebrospinal fluid (CSF) and pleural effusions. Many of these have demonstrated great potential as a source of cancer biomarkers [96, 97].

Urine: Urine is a valuable source of ctDNA for urogenital cancers such as prostate, bladder and cervical cancers and non-urogenital malignancies such as NSCLC, Colorectal Cancer (CRC) and gastric cancer [98], and its EV content is source of several candidate biomarkers [99]. The first liquid biopsy test on urine, Progensa® PCA3 Assay was~~,~~ FDA approved in 2012[iii] to aid the decision-making of a repeat prostate biopsy in case of a first negative biopsy. This test measures the level of Prostate Cancer Antigen 3 (PCA3) lncRNA, which is increased in ~~more~~ ~~over~~than 95% of primary prostate tumors [100]. Another promising urine-based test is the ExoDx® Prostate(IntelliScore)[iv], a **Laboratory-Developed Test (LDT)** based on the analysis of the levels of three exosome-associated RNAs overexpressed in high-grade prostate cancer which is used to "rule-out" potentially unnecessary prostate biopsies.

Saliva: Salivary biomarkers include ctDNA for head and neck squamous cell carcinoma [101], and microRNAs (miRNAs) for detecting early malignancy in potentially malignant oral cancers

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[102]. Furthermore, EV-associated miRNAs have the potential to be used as biomarkers of oral squamous cell carcinoma [103].

CSF: CSF, thanks to its direct contact with the central nervous system (CNS), is set to become an important source of biomarkers for CNS-restricted cancers, potentially overcoming the relative scarcity of circulating biomarkers (especially ctDNA) in these diseases caused by the blood-brain-barrier [104]. CSF-derived ctDNA has proven to represent genetic alterations of brain tumors better than plasma ctDNA [105], and a miRNA CSF signature for glioblastoma has been recently reported [106].

Other bodily fluids: The analysis of stool-derived DNA has been recently validated as a powerful diagnostic tool for colorectal cancer [107], while sputum DNA and protein content has promising potential in the context of lung cancer [108]. Pleural effusions, finally, are source of DNA biomarkers for lung cancer [109] and malignant pleural mesothelioma [110], and the presence of Epithelial Cell Adhesion Molecule (EpCAM)$^{+}$ microparticles allows distinction between malignant and non-malignant pleural effusions [111].

The studies reported here are examples of the enormous potential of non-blood liquid biopsies as biomarkers trove. The association between information obtained from blood and non-blood samplings will surely represent a precious added value in the field of liquid biopsy.

Box 2 - Strategies for CTC isolation, enrichment and identification

Based on biological properties: Approaches based on biological properties of CTCs exploit the expression of cell surface markers for their isolation and selection [34]. The most used marker for positive selection is EpCAM, which is a calcium-dependent transmembrane glycoprotein

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that mediates cell adhesion in epithelia and is~~,~~ detected by the CellSearch® platform [36]. CellSearch® integrates immunomagnetic enrichment of $EpCAM^+$ cells and staining with anti-cytokeratine (CK), anti-CD45 and **DAPI** [36]. A limitation of EpCAM-dependent approaches is that many CTCs don't express EpCAM ~~-~~as they undergo Epithelial-to-Mesenchimal Transition (EMT), thus underestimating CTC counts [112]. To overcome this, other markers must be considered. For example, Gao and colleagues enriched lung cancer CTCs using anti-EpCAM, anti-Mucin 1(MUC1) and anti-EGFR antibodies [113]. Alternative approaches, preferable because they include $EpCAM^{low}$ and $EpCAM^-$ populations, leaving CTCs unstained, involve the immunomagnetic depletion of $CD45^+$ leukocytes [114]. Other EpCAM-based strategies include microfluidic platforms such as the CTC-Chip [115] and the NanoVelcro system [116], and devices for the *in vivo* capture of CTCs directly into patients' veins, such as the GmbH CellCollector device [117].

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Based on physical properties: Other enrichment technologies exploit CTCs' physical properties. CTC isolation by size is possible as CTCs are generally larger than leukocytes [118]. Size-based approaches include filtration methods, such as the Isolation by Size of Tumor cells (ISET®) [118] and Metacell® [119] devices. Limitations of filtration include difficulty of detaching cells from the filter and loss of cell viability [37]. To overcome these, label-free microfluidics devices were developed, such as Parsortix™[120]. More recently, CellSearch® and Parsortix™ were sequentially combined to separately isolate $EpCAM^+$ and $EpCAM^{low/-}$ CTCs from blood of metastatic breast cancer patients, simultaneously analyzing the matched CTC populations [121]. Another example of size- based CTC enrichment is the high-throughput spiral inertial microfluidic biochip, which has been clinically validated in breast and lung cancer [122].

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Technologies exploiting density include differential centrifugation [123] and platforms such as MagDense [124]. Other technologies involve exploiting the differences between dielectric properties of CTCs and white blood cells (WBCs) and form the basis of Dielectrophoresis (DEP)-based platforms [125]. DEP ~~can be~~has been associated with Lateral Field Flow Fractionation (LFFF) to improve the isolation of spiked breast cancer cells from regular blood cells [126]. A similar technology is the DEPArray, a microfluidics platform that combines DEP and imaging and has been used to accurately sort single breast cancer cells following CellSearch® enrichment [127].

CTC functional assays support CTCs identification based on functional characteristics, overcoming limitations attributed to their heterogeneity. The Vita-Assay™ exploits the ability of CTCs to digest a Cell Adhesion Matrix (CAM), measuring the uptake of CAM proteins on a coated culture dish via fluorescence. [128]. The EPISPOT Assay detects viable CTCs through detection of specific proteins released following culture on substrates functionalized with specific antibodies [129]. The TelomeScan® platform exploits the activation of telomerase in most cancers, allowing identification of CTCs by using adenoviruses that selectively replicate in cells expressing functional **hTERT**, expressing GFP [130].

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Likewise, the next sentence seems very incomplete… DEPArray helps how?

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Table 1 – Advantages of Liquid Biopsies over conventional Tissue Biopsies

	Tissue Biopsy	**Liquid Biopsy**
Invasiveness	High	Minimal
Pain	Yes	No
Risk of complications	Yes	No
Time needed	Time-consuming	Quick
Tumor heterogeneity representation	Low/null	High/total
Tumor region selection bias	Yes	No
Compatibility with longitudinal monitoring	No	Yes

501 **Table 2 – Examples of CTCs isolation, enrichment and identification strategies**

Category	Principle	Technique / Platform	Ref.
Biological Properties	Surface marker expression	$EpCAM^+$ Enrichment / CellSearch®	[36]
		$EpCAM^+$ + other surface markers	[113]
		$CD45^+$ Depletion	[114]
		GmbH Cell Collector (*in vivo* capture)	[117]
	Surface marker expression - Microfluidics	CTC-Chip	[115]
		NanoVelcro	[116]
Physical Properties	Size - Filtration	ISET®	[118]
		Metacell®	[119]
	Size - Microfluidics	Parsortix™	[120]
		Spiral inertial microfluidic chip	[122]
	Density	Differential Centrifugation	[123]
		MagDense	[124]
	Dielectric Properties	DEP	[125]
		DEP-LFFF	[126]
		DEPArray	[127]
Functional Assays	CAM digestion	Vita-Assay™	[128]

	Protein Release during Culture	EPISPOT Assay	[129]
	Telomerase Expression	TelomeScan®	[130]

502

503 **Table 3 – Limitations of Liquid Biopsies**

Liquid Biopsy Component	Limitations	Solutions	Ref.
Circulating Tumor DNA (ctDNA)	Low Sensitivity of mutation detection (when MAF is low)	Unique Molecular Identifiers Nuclease digestion of non-mutated DNA Sampling of alternative body fluids	[26] [27] [28]
	Low predictive value of single / small sets of mutations	Analyze large mutations sets and / or associate mutations with other classes of biomarkers (e.g. proteins)	[29]
	Lack of standardized pre-analytical handling protocols, sample degradation,poor reproducibility	Layout of standardization guidelines Automated purification / analysis chips minimizing sample handling	[30] [5]
Circulating Tumor Cells (CTCs)	Poor efficiency of isolation from blood because of marker rarity, fragility, physical and phenotypic heterogeneity	Combined use of different methodologies for enrichment / isolation (e.g. CellSearch + Parsortix)	[121]
Extracellular Vesicles (EV)	High variability between isolation techniques – lack of standardized protocols	Comprehensive standardization guidelines (e.g. MISEV 2018) Automated purification / analysis chips (e.g. ACE Chips)	[54] [59]
	Lack of single-EV protein expression analysis techniques (especially for exosomes)	Development of high-resolution flow cytometers Fluorescence-based Nanoparticle Tracking Analysis	[6] [65]

Circulating Tumor RNAs (ctRNAs)	Pre-analytical handling variability,RNA instability	Layout of sample handling standardization guidelines Automated purification / analysis chips minimizing sample handling	[85] [87]

Abbreviations used:- ACE: Alternating Current Electrokinetic; MAF: Mutant Allele Fraction; MISEV: Minimal Information for Studies of Extracellular Vesicles, ~~ACE:~~

GLOSSARY:

~~**Allele**: variant form of a given gene~~

BEAMing; Beads, Emulsion, Amplification, Magnetics, highly sensitive dPCR method which combines emulsion PCR and flow cytometry to identify and quantify DNA mutations

CA 19-9~~,~~: Cancer Antigen 19-9. It is a portion of the Sialyl-Lewis A antigen. Its presence is highly correlated with advanced epithelial cancers.

Clonal hematopoiesis, the condition in which a substantial proportion of mature blood cells is derived from a single dominant hematopoietic stem cell lineage. Clonal hematopoiesis has been linked to a greater than 10-fold increased risk of developing a ~~hmtological cancer~~hematological cancer

CAPP-Seq; Cancer Personalized Profiling by deep Sequencing, a sensitive method of analysis consisting of the sequencing of cancer-specific (personalized) panels of genes to identify cancer-specific mutations

Companion diagnostic~~:~~, medical device, often an *in vitro* test, providing information that is essential and required for the safe and effective use of a corresponding drug. It is often developed simultaneously with the corresponding drug. ~~-~~ The cobas® EGFR Mutation Test v2 described in the text, for example, consists of a PCR-based analysis of a set of mutations, insertions and deletions on the EGFR gene and is used to inform on the use of erlotinib and osimertinib in NSCLC

DAPI~~:~~, 4',6-diamidino-2-phenylindole. ~~It is a~~A fluorescent dye that binds to AT-rich regions on DNA and is used to stain nuclei.

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DNA, without the need of a calibration curve with samples of known quantities. In dPCR, the initial sample mix (which is prepared like a common qPCR) is split into several individual wells before the amplification step. Following PCR amplification, the absolute quantification of the target is calculated using Poisson statistics, based on the number of positive and negative wells for the target sequence.

ddPCR; Droplet Digital PCR, a variation of dPCR in which the sample is partitioned in a large number of tiny water-oil emulsion droplets, containing on average one fragment of starting material each, before the analysis. The partition of the sample in small droplets in emulsion has the advantage, compared to dPCR, to increase the number of partitions analyzed and, therefore, the resolution of the analysis

EMT, Epithelial-to-Mesenchymal Transition: a process in which epithelial cells lose their polarization and adhesion properties, gaining migratory properties and thus differentiating in mesenchymal cells.

Genetic Heterogeneity: the presenceof different genetic clones, within the same tumor

~~**GFP**: Green Fluorescent Protein~~

Intravasation: the process by which cancer cells invade blood or lymphatic vessels through the basal membrane

~~**hTERT**: human Telomerase Reverse Transcriptase: the catalytic subunit of the Telomerase Complex~~

LDT: Laboratory-Developed Test, a type of in vitro diagnostic test that is designed, manufactured and used within a single laboratory

~~**Loci**: (plural of locus) particular positions of genes on a chromosome~~ serum concentration of a protein) in the same patient over a period of time.

~~**Methylation**: transfer of a methyl (-CH_3) group on a molecule~~

Necrosis~~:~~, traumatic cell death resulting from acute cellular injury

~~**Next-Generation Sequencing**: high-throughput DNA sequencing technologies~~

PSO; Particle-Swarm Optimization, is a population based stochastic optimization technique that shares many similarities with Genetic Algorithms.

~~**PCR**: Polymerase Chain Reaction: technique used to amplify (increase the number of copies of) a specific DNA sequence~~

Sensitivity~~:~~, ~~proportion of positive individual/samples correctly identified as positive~~in a binary classification test (positive/negative; healthy/diseased) the sensitivity measures the proportion of actual positives that are correctly identified as positive by the test. It is also called true positive rate (TPR)

Specificity: in a binary classification test, the specificity measures the proportion of actual negatives that are correctly identified as negative by the test. It is also called true negative rate (TNR)~~proportion of negative individual/samples correctly identified as negative~~

WES; Whole Exome Sequencing, a genomic technique used for sequencing all the protein-coding genes in a genome (exome)

RESOURCES

i) http://www.who.int/news-room/fact-sheets/detail/cancer

ii) https://www.cancer.gov/about-cancer/understanding/statistics

iii) https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P100033

iv) http://www.exosomedx.com/news-events/press-releases/exosome-diagnostics-announces-launch-exodxr-prostateintelliscore-completely

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860 conditionally telomerase-selective replicating adenovirus in non-small cell lung cancer patients.
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Figure Legend:

Figure 1, Key Figure– Components of the Tumor Circulome

The Tumor Circulome comprises all the tumor-derived elements circulating in the bloodstream that can be used, directly or indirectly, as a source of cancer biomarkers. It includes circulating tumor proteins~~, TEPs~~, ctDNA~~, tumor-derived EVs~~, CTCs, tumor-derived EVs and their constituents, ~~and~~ ctRNAs and TEPs. Each of these components ~~has~~ provide one or more levels of information.

The measurement of the concentration of single proteins or panels composed of multiple tumor proteins is the current gold standard used in cancer management. ~~for relapse detection. As single proteins may not be totally informative, the use of panels of proteins is a current trend.~~ The information detectable from ctDNA include~~s~~ mutations, deletions, gene amplifications, methylation patterns and translocations. CTCs provide a rich source of genomic, proteomic, transcriptomic and cytogenetic information and can be cultured *ex vivo* to perform personalized drug sensitivity testing The ~~The *ex vivo* culture of CTCs allows clinicians to perform personalized drug sensitivity tests to help in the treatment decision-making process.~~ constituents of Extracellular Vesicles (EV) provides a "molecular fingerprint" of the tumor cells of origin, and their DNA~~-~~, RNA and protein (both surface and intraluminal) content provides a rich source of cancer biomarkers. ctRNA, including EV-associated circulating RNA, includes different RNA classes. Among these, miRNA expression panels and lncRNA expression are good sources of quantitative biomarker information. ~~Furthermore,~~ ~~q~~Qualitative information such as the presence of tumor-specific ~~alternatively spliced transcripts and gene fusion transcripts~~alternative splicing and gene fusions can also be obtained from this source. The platelets of cancer patients or tumor educated platelets (TEPs)

Commented [MK(58]: I would suggest modifying this sentence and the Figure such that the authors start with ctDNA, CTCs, EVs, ctRNAs, TEPs and then tumor proteins.

The logic is that the Figure and consequently its legend follow the description in main text. While a small and more of a stylistic issue, I believe this has the potential to increase the ease of readability of the manuscript and would encourage the authors to incorporate this.

Commented [GDR59R58]: Done. We modified the sentence and the Figure showing the different components in the same order in which they're described in the text

Commented [MB60]: Are you measuring concentrations?

Commented [GDR61R60]: yes

Commented [MK(62]: This is the information on tumor proteins I earlier mentioned. Would it be right to make a separate section for this in the main text and elucidate a bit?

This can then be shortened here.

Commented [GDR63R62]: Done. We reduced this sentence and added a small paragraphs on circulating tumor proteins. Please see comment on the TEP chapter for more detailed explanation

Commented [MK(64]: Please recheck…the Figure only says luminal?

Commented [GDR65R64]: The Figure already said "Surface and luminal proteins". We changed "luminal" with "intraluminal", which is more accurate

Commented [MK(66]: Please involve this information in the main text too where I suggest more correlation of the main text with the Figure.

Commented [GDR67R66]: It is already included in the main text

Commented [MK(68]: This would be incorporated in the main text and shortened here.

Commented [GDR69R68]: It was already in the main text. We shortened the sentence a bit

886 are also source of biomarker information, containing tumor-derived RNAs~~, generating~~
887 ~~specific platelet cancer signatures,~~ and tumor-induced alternatively spliced transcripts.
888 Abbreviations used- CTC: Circulating Tumor Cells; ctDNA: circulating tumor DNA; ctRNA:
889 circulating tumor RNA; EV: Extracellular vesicle; lncRNA: long non-coding RNA; miRNA:
890 microRNA; mRNA: messenger RNA; nc-RNA: non-coding RNA; TEP: tumor-educated platelet.
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