Liquid biopsies in personalised medicine: future and pitfalls

Historically, the analysis of tumour tissue has been the primary mean through which cancers have been diagnosed, characterised, and subclassified. Nevertheless, the increasing importance of personalised cancer medicine is creating a heightened need for sufficient and recent high-quality diagnostic material, which often must be obtained through surgical biopsy procedures. Given the associated costs, risks, and logistical hurdles, recent technological advancements in the use and analysis of liquid biopsies present an attractive opportunity for minimally invasive genomic diagnostics and tumour monitoring. In this whitepaper, the potential applications of liquid biopsies in personalised medicine and its current pitfalls are described.

Introduction

Liquid biopsy refers to the sampling and analysis of body fluids such as blood, urine, and cerebrospinal fluid. Due to its limited invasiveness, liquid biopsy analysis allows for the obtention of a large amount of information at multiple timepoints, letting medical practitioners monitor tumour behaviour and molecular landscape across timescales much longer than classical biopsies. In addition, this technology can capture the heterogeneity of a tumour by providing a composite of molecular alterations across distinct tumour-cell subpopulations. This is a major advantage compared to tissue biopsies that only offer a snapshot of a particular section of a tumour at a given timepoint, albeit at higher resolution.

The main analytes present in a liquid biopsy sample are circulating cell-free nucleic acids (cfDNA and cfRNA), circulating tumour cells (CTCs), extracellular vesicles, and tumour-educated platelets (TEPs) (**Figure 1**). Interestingly, part of the circulating nucleic acids in cancer patients is tumour-derived: **the circulating tumour DNA (ctDNA) and the circulating tumour RNA (ctRNA)**, which can provide information about the tumour's genome, transcriptome, and epigenome allowing the assessment of cancer-associated alterations and of the cancer subtype. **CTCs**, on the other hand, can reveal the morphology and metabolism of tumour cells, while extracellular vesicles such as **exosomes** can provide insights into the communication between cancer cells and the tumour microenvironment. In addition, both CTCs and exosomes can be used for (epi)genome and transcriptome analysis. Finally, **TEPs** are blood platelets that have absorbed tumour-associated biomolecules, including RNA, resulting in altered RNA profiles. Therefore, TEPs provide potential interesting signatures regarding cancer status.

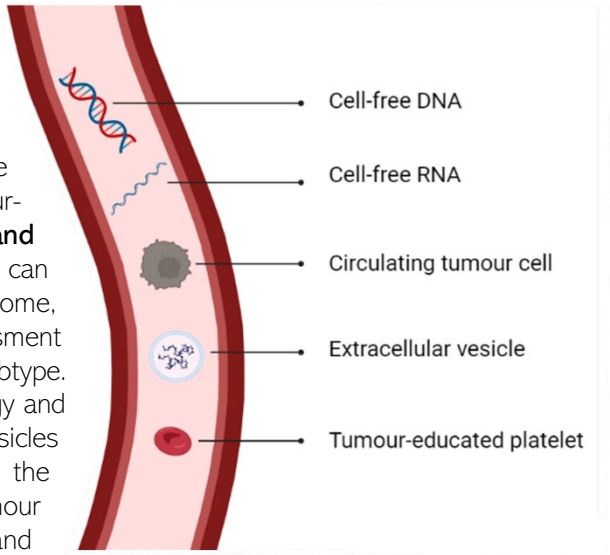

Figure 1. Main analytes present in a liquid biopsy

Liquid biopsy applications

The benefits associated with liquid biopsies open the door to many clinical applications, with a potential role throughout all disease stages (**Figure 2**). Despite some challenges that are still ahead, these applications render liquid biopsies as a very useful tool in the field of (personalised) immunotherapy.

- Cancer screening and diagnosis

Due to the limited invasiveness of liquid biopsy sampling, its implementation as a screening methodology seems attractive. This potential was demonstrated by a validation study using a multi-cancer early detection test based on cfDNA analysis, which reached a very high specificity[1]. Nevertheless, the low levels of tumour-related analytes associated with early stage cancer and the relatively low incidence of cancer in the general population remain an important challenge for screening[2]. Moreover, similar to current screening

approaches, the risk of false-positive results and of overdiagnosis – due to the detection of indolent and non-life threatening cancers – has obvious implications for healthcare resources as well as for patient well-being[3]. Therefore, well-defined recommendations on how to implement liquid biopsies as cancer screening strategy in a clinical workflow are required. For example, the US Preventive Service Task Force has already defined a set of recommendations for prostate cancer screening through serum PSA testing[4].

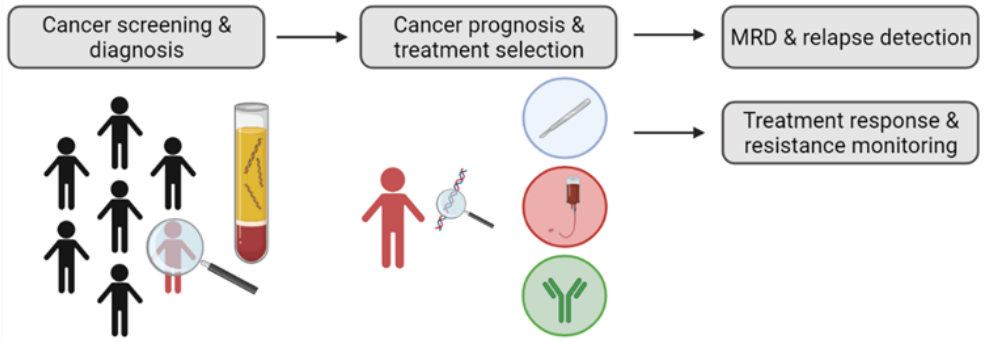

Figure 2. Clinical applications of liquid biopsies (MRD, minimal residual disease)

Furthermore, liquid biopsy analysis could play a role in cancer diagnosis, including in **cancer classification** by molecular analysis of cancer-associated analytes[5]. Especially in cases where a tissue biopsy cannot be performed due to localization of the tumour or physical condition of the patient, or where the tissue biopsy was inconclusive.

- Cancer prognosis and treatment selection

When a patient is diagnosed with cancer, the molecular profiling of the tumour is key for cancer prognosis and treatment selection. Molecular profiling is still mainly performed on tumour tissue, despite the risks associated with obtaining these tissue biopsies. Because of technical advances, it now becomes possible to also perform these assays in liquid biopsies, which could complement or even replace tissue analysis. Especially in patients without accessible lesions for tissue biopsy, ctDNA in the blood for example could serve as an alternative source of genetic material for variant identification. Therefore, ctDNA genotyping is due to its **limited invasiveness** an attractive alternative to tumour tissue profiling. This allows for example for frequent blood sampling & analysis, and could thus be considered before every treatment switch.

- Minimal residual disease and early relapse detection

Most common first-line treatments for early-stage cancer are local therapies, such as surgery and radiation therapy, that aim at completely curing the patient. In this context, liquid biopsy analysis could provide a useful strategy for monitoring minimal residual disease (MRD), indicating the **presence of residual cancer cells after treatment**, and thereby identifying patients that could benefit from adjuvant therapy[6]. Indeed, studies in different cancer types showed that ctDNA and CTC analysis during follow-up were able to assess MRD and predict disease relapse with a high sensitivity and specificity several months before disease recurrence could be observed through radiological assessment[6,7]. Data from multiple studies demonstrated that the median time from ctDNA detection to radiological detection of relapse was up to 11 months, thereby highlighting the potential of ctDNA analysis for early relapse detection[2].

- Treatment response and resistance monitoring

Assessing the efficacy of therapy can be challenging, especially for immunotherapies where pseudoprogression, an initial increase in total tumour size owing to tumour immune infiltration subsequently followed by tumour shrinkage, has been observed. As a result, it might be difficult to assess the response to immunotherapy using conventional imaging techniques. To address this problem, **monitoring tumour-associated components in liquid biopsies for the assessment of tumour responsiveness to therapy** might offer an intriguing alternative. Indeed, recent studies have reported that

for example ctDNA levels in the blood can be used as a biomarker for treatment response[7]. It is important, however, to take into consideration the time of sampling as therapies can cause a transient increase due to release of tumour components from dying cancer cells, which could give false positive results[8]. In most responders, ctDNA levels are undetectable or have decreased significantly at 3-4 weeks after first treatment administration, and therefore, the variation in ctDNA levels at this timepoint seems to be the most clinically relevant parameter, although this also depends on the treatment[7,8].

Besides treatment response, **long-term monitoring** using liquid biopsies could also allow identification and further characterisation of therapy resistance. For therapies where resistance mechanisms are well described, assays targeting specific alterations could be implemented, provided that a 'rescue' therapy tailored to overcome the resistance mechanism is available. For unknown resistance mechanisms, large gene panels could be used to elucidate new mechanisms of resistance[2].

ctDNA analysis

Research into liquid biopsies has mostly focused on the analysis of cfDNA which is a mixture of normal circulating DNA from healthy cells and, more interestingly, tumour-derived DNA, i.e. the ctDNA. This ctDNA is released from cancer cells as a result of apoptosis, necrosis, and/or active secretion which results typically in short DNA fragments (average length of 120–160 bp)[7]. Due to the limited half-life of cfDNA (between 16 minutes and 2.5 hours), ctDNA analysis can be considered as **a real-time snapshot of the cancer's genomic profile**[5]. Two main goals of ctDNA analysis can be distinguished being (1) molecular characterisation of the tumour, and (2) estimation of the amount of ctDNA, i.e. the ctDNA level, as a marker for tumour burden.

- Targeted and genome-wide mutation analysis of cfDNA

A variety of techniques are available for the molecular analysis of cfDNA, ranging in scale from single base pair to whole-genome analyses. Targeted approaches are designed to evaluate one or multiple alterations using mostly droplet digital PCR (ddPCR) or a targeted next-generation sequencing (NGS) approach. **ddPCR** can analyse one to several patient-specific or common alterations. Targeted **NGS**, using a PCR amplicon or hybrid capture strategy, can target both large predefined and personalised gene panels. For example, the MSK-ACCESS cfDNA assay is FDA approved as a tumour profiling test to provide information on somatic mutations and microsatellite instability (MSI) by targeting 129 key cancer-related genes (e.g. *EGFR*, *KRAS*, etc.). Using this information, the tumour can be characterised and an appropriate treatment strategy can be selected. Indeed, the feasibility of applying cfDNA assays in the clinical setting and the ability of such assays to identify common mutations that can guide the use of molecularly targeted therapies has been well demonstrated[2]. For personalised cfDNA analysis, on the other hand, prior tumour-versus-normal sequencing (targeted, exome (WES) or genome (WGS)) is required to identify a set of somatic mutations to design the personalised gene panel. These **personalised assays targeting patient-specific alterations** could then be used for example as a complementary follow-up strategy for MRD detection. Next to characterisation of the tumour, molecular profiling also allows estimation of the ctDNA level which is associated with tumour burden and is therefore useful for treatment monitoring.

Apart from targeted approaches, it is also possible to perform a genome-wide analysis of ctDNA using **WES or WGS**. These genome-wide approaches allow the investigation of the complete genome, and therefore, enable *de novo* mutation detection. Nevertheless, a major pitfall of this approach is the limit of detection of mutations with low variant allele frequencies (VAFs) and its limited sensitivity in case of low ctDNA levels. In addition, the aforementioned sequencing approaches as well as shallow WGS allow detection of copy number alterations that can aid in characterisation of the tumour and estimation of ctDNA level[5,9].

- CfDNA methylation analysis

Next to detection of alterations in the DNA sequence, ctDNA analysis also allows detection of DNA methylation alterations, an important cancer hallmark. DNA methylation analysis is mostly based on bisulfite

conversion followed by ddPCR, targeted sequencing, or genome-wide sequencing. DNA methylation analysis could also play a role in screening, characterisation of the tumour, MRD detection, and treatment monitoring[10].

- CfDNA fragmentation analysis

In addition to the mutation and methylation analysis of ctDNA, the analysis of the cfDNA "fragmentome" represents a novel genome-wide approach for cancer detection. Indeed, Cristiano *et al.* have shown that cfDNA fragmentation patterns are altered in cancer patients, potentially reflecting changes in chromatin structure as well as other genomic and epigenomic abnormalities[11]. Using WGS of cfDNA of a set of cancer patients and healthy individuals, they developed a machine learning model that was able to detect cancer patients with a high sensitivity and specificity over seven cancer types. Moreover, using their model the fragmentation profiles could be used to identify the tissue of origin of the cancers. Combining this approach with mutation-based cfDNA analyses could therefore increase the sensitivity of cfDNA screening.

- Challenges

Evaluating a tumour's genomic profile via ctDNA analysis also represents some challenges. First of all, the variable level of ctDNA in the blood is highly dependent on the cancer indication, stage, tumour burden, as well as the level of proliferation, apoptosis, and vascularisation of the tumour. Especially in early stage cancers, low ctDNA levels are observed[12]. Low ctDNA levels might lead to false negative results when the limit of detection is not reached, decreasing the sensitivity of cfDNA analysis for screening. Therefore, **if a defined mutation is not detected via cfDNA analysis, it does not implicate the absence of the mutation in the associated tumour**. This is especially important for mutations with lower variant allele frequencies, which need even higher ctDNA levels to be detected. Therefore, very sensitive techniques are required for cfDNA analysis.

On the other hand, there is a risk of detecting mutations that are not actually present in the cancer cells, but that are rather attributable to clonal haematopoiesis. The latter phenomenon is associated with aging and is characterised by clonal populations of myeloid cells in the bone marrow or blood that harbour an acquired mutation, often in leukaemia-associated driver genes and other genes such as *JAK2*, *TP53* and *TET2*[13]. Correction for these mutations is possible by sequencing DNA extracted from peripheral blood cells.

An additional challenge in the liquid biopsy field is the lack of standardisation regarding pre-analytical and analytical conditions, which makes retrospective cross-cohort analyses extremely challenging due to their effects on sensitivity. However, the establishment of consortia like the 'International Liquid Biopsy Society' and the 'International Liquid Biopsy Standardisation Alliance' are expected to lead to protocol standardisation and, together with additional large-scale validation studies, to further pave the way for clinical implementation of liquid biopsies.

Going beyond ctDNA

As described earlier several other interesting analytes are present in liquid biopsies, each associated with their own advantages and challenges. **CTCs** are actual tumour cells and can therefore provide both phenotypic and molecular information. In addition, CTC counts have been associated with prognosis and could play a role in disease monitoring. Challenges associated with CTC analysis include diversity in CTC extraction and characterisation methods as well as the low number of CTCs present[14]. **Exosomes** are extracellular vesicles that play a crucial role in intercellular communication and can contain nucleic acids, proteins, and lipids. Hence, exosomes released by living cancer cells contain a lot of valuable information and might have applications in diagnosis, treatment monitoring, and prognosis. Due to their lipid bilayers exosomes are very stable. The biggest challenge remains the isolation of exosomes, which is not standardised and has difficulties in reaching high efficiency and purity. Furthermore, tumour-derived exosomes only represent a small part of all the exosomes, requiring very sensitive analytical techniques[15].

It should also be mentioned that the expression profile of cancer cells is drastically altered, which can be evaluated in liquid biopsies as well. RNA profiling in liquid biopsies and especially the expression of non-coding RNA, such as microRNA, circular RNA, and long non-coding RNA, has been extensively studied for its biomarker potential[16,17]. Besides cfRNA, which is generally less stable, other analytes including exosomes, CTCs, and TEPs can also be used as a source of tumour-derived RNA.

Conclusion

It can be concluded that liquid biopsies offer a wide range of potential clinical applications that could benefit patients' health and provide important advantages over tissue biopsies, including minimal invasiveness. Which analyte will play the most important role, remains to be determined, although the current increase in commercially developed ctDNA panels gives a strong edge to the implementation of ctDNA analysis. Nevertheless, an approach where different analytes would complement each other could also be envisioned. Whilst some challenges are still lying ahead, the implementation of liquid biopsies in a clinical setting could go hand in hand with a further adaptation of personalised therapy.

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About myNEO

myNEO (Ghent, Belgium) developed a platform enabling genomic-informed drug discovery in the key therapeutic areas of oncology and immunology. The data-driven ImmunoEngine identifies the most efficacious targets (epitopes) for each cancer patient, uniquely presented on the tumour cells and capable of redirecting a patient's immune system, leading to elimination of the cancer cells. The discovery platform enables targets to be identified even in hard-to-treat tumours with a cold/lowly mutated profile. Similarly, the company has applied its technology to identify immunogenic sequences in infectious diseases, capable of protecting populations with strong broad immune responses. myNEO is one of the companies that emerged from the Novalis biotech incubator fund at the end of 2018, founded by two leading entrepreneurs already known for several successes in the biotech industry: Wim Van Criekinge, professor of computational biology at Ghent University, and childhood friend Jan Van den Berghe.

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