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Front Line Genomics

Liquid Biopsy:

Current Status and Future Directions

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** Contribution made by approved comments via webinar: Liquid Biopsies in a clinical setting, 15th October, available here.*

FOREWORD

A cancer diagnosis and treatment journey is an overwhelmingly painful and traumatising experience for patients. Global researchers have been uniting on the mission to improve our ability to diagnose and treat even the most aggressive cancers.

The gold standard method of diagnosis is often imaging-based, followed by a tissue biopsy to understand the pathology of the tumour and undergo molecular testing. However, when a tumour is in a difficult-to-reach place, or it has significantly shrunk in response to treatment, collecting a sample for analysis can be extremely challenging.

Over a decade ago, Klaus Pantel and Catherine Alix-Panabieres introduced the concept of a "liquid biopsy" - the ability to analyse tumour cells and tumour-derived products in the blood and other bodily fluids[1]. Since then, this definition has been extended to include non-tumour cells such as circulating immune cells or endothelial cells, and also products derived from host cells. The vision is to get more comprehensive and real-time information on the tumour burden via the analysis of blood samples and other bodily fluids.

Liquid biopsies hold a lot of promise for the field of oncology, easing patient diagnosis and monitoring. These benefits can be both from the patient perspective (being non-invasive), but also from an economic standpoint, enabling less-trained clinicians to test patients locally, rather than at a specialist centre.

A few decades ago, the idea of a liquid biopsy seemed too good to be true. Fast forward to 2020 and liquid biopsies seem to be just around the corner, ready to transform cancer care forever. But, as with all new and amazing technologies, there are challenges preventing adoption.

This report will cover the applications and promise of liquid biopsies, their current status of development and translation, and future directions.

We would like to thank our contributors and advisors for their input in helping to put this report together, and - as with all the reports we produce - we hope you find this report useful.

Diana Georgi, Front Line Genomics

References:

1. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med. 2010;16(9):398-406. doi:10.1016/j.molmed.2010.07.001

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This chapter introduces the basic biology of cancer that enables liquid biopsy technologies to be on the cusp of transforming cancer care.

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As with all new technologies, there are challenges preventing initial adoption – with both scientific and social barriers to adoption, this chapter highlights the challenges and work being done to enable clinical adoption of liquid biopsies.

- **Technical challenges and (Pre)analytics**
  This part of the chapter will focus on the pre-analytics and technical challenges of liquid biopsy.
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  This part of the chapter focuses on the scientific and social barriers to adoption.

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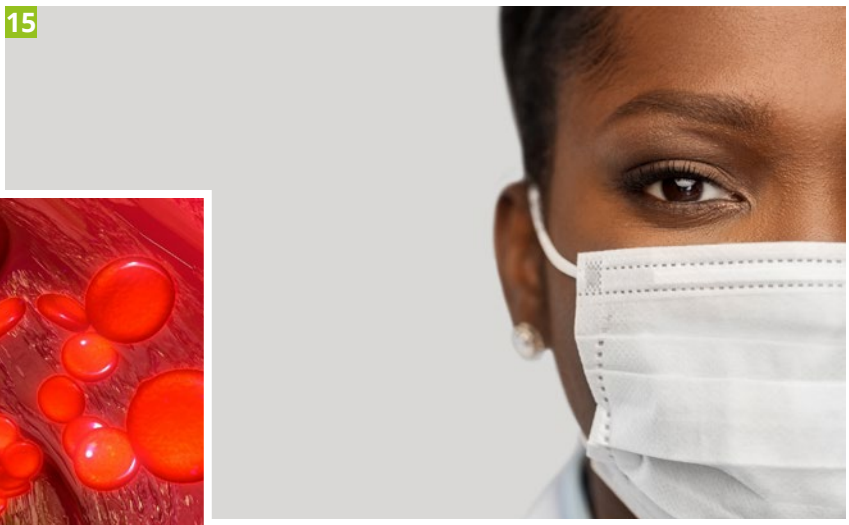

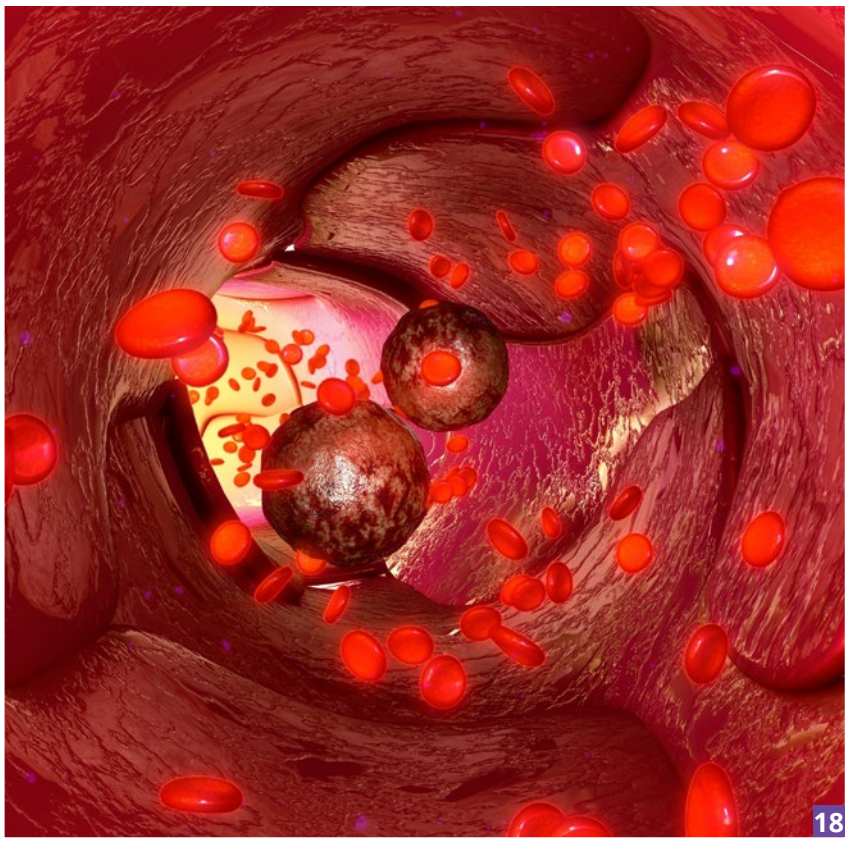

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CHAPTER 1

INTRODUCTION AND BIOLOGY

IN 2015, A STATISTIC PUBLISHED IN THE BRITISH JOURNAL OF CANCER SHOCKED THE WORLD: THAT ONE IN TWO OF US WILL GET CANCER IN OUR LIFETIME. WHILE CANCER IS BECOMING MORE PREVALENT, RESEARCHERS ARE INCREASINGLY LOOKING FOR WAYS TO IMPROVE OUR ABILITY TO DETECT, DIAGNOSE AND MONITOR CANCER TO ACHIEVE BETTER PATIENT OUTCOMES. IT IS ALSO WIDELY AGREED THAT AN UNDERSTANDING OF THE PATHOGENESIS OF CANCER CAN INFLUENCE BETTER TREATMENT DECISIONS AND PATIENT PROGNOSIS.

Recently, a lot of attention has been given to liquid biopsies – non-invasive tests that can analyse circulating biomarkers from tumours in bodily fluids.

DEFINITION

Defined by the National Cancer Institute as: "A test done on a sample of blood to look for cancer cells from a tumour that are circulating in the blood or for pieces of DNA from tumour cells that are in the blood. A liquid biopsy may be used to help find cancer at an early stage. It may also be used to help plan treatment or to find out how well treatment is working or if cancer has come back. Being able to take multiple samples of blood over time may also help doctors understand what kind of molecular changes are taking place in a tumour"[1].

While this definition refers only to diagnosis via blood, the term liquid biopsy is being used to refer to a non-invasive diagnostic test carried out on a variety of bodily fluids, including urine, saliva, or even cerebrospinal fluid.

This report focuses mainly on the applications of liquid biopsies via a blood draw, but will also touch upon some of the ongoing research to search for tumour material in other fluids.

CANCER BIOLOGY – THE COMMON ANALYTES

Before we address the potential of liquid biopsies, we must understand the biology of cancer and how this enables liquid biopsies to monitor the molecular information of distant tumours from a simple blood draw. The two main analytes liquid biopsies study are circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) – the biology of which is addressed here:

One of the hallmarks of cancer is the ability to metastasise and distally spread. CTCs are intact tumour cells which have broken off from the primary tumour and have entered the blood.

Cell-free DNA (cfDNA) is fragmented DNA comprising of both germline DNA and – potentially – ctDNA. ctDNA is detectable DNA that is thought to be released following tumour cell apoptosis and/or necrosis[2].

While CTCS and ctDNA remain the most common analytes, researchers are also starting to examine exosomes and RNA as biomarkers that can be studied. There are several isolation methods for exosomes, all of which have unique advantages and disadvantages, but there is no current universal standard for exosome extraction, and the clinical utility of nucleic acids from exosomes is still in its infancy[3].

CTCs are thought to stem from the biological nature of cancers to metastasise, and normal cellular apoptosis of tumour cells enables the detection of cfDNA, which can include ctDNA. Thus, with the right technologies in place, the biological characteristics of cancer enable detection via liquid biopsies.

However, analysing tumour material from a blood draw is likened to finding a needle in a haystack, and the analytes themselves can be challenging to work with.

In the case of cfDNA, it is present in plasma, urine and other bodily fluids, typically in low concentrations and short fragments. This presents a challenge, as a 1ml blood sample only contains 1-50ng of cfDNA, of which only 0.01-0.1% is expected to be tumour ctDNA. Therefore, highly efficient extraction reagents and process automation are critical requirements for studying cfDNA in liquid biopsies.

Here, we present a case study from Beckman Coulter on a new scalable and automatable method for extracting cfDNA for research applications:

CASE STUDY: A NEW SCALABLE AND AUTOMATABLE METHOD FOR THE EXTRACTION OF CFDNA

LAUREN P. SAUNDERS, ANTONIA HUR, BRITTANY NICCUM, AND ASMITA PATEL • BECKMAN COULTER LIFE SCIENCES

INTRODUCTION

Liquid biopsies represent a promising area of cancer testing as taking blood is less invasive than tumor biopsies. The cell free DNA (cfDNA) present in the blood includes DNA derived from cancer cells and cancer biomarkers can be detected in the extracted cfDNA. Whole blood also contains genomic DNA, and can be removed by centrifugation, resulting in plasma. cfDNA is present in very small amounts in blood or plasma, and thus larger amounts of plasma are required for many applications. Larger extractions are more challenging to automate, as they require additional pipetting steps. Here we present a novel cfDNA extraction kit and show its compatibility with extractions from 200 µl – 5 mL. We discuss the optimisation of the method and demonstrate automation on a KingFisher Duo. This workflow can also be automated on a Biomek i7 Automated Workstation. We demonstrate that this kit can be used for NGS and produce results comparable to other commercial kits.

SCALABLE CHEMISTRY WITH INPUTS FROM 200 ML TO 5 ML

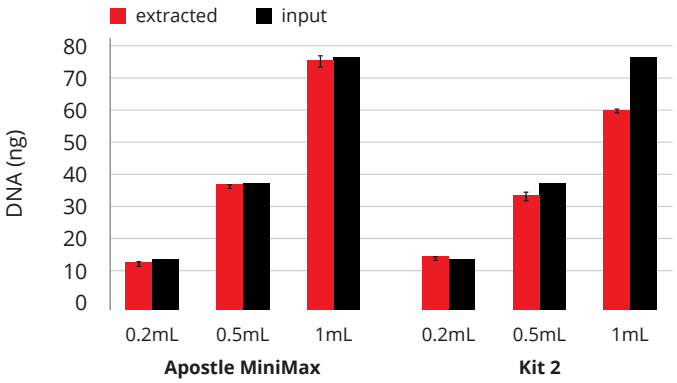


Figure 1. *DNA isolation from varying plasma amounts. Nuceleosomes were spiked into plasma to ensure enough DNA was present for QC. The Apostle MiniMax kit isolated nearly all the input DNA, while Kit 2 isolated less from larger plasma volumes.*

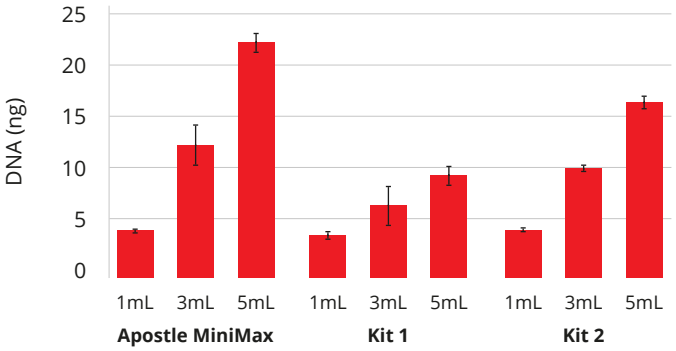
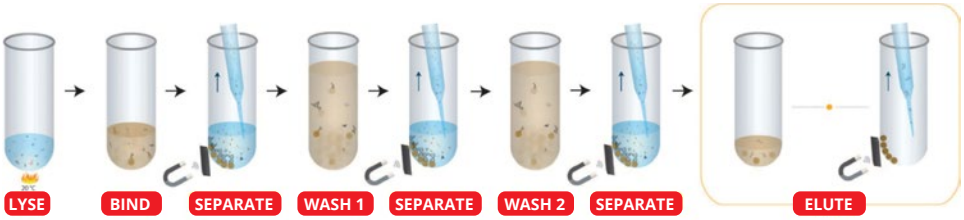


Figure 2. *Isolation of DNA from 1 – 5 mL of EDTA plasma. Yields were highest with the Apostle MiniMax kit for 3-5 mL, and yields were similar across all kits at 1 mL.*

APOSTLE MINIMAX WORKFLOW

Figure 3. *Apostle MiniMax Workflow. The Apostle MiniMax kit involves a lysis step, then the addition of magnetic beads to bind the DNA. Once the DNA is bound, it is washed with various wash buffers and finally eluted from the beads.*



REMOVAL OF PCR INHIBITORS AND GDNA

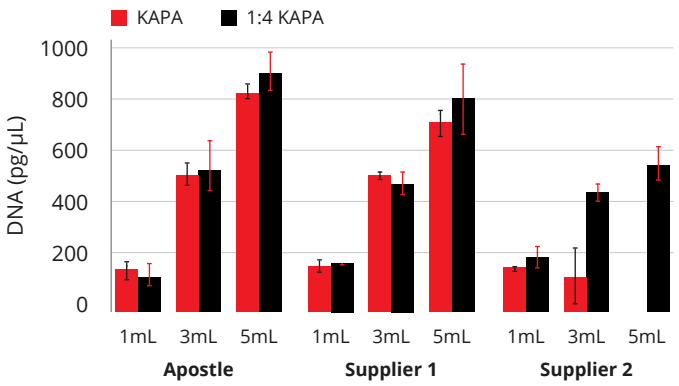


Figure 4. *Comparison of PCR inhibition. The p41 primers and the KAPA hgDNA Quantification and QC kit was used to estimate [DNA]. Undiluted samples were compared to samples diluted 1:4 to measure the effect of PCR inhibitors. If PCR inhibitors are present, the estimated concentration will be higher in more dilute samples. Similar concentrations estimated from the 1:1 and the 1:4 dilution KAPA is a sign of low inhibition. As such, Apostle MiniMax and Kit 1 have low inhibition and significant PCR inhibition is seen in Kit 2.*

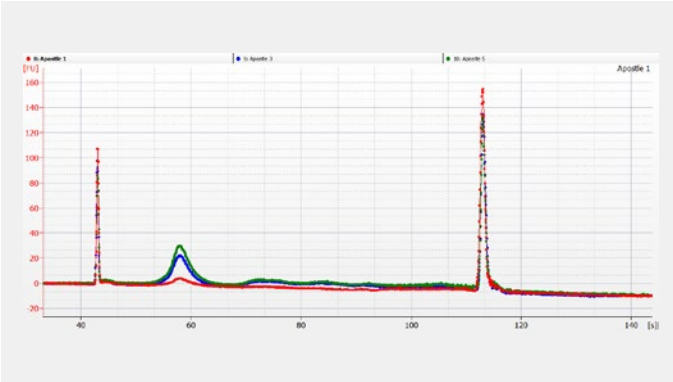


Figure 5. *Increasing input amounts yield increasing amounts of cfDNA. Bioanalyzer traces show that the increase in DNA yield is due to increasing amounts of a small DNA peak. No contaminating genomic DNA was seen. The high peaks at the beginning and end of the trace are high and low markers.*

PROTOCOL OPTIMIZATION

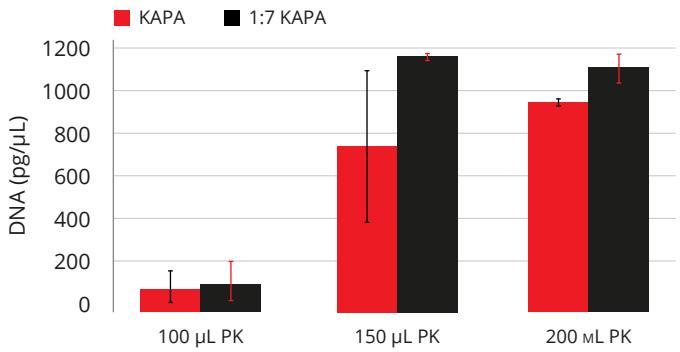


Figure 6. *Optimization of Proteinase K. Changes in Proteinase K concentration have significant effects on final [DNA]. Increasing the amount of Proteinase K to 150 µL results in significantly more yield in EDTA plasma tubes.*

AUTOMATION ON KINGFISHER DUO

The extraction was automated on the KingFisher Duo instrument after the addition of the binding/ nanoparticle solution. The automated portion of the extraction was 37 min.

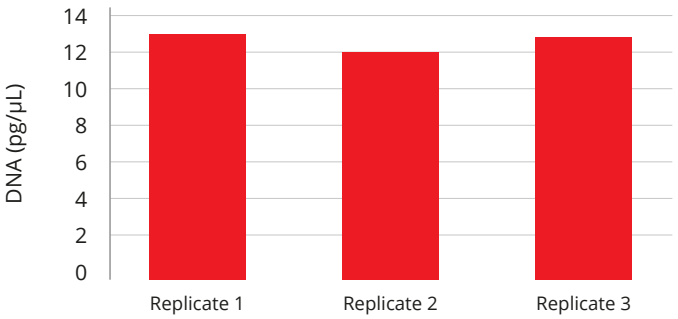


Figure 7. *cfDNA was extracted from 1 mL of plasma in concentrations consistent with manual extractions. Little variability was seen between the replicates (standard deviation of 1.0).*

AUTOMATION ON THE BIOMEK I7 AUTOMATED WORKSTATION

The Apostle MiniMax kit is compatible with automation on Beckman Coulter's Biomek i7 Automated Workstation instrument with integrated KingFisher Presto. While the method can be automated on the Biomek i7 Automated Workstation alone, the run time is prohibitively long (4.5 hr). With the KingFisher integration, run time is expected to be 2.5 hr.

Sample Name	Percent Aligned Reads	Read Enrichment	Uniformity of Coverage (Pct > 0.2*mean)	Target Coverage at 1X	Target Coverage at 20X
EDTA donor 1 Apostle MiniMax	99.80%	70.10%	98.40%	100.00%	99.90%
EDTA donor 2 Apostle MiniMax	99.80%	70.80%	97.90%	99.90%	99.80%
EDTA donor 1 Kit 1	99.70%	67.90%	97.80%	100.00%	99.90%
EDTA donor 2 Kit 1	99.80%	65.10%	95.70%	99.90%	99.70%
cfDNA Tube 1 donor 1 Apostle MiniMax	99.80%	72.10%	98.60%	100.00%	99.90%
cfDNA Tube 1 donor 2 Apostle MiniMax	99.80%	71.90%	98.50%	100.00%	99.90%
cfDNA Tube 1 donor 1 Kit 1	99.80%	71.10%	97.50%	100.00%	99.80%
cfDNA Tube 1 donor 2 Kit 1	99.80%	70.20%	95.90%	100.00%	99.90%
cfDNA Tube 2 donor 1 Apostle MiniMax	99.80%	68.10%	97.90%	100.00%	99.90%
cfDNA Tube 2 donor 2 Apostle MiniMax	99.80%	69.60%	98.40%	100.00%	99.90%
cfDNA Tube 2 donor 1 Kit 1	99.80%	68.40%	96.70%	100.00%	99.90%
cfDNA Tube 2 donor 2 Kit 1	99.70%	67.20%	96.30%	100.00%	99.90%

Table 1. *Quality Control of NGS run. Libraries were prepared from 25 ng DNA with the Accel-NGS 2S Hyb DNA Library prep kit for NGS and a target capture library was prepared from that library using the IDT xGen Pan-cancer panel. Libraries were pooled and run on an Illumina NextSeq. Data was analyzed via BWA enrichment. Human genome UCSC hg19 was used as the reference genome. Quality control metrics from all runs are good and comparable between extraction methods.*

DETECTION OF CANCER MUTATIONS

Sample Name	Indels	Indel Het/Hom Ratio	SNVs	SNV Het/Hom Ratio	SNV Ts/Tv Ratio
EDTA donor 1 Apostle MiniMax 1	08	4.4	374	1.7	2.3
EDTA donor 2 Apostle MiniMax	127	4.5	453	1.9	2.8
EDTA donor 1 Kit 1	110	4.8	373	1.7	2.3
EDTA donor 2 Kit 1	126	4.3	452	1.9	2.7
cfDNA Tube 1 donor 1 Apostle MiniMax	108	3.3	398	1.7	2.6
cfDNA Tube 1 donor 2 Apostle MiniMax	99	3.1	403	1.7	2.2
cfDNA Tube 1 donor 1 Kit 1	108	3.3	401	1.7	2.5
cfDNA Tube 1 donor 2 Kit 1	99	3.3	402	1.7	2.3
cfDNA Tube 2 donor 1 Apostle MiniMax	115	4.8	410	2.1	2.2
cfDNA Tube 2 donor 2 Apostle MiniMax	112	4.3	434	2.5	2.6
cfDNA Tube 2 donor 1 Kit 1	116	4.8	414	2.1	2.2
cfDNA Tube 2 donor 2 Kit 1	113	4.4	427	2.5	2.6

Table 2. *Mutation Detection with Different Extraction Methods. The detection of indels and SNVs was similar with both extraction methods.*

Sample Name	Indels	Indel Het/Hom Ratio	SNVs	SNV Het/Hom Ratio	SNV Ts/Tv Ratio
cfDNA Tube 1 donor 2 Apostle MiniMax Run A	99	3.1	403	1.7	2.2
cfDNA Tube 1 donor 2 Apostle MiniMax Run B	101	3.4	399	1.7	2.3
cfDNA Tube 1 donor 2 Kit 1 Run A	99	3.3	402	1.7	2.3
cfDNA Tube 1 donor 2 Kit 1 Run B	104	3.5	402	1.7	2.3
cfDNA Tube 2 donor 2 Apostle MiniMax Run A	112	4.3	434	2.5	2.6
cfDNA Tube 2 donor 2 Apostle MiniMax Run B	112	4.1	432	2.5	2.7
cfDNA Tube 2 donor 2 Kit 1 Run A	113	4.4	427	2.5	2.6
cfDNA Tube 2 donor 2 Kit 1 Run B	114	4.4	431	2.5	2.7

Table 2. *Run Variation. The duplicate libraries were sequenced to determine the amount of intra-run variation. As you can see, the variation between runs A and B of the same library have equal or greater variation than the runs observed for the different extraction methods, implying that the two methods sequence equaling well with NGS.*

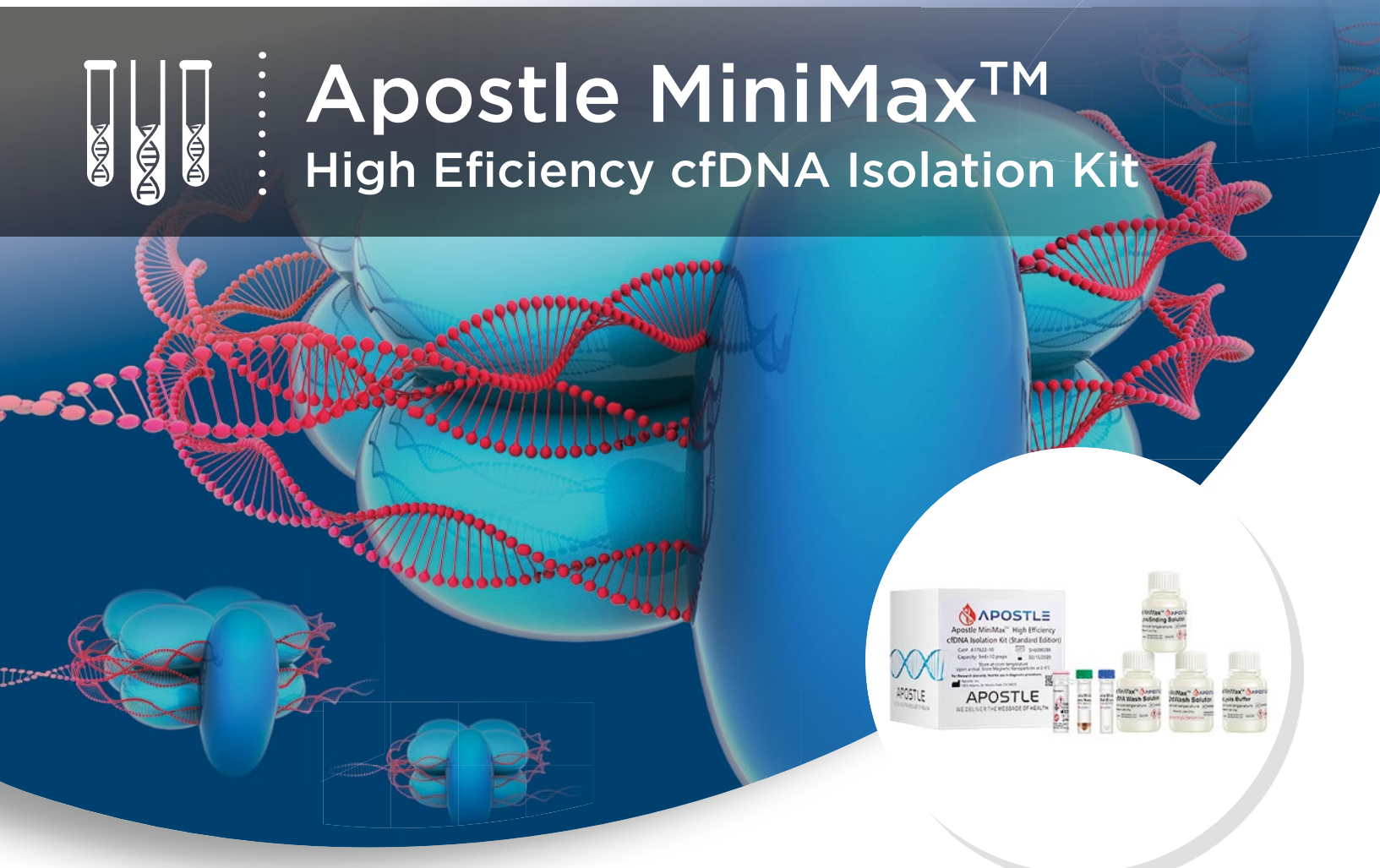
CONCLUSIONS

- DNA can be extracted from 200 µL to 5 mL of plasma
- The Apostle MiniMax kit removes the PCR inhibitors present in plasma
- Genomic contamination is not present in the extracted cfDNA
- Extraction of 1 mL plasma can be automated on a KingFisher instrument with yields similar to manual extraction
- Similar numbers of mutations were found in cancer plasma with the Apostle MiniMax kit and another commercial kit.

The Apostle MiniMax kit is a versatile new cfDNA kit that can extract from a wide range of sample amounts and be run either manually or on a variety of automation systems.

Apostle MiniMax™

High Eficiency cfDNA Isolation Kit



Sample Types

- Plasma
- Urine
- Cerebrospinal fluid
- Serum
- Saliva
- Pleural effusion

Downstream Application Compatibility

- NGS and PCR-based assays

Demonstrated Key Features

- Input volumes: 200 µL – 5 mL
- Demonstrated to outperform the leading column-based kit
- Linear increase in DNA yield with increased sample input
- Magnetic bead-based technology, scalable and automatable

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LIQUID VS TISSUE BIOPSIES

The motivation to develop liquid biopsies comes from the potential benefits to patients of utilising these diagnostic tests, plus the economic benefit over using standard tissue biopsies. Here, we will compare a few of the advantages and disadvantages of liquid biopsies, compared to tissue biopsies.

One of the most notable advantages of a liquid biopsy in comparison to traditional tissue biopsies is that the collection of tissue biopsies are invasive procedures, often requiring a surgeon or other highly trained medical professional. In addition, with a tissue biopsy, the tissue sample that is analysed may not be representative of the overall heterogeneity within the tumour, and the sample cannot be reanalysed over time. However, by monitoring cancer progression with liquid biopsies, you can achieve something closer to real-time monitoring of the disease. A liquid biopsy, in this regard, is more representative of the heterogeneity of the tumour.

From an economic standpoint, the cost of collecting a blood sample is a much cheaper procedure than tissue biopsy collection. One of the potential benefits of utilising liquid biopsies would be seen in cancer diagnoses in poorer countries, where access to medical care is limited. In this instance, a more junior technician can administer the blood draw. This sample would be analysed and could diagnose cancer, potentially at an earlier stage than would be possible via tissue biopsy. This can then be followed up with the necessary medical care.

The benefit of having a less-skilled medical professional being able to administer liquid biopsies is not only a benefit for poorer countries. The potential of this became especially apparent during the COVID-19 pandemic, where we saw a decline in the number of patients visiting hospitals. Blood testing at home could be a transformative option for high-risk patients to enable safe monitoring, and to enable clinicians to advise patients when hospital visits are necessary.

However, tissue biopsies have been the gold standard biopsy procedure for a long time and have decades of evidence proving their validity. One of the major challenges that is explored in more depth throughout this report, is that current studies to prove the clinical utility of liquid biopsies are not exemplary; they do not include many patients, and very few have international cohorts. The trials need to prove that the information obtained from a liquid biopsy is informative enough to the clinician to change the course of treatment, and ultimately lead to better patient outcomes. The next step in moving towards the clinical utility of liquid biopsies is proving that monitoring in this way can improve patient survival.

Finally, and perhaps most importantly, is the patient perspective. Tissue biopsies can be a painful and often traumatic process, which liquid biopsies would be able to remedy.

It is important to note though, that the information gathered by liquid and tissue biopsies differ, and thus it is likely that these types of testing will be used in conjunction with each other in the future.

With so many potential benefits of utilising liquid biopsies, it is easy to understand why the field has attracted some of the greatest minds to form groups and collaborations to make progress in this area. One of whom is Klaus Pantel.

In recognition of the advances the field has made since conception, we caught up with Klaus Pantel to reflect on a decade of research. ■

INTERVIEW WITH KLAUS PANTEL

WE RECENTLY HAD THE OPPORTUNITY TO SPEAK WITH DR KLAUS PANTEL.

During the decade since he and Dr Catherine Alix-Panabieres coined the term "liquid biopsy", we have seen many assays enter development, FDA approval of the first liquid biopsy, and a lot of concerted efforts to build a liquid biopsy community. Having said that, confidence in the technology that drives liquid biopsies has also been knocked during the last decade. We took the time to review how far we have come over the last decade and cast an eye on what needs to happen next.

FLG: Since you and Dr Catherine Alix-Panabieres first introduced the term "liquid biopsy" in 2010, the liquid biopsy field and community have progressed rapidly. What, in your opinion, are the biggest milestones that we have overcome since its conception?

Klaus: Well, I think one of the milestones that has been overcome but is still ongoing is assay standardisation and validation. I think that ten years ago we were impressed by each new assay that was published, but there were little efforts to independently validate the assays and there was no effort to do assay standardisation, including the pre-analytical and the analytical steps of each assay. I think we have now done quite a bit with our European Cancer-ID network, and now with the ELBS network. There are more and more publications on the standardisation of liquid biopsy assays, and now the definition of the variable guidelines are probably the necessary next steps.

The second achievement is that we've seen a lot of clinical trials on clinical validation of liquid biopsy assays in different tumour types and for different clinical applications. We have also seen that a few interventional clinical trials have been started or have been completed now, and these trials are very important to show clinical utility because, at the end of the day, the clinical collaborators want to know what the change in the management of the patient is based on the liquid biopsy assay results. So, the results must induce a change in the management, for example, of therapy of cancer patients and then you must prove that this change also leads to a better clinical outcome for the patient. There are several of those trials running now and several that have been completed over the past five years, which I think is a big step forward.

FLG: We don't have as many interventional trials being run as might be expected for liquid biopsies, given the interest in the technology. Why do you think that is?

Klaus: I think it is still very cumbersome to set up these interventional trials because you have to decide which type of assay you should use and combine it with the therapeutic intervention that has a chance to lead to a better outcome.

Let me give you one example of an interventional trial that was a failure; which involved using CTC counts to decide whether breast cancer patients needed a more intense therapy or a change in chemotherapy. The trial failed because the change in chemotherapy did not change the course of the disease of the patients, probably because the patients had very aggressive tumours and no treatment in the world can change that. So, the liquid biopsy assay was able to identify patients with non-responding tumours to chemotherapy, but there was no alternative treatment that could help the patient. When you do an interventional trial you also have to make sure that the change in your patient management has the chance to lead to a better clinical outcome.

So, in a certain way, the success of your liquid biopsy marker is coupled to the success of your clinical intervention. You can imagine that setting up these trials is much more work than just setting up an observational trial where you measure a liquid biopsy marker and then see what the outcome is based on the results of your liquid biopsy marker selection.

FLG: So, where are we now in the movement towards proving the clinical utility? We have a few trials out there, but what's needed to bring liquid biopsies into routine care?

Klaus: First of all, I think we need more interventional clinical trials. We have some new trials, such as in breast cancer, that help to select patients for either chemotherapy or endocrine therapy. We have interventional trials on the ctDNA level as well. But we need many more to convince the medical community that liquid biopsy assays have clinical utility. I think once we have these results, there is an additional effort needed to get the validated assay - in these clinical contexts - into reimbursement schemes. This is not going to happen by itself. It is not the case that somebody is going to read the nice paper about the interventional trial, and it will automatically lead to reimbursement. It's quite some work to get an assay into the national reimbursement schemes. I think for that, we also need a "hub" of organisations and concerted actions from protagonists of these clinical assays that lead to reimbursement. I also think reimbursement is probably the key to the long-term introduction to liquid biopsy assays into routine clinical care.

FLG: Although liquid biopsies can quite reliably detect late-stage cancers, we are seeing a lot of movement towards early-stage cancer detection. What is needed to achieve this?

Klaus: I think early cancer detection is a little bit like the "holy grail" of liquid biopsy analysis, which of course has attracted a lot of public attention. The most important thing is that we have assays with a particularly high specificity for detecting early tumour stages. We want to detect early tumour stages because the vision is - if we detect them early, we can cure them by local intervention, and that is what we want to achieve with early detection. We want to cure patients.

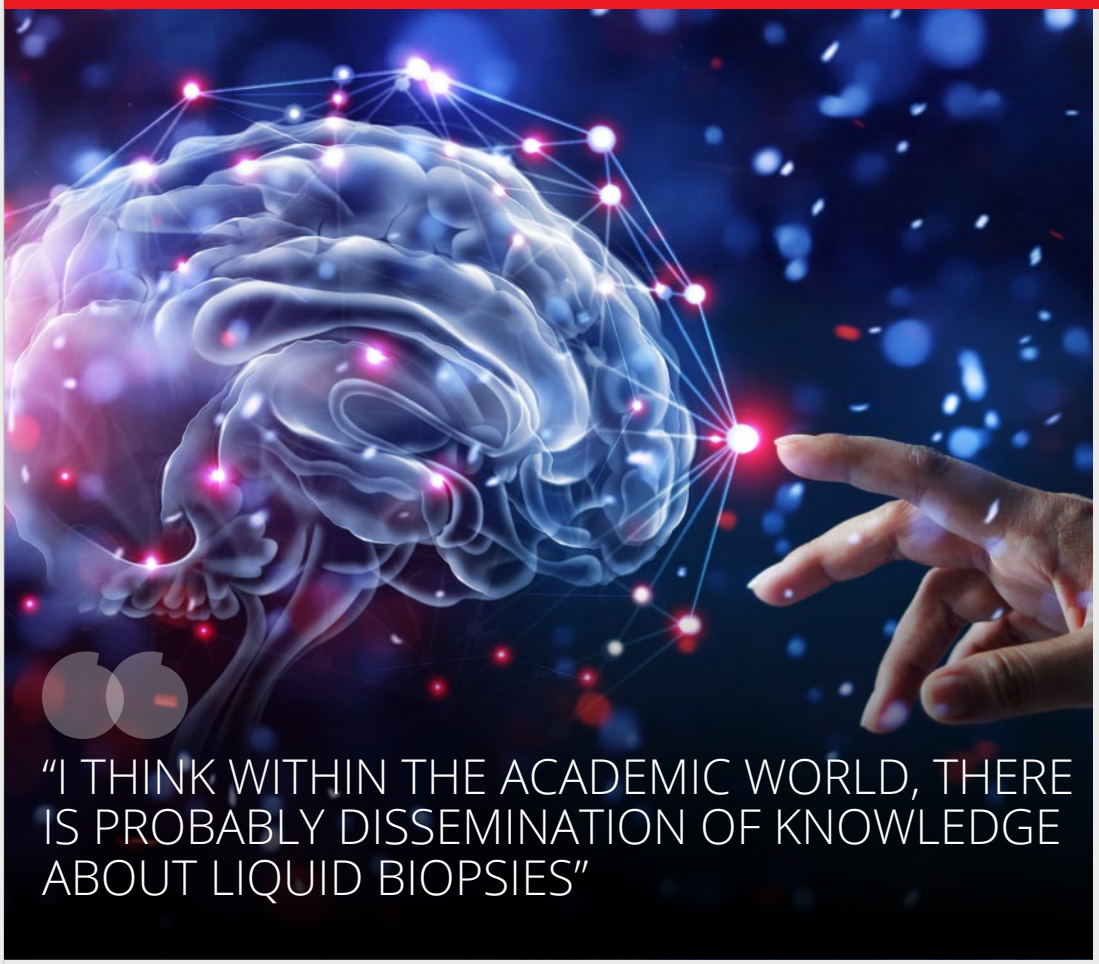
There is also a wish to detect pre-cancerous lesions, for example, high-grade colon adenomas to intervene with surgery and thus, prevent those adenomas from becoming carcinomas. So, this kind of early interception could also be a common goal for future liquid biopsy assays, because preventing cancer development is, of course, going to be even better than treating early cancers. But I think both should be the goal of early cancer detection programmes.

And, whatever is circulating in the blood should also give you some information about where the neoplastic lesion is located, because it's obvious that after you get a positive blood test, you also want to know where the neoplastic lesion is located, e.g. in the brain, breast, liver or lung. This is important because it will lead to subsequent clinical evaluation of organs, and this might be more specific if you have an idea of which organ could be affected. So, there is also ongoing work to get more information on the liquid biopsy markers with regards to the localisation of the neoplastic lesion.

FLG: How do collaborations such as ELBS help to advance liquid biopsies?

Klaus: Well, I think we can look at our goals of the ELBS. It is very important to induce a concerted action on technical and clinical validation. There are many studies published and for people who are not working in the liquid biopsy field themselves, it can be very confusing. Therefore, I think it is important to create a hub that gives you updates on the technical and clinical validation of each assay for specific tumour types and specific applications. It can also develop guidelines on how to do quality assurance of these assays and which assay is applicable to which clinical application. That could help the field to avoid the use of, let's say, not-applicable assays, and to provide guidelines for the use of liquid biopsies in cancer patients.

Another important thing that ELBS is doing, is to bridge academic and industrial partners. It is obvious that once you've published a nice liquid biopsy assay, to turn it into a product that can be used for patients, there needs to be a bridge between academic and industrial partners.



"I THINK WITHIN THE ACADEMIC WORLD, THERE IS PROBABLY DISSEMINATION OF KNOWLEDGE ABOUT LIQUID BIOPSIES"

assay then that's probably a bit prohibitive.

I also think the results should arrive within a short turnaround time of a few days. When we want to treat cancer patients, we need to get this information quickly to start the therapy. If the results take four weeks or longer, then the result is going to come too late.

The most important thing is that the data generated from the liquid biopsy assay informs the clinician on clinical decision-making. And, of course, we need to develop assays for specific tumour types and specific clinical applications. There will not be one assay that fits all tumour types or all clinical applications, so we must make our ELBS recommendations on which assays can be recommended in which types of tumour, and for which applications. We can then introduce these assays step-by-step into clinical trials and clinical practice.

The next point is the dissemination of knowledge about liquid biopsies. I think within the academic world, there is probably dissemination of knowledge about liquid biopsies. But outside of the academic world there is very little knowledge about the possibilities of liquid biopsy, and I think that is very important too.

Finally, there needs to be a partner organisation to interact with the regulatory agencies. At present, there is no real partner organisation for that. It will be very important that there is a big hub, and ELBS has more than 50 partners from academia and industry that can give guidelines, but can also talk to regulatory agencies when it comes to the approval of liquid biopsy assays. So, I think it is a wonderful time now, to bring liquid biopsy into clinical practice. If we act as a team in terms of collaborations, we have a much higher chance to increase our visibility and support this common goal of bringing liquid biopsy to clinical practice.

FLG: What do you think is needed next to progress the future of liquid biopsies in the short and long term?

Klaus: We must have reliable and robust assays that reduce any kind of variance in results and make it possible to measure both the biology of tumour evolution and the response to therapy. The assays need to be very reproducible to be able to accomplish that.

It's also important that the assays are not too expensive, because if it will cost thousands of dollars or euros to start with a liquid biopsy

FLG: What else is important for the liquid biopsy community to know?

Klaus: For me, it is also important that we understand that we need to establish a liquid biopsy community. Many times, in the past I have seen that different groups or assay providers see each other as competitors, which of course is true. But my philosophy is that the combination of different liquid biopsy assays in specific tumour types and specific applications can be very important. We must see that, as liquid biopsy providers, we have a common goal.

The common goal must be to introduce liquid biopsies into clinical trials and clinical practice. So, I guess this kind of complementarity is a very important point, rather than to compete on which assay is the best. We are really at a point in liquid biopsy development where we have the chance to bring assays into clinical practice, step-by-step. But, there's also still a great world of non-believers who want to keep cancer diagnostics the way it already is. So, I feel that the complementarity of liquid biopsy makers is very important, as well as the development of international team effort.

That's why we, for example, have also joined the International Liquid Biopsy Standardisation Alliance, together with BloodPac and other organisations; because I feel that this is a worldwide, international topic and we can learn from and support each other. ■

Global collaborations

The rapid speed of advances seen in liquid biopsies has been made possible through global collaborations. Two groups of collaborators are mentioned frequently during this report: The European Liquid Biopsy Society and BloodPac. More information on these can be found here.

THE EUROPEAN LIQUID BIOPSY SOCIETY

GOALS

- Foster the introduction of liquid biopsy into clinical practice
- Encourage interactions between academia and industry
- Provide a partner for regulatory agencies, healthcare providers and patient advocacy groups
- Support the implementation of liquid biopsy tests into clinical trials
- Develop guidelines and provide training in liquid biopsy
- Disseminate the knowledge about liquid biopsy
- Increase visibility of Europe as leading hub for liquid biopsy research
- Outreach to non-EU networks

"ELBS IS A FOUNDING MEMBER OF THE INTERNATIONAL LIQUID BIOPSY ALLIANCE COORDINATED BY FOUNDATION OF THE NATIONAL INSTITUTE OF HEALTH (NIH), USA."

BloodPAC
BLOOD PROFILING ATLAS IN CANCER

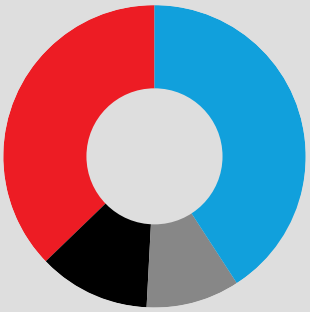
OUR COMMUNITY

Members
American Cancer Society
Arkansas Bioinformatics Consortium
Association for Molecular Pathology
AstraZeneca
Bio-Rad Laboratories
Breast Cancer Research Foundation
Bristol Myers Squibb
Center for Translational Data Science at the University of Chicago
Center for Genetic Medicine Research at Children's National Medical Center
Ceres Nanosciences
Chan Soon-Shiong Institute of Molecular Medicine at Windber
Eli Lilly and Company
Epic Sciences
Fluxion Biosciences
Focused Ultrasound Foundation
Foundation Medicine, Inc.
Freenome
Friends of Cancer Research
GlaxoSmithKline

Collaborators
AACR
Center for Medical Technology Policy
College of American Pathologist / Arizona State University
U.S. Department of Defense
Guardant Health
Horizon Discovery Ltd.
Illumina, Inc.
Inivata
LGC/SeraCare
Memorial Sloan Kettering Cancer Center
Movember Foundation
National Cancer Institute at the National Institutes of Health
Novartis
Open Commons Consortium
Personal Genome Diagnostics
Pfizer, Inc.
The Prostate Cancer Clinical Trials Consortium
Prostate Cancer Foundation
SolveBio
Streck
Sysmex Corporation
Tempus Labs
Thermo Fisher Scientific
Thrive Earlier Detection
University of Southern California
U.S. Food & Drug Administration
FNIH

External Data Contributor
University of California, Los Angeles

MEMBERSHIP BREAKDOWN



41% **Diagnostic / Industry**
10% **Government Agencies**
12% **Pharmaceutical**
37% **Academic / Non Profit**

"EVERY TIME A PATIENT IS DIAGNOSED WITH CANCER, IT ELICITS AN ARRAY OF QUESTIONS THAT CLINICIANS STRIVE TO ANSWER TO ACHIEVE THE BEST OUTCOME. THESE LARGE, SHARED DATABASES AND PROTOCOLS FOR VALIDATION ARE EXACTLY THE INFRASTRUCTURE NEEDED TO PUT ANSWERS WITHIN REACH OF CLINICIANS – ANSWERS THAT ULTIMATELY IMPROVE PATIENT CARE."

Howard Scher, Physician and Head, Biomarker Development Initiative, **Memorial Sloan Kettering Cancer Center**

However, there are many more groups who work together to help advance liquid biopsies, including (but not limited to):

- International Society of Liquid Biopsy
- FNIH Biomarkers Consortium ctDNA Quality control materials project
- Friends of Cancer Research
- The Japanese bio-Measurement and Analysis Consortium
- Medical Device Innovation Consortium
- NIBSC

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PRODUCT SPOTLIGHT FROM OUR SPONSOR NATERA: SIGNATERA

NATERA'S SIGNATERA ASSAY IS THE FIRST MOLECULAR RESIDUAL DISEASE (MRD) TEST TO RECEIVE BREAKTHROUGH DEVICE DESIGNATION BY THE FDA AND THE CE MARK IN EUROPE. UNLIKE MOST LIQUID BIOPSIES THAT EMPLOY A FIXED PANEL APPROACH, SIGNATERA DESIGNS A TUMOUR-INFORMED PRIMER SET FOR EACH PATIENT.

TUMOUR-INFORMED VS TUMOUR-NAIVE ASSAYS

Tumour-naïve fixed panels face limitations that can be avoided with a tumour-informed bespoke assay:

- trade-offs in sensitivity and specificity: fixed panels can increase the number of tracked genes to boost sensitivity but at the cost of specificity (the more you look the more you find, but not necessarily what you are looking for, so false positives increase)
- accurate measurement of circulating tumour DNA (ctDNA) quantity
- higher cost for repeat testing with a fixed panel.

A tumour-informed approach focuses on clonal mutations in a patient's tumour, effectively creating a unique tumour biomarker to track ctDNA. Even for patients with the same cancer type, tracked assay variants rarely if ever overlap. The tumour-informed assay can be used for the rest of the patient's life from diagnosis to surveillance to monitoring metastatic disease if it occurs.

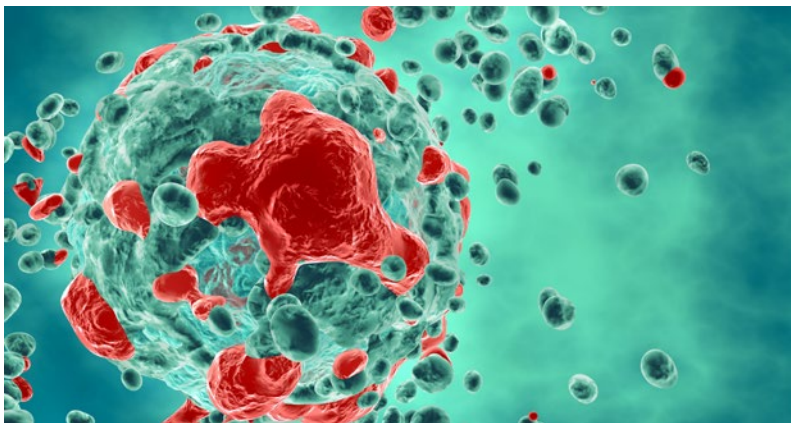
In order to achieve this personalised approach, a sample of the patient's tumour tissue is required along with the patient's blood sample. Knowledge of the patient's tumour's specific mutations permits a more accurate assessment of the quantity of circulating tumour DNA in the patients' blood. This allows a deeper view into the circulating cell free DNA to find the molecule of ctDNA.

LIQUID BIOPSIES IN AIDING CLINICAL TRIAL DESIGN

As liquid biopsies move towards clinical utility, they can improve clinical trial design and drug discovery. We provide examples of how ctDNA could inform all treatment settings: neoadjuvant, adjuvant and metastatic.

Neoadjuvant

Breast cancer is a good example of how liquid biopsies can be impactful in the neoadjuvant setting. Preoperative systemic therapy is often administered when the tumour is too large to resect. An effective



in vivo response shrinks unresectable tumours to resectable scale. Neoadjuvant therapy gives patients the chance to conserve breasts and avoid mastectomies.

However, the current surrogate endpoint for assessing neoadjuvant treatment prognosis - pathological complete response (pCR) - has been shown less effective than ctDNA in the recently published I-SPY 2 trial (more trial data to come at the San Antonio Breast Cancer Symposium mid December 2020). In the I-SPY2 trial, ctDNA levels were examined using Natera's Signatera tumor-informed bespoke assay at multiple time points throughout neoadjuvant therapy: at baseline, after 3 weeks, after completion of taxane therapy, and after completion of systemic therapy before surgery. ctDNA levels at these time points were then compared to pCR and clinical outcome.

- Results showed that although some patients demonstrated pCR on MRI's, if they had detectable ctDNA, they were still at a high risk of relapse.
- Patients who did not achieve pCR but were ctDNA negative had as good an outcome as patients who achieved pCR.[1]

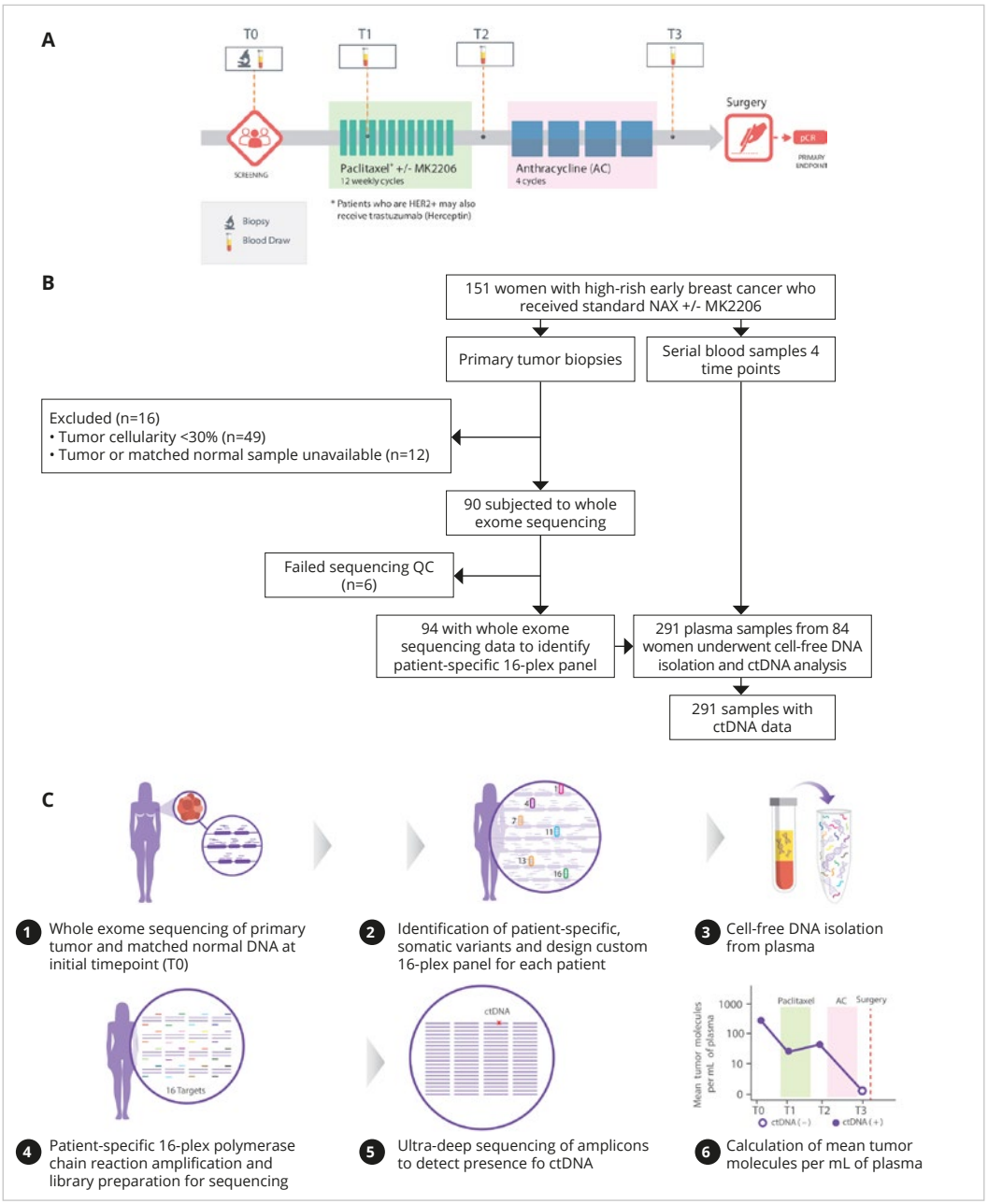


Figure 1:
A figure showing the study design of Magbanua MJM, Swigart LB, Wu H-T, et al.

0.43 – 0.79]; p=0.0005). Interim analysis of overall survival (OS) also favored atezo in patients with detectable ctDNA (HR=0.59 [95% CI 0.41 – 0.86]; p=0.0059), with median OS 25.8 months with atezo vs 15.8 with observation alone. Curiously, IMvigor010 found no benefit in treating patients with undetectable ctDNA.[2]

For more information about this study, please see the case study

Other potential adjuvant trial designs leveraging ctDNA could include timely switching of therapies for patients not responding to treatment according to ctDNA dynamics. Additionally, companies evaluating an array of regiments could quickly select the winning combination by leveraging ctDNA.

Overall, study results point to serial ctDNA testing being a more reliable prognostic factor than pCR. Potentially, ctDNA identifies micrometastatic disease that was not eradicated with standard of care treatment.

Adjuvant

In the post-surgical, adjuvant setting clinicians struggle to accurately identify patients who would benefit from timely treatment. A great example of application comes from the recently published abstract of the IMvigor010 trial for adjuvant treatment with atezolizumab (atezo) in muscle-invasive urothelial carcinoma (MIUC). While IMvigor010 did not meet its primary endpoint of disease-free survival (DFS) in the intent to treat population (IIT), those patients who had detectable ctDNA by Signatera's assay showed substantial disease-free survival (DFS) benefit with atezo vs observation alone (HR=0.58 [95% CI

Surveillance and Metastatic

Finally, using liquid biopsies for longitudinal monitoring can identify patients with early molecular relapse before the metastasis is detectable radiographically. On average, ctDNA detection precedes radiographic relapse by 8 months, but can be up to 1 year. This could allow clinicians to treat lower disease-burden patients with better outcomes.

As well as predicting tumour response in clinical trials, liquid biopsies can help identify ineffective treatments and will undoubtedly continue to help support the drug approval process.

CIRCULATING TUMOR DNA IN NEOADJUVANT TREATED BREAST CANCER REFLECTS RESPONSE AND SURVIVAL (FROM REFERENCE 2 IN NEOADJUVANT)

Abstract 10

Background:
MIUC carries a substantial risk for death; nearly 50% of patients (pts) develop recurrence within 2 years of cystectomy. IMvigor010, a global phase III trial, evaluated adjuvant treatment with atezolizumab (atezo) (anti-PD-L1) compared with observation (obs) in MIUC. Circulating tumor DNA (ctDNA) has been shown across multiple indications to be a strong predictor of recurrence. A ctDNA exploratory analysis was included prospectively to evaluate if: 1) atezo provides clinical benefit vs obs in pts with detectable ctDNA (ctDNA+) and 2) ctDNA clearance occurs at a higher rate with atezo vs obs.

Methods:
Biomarker evaluable pts (BEP) had evaluable tissue whole exome sequencing (WES) and C1D1 plasma (median 11 weeks post-cystectomy) for ctDNA analysis by Natera's Signatera assay (n=581; 72% of intent to treat population [ITT]). Baseline characteristics were similar between ITT and BEP. Tumor mutational burden (TMB) status and PD-L1 status were determined by WES and Ventana SP142 antibody.

Results:
Prevalence of ctDNA+ was 37% (n=214); positivity was associated with nodal status (p<0.001) and to a lesser extent tumor stage (p=0.09) but no other baseline factors. While IMvigor010 did not meet its primary endpoint of disease free survival (DFS) in the ITT, ctDNA+ pts showed substantial DFS benefit with atezo vs obs (HR=0.58 [95% CI 0.43 – 0.79]; p=0.0005). Interim analysis of overall survival (OS) also favored atezo in ctDNA+ pts (HR=0.59 [95% CI 0.41 – 0.86]; p=0.0059), with median OS 25.8 months with atezo vs 15.8 with obs. No benefit was noted for ctDNA ➔ pts. Within the ctDNA+ pts, additional survival benefit was derived for PD-L1-high pts (HR=0.52 [95% CI 0.331-0.82]; p=0.004), and TMB-high pts (HR=0.34 [95% CI 0.19-0.6]; p<0.0001]). The rate of ctDNA clearance from C1D1 to C3D1 was higher in atezo (18.2%) vs obs (3.8%) (p=0.0048).

Conclusions:
Post-surgical ctDNA positivity, associated with high risk for recurrence and death, identified MIUC patients likely to benefit from adjuvant atezo. Detection of minimal residual disease in an adjuvant setting will allow personalised treatment selection for patients.

Clinical trial identification:
NCT02450331.

Editorial acknowledgement:
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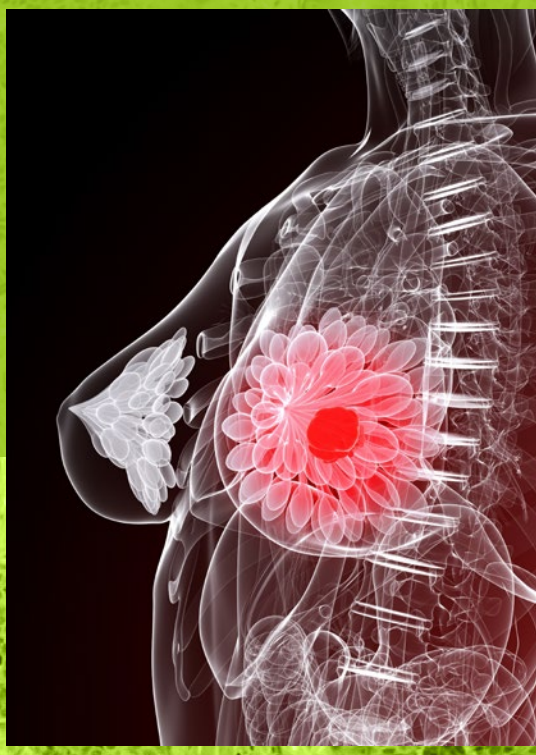
Legal entity responsible for the study:
F. Hoffmann-La Roche, Ltd.

Funding:
F. Hoffmann-La Roche, Ltd.



Next, we delve deeper into the specific applications of how liquid biopsies can benefit patients.

EARLY CANCER DETECTION AND SCREENING

The early detection of cancer greatly increases the chances of successful treatment and improved patient prognosis.

A report written in 2020 on the applications of liquid biopsies would not be complete without addressing the cancer diagnostic challenges that the COVID-19 pandemic has caused. In March, following the national lockdown in England, there was an immediate reduction in routine diagnostic testing, resulting in a later-stage diagnosis of cancer in many patients. A study on the impact of the pandemic on cancer deaths suggested that this will result in a significant increase in avoidable cancer deaths[3].

Tissue biopsy procedures require specialist staff and equipment. During the pandemic, many people have been anxious to visit healthcare establishments. Liquid biopsies would have been hugely beneficial, whereby a member of staff would have been able to carry out a diagnostic test remotely.

Therefore, as you can imagine, one of the ultimate goals of liquid biopsies is to enable earlier cancer detection and intervene therapeutically before the cancer metastasises.

The hope of using liquid biopsies for healthy, but potentially high-risk, individuals to detect cancer early has implications that include better treatment options for them. This would be of huge benefit, both economically and – most importantly – to patients.

Challenges of using liquid biopsy for early detection include[4]:

1. **Biomarker choice**
   It is important to choose the right biomarker to analyse for early detection because, while some biomarkers may be validated in advanced cancers, they may lack specificity or sensitivity for early detection

2. **Biomarker concentration**
   In the earlier stages of cancer progression, the biomarkers would be circulating in much lower concentrations, presenting a higher opportunity for false-negative tests.

SO, WHERE ARE WE NOW?

In 2014, Ilie et al. demonstrated proof of concept for using liquid biopsies for early disease detection in lung cancer by using CTC detection as an early indicator, and for monitoring of patients with chronic obstructive pulmonary disease (COPD)[5]. Tissue biopsies of lung cancers are very invasive and uncomfortable for the patients and being able to identify high-risk patients and prioritise monitoring for an early diagnosis is likely to have a big impact on the patient prognosis.

Despite this, to be able to use liquid biopsies for screening or early diagnosis the biopsy must be incredibly sensitive. Currently, a positive result must be investigated further with follow up testing, but there are many false-negative tests and thus, the clinical use of liquid biopsies for this is not currently reliable.

DETECTION OF ACTIONABLE MUTATIONS FOR THERAPY SELECTION

One of the major advantages of using liquid biopsies is to be able to access the molecular information of the tumour, and potentially discover actionable mutations of the tumour which can influence therapy selection.

This use of liquid biopsies has been shown to hold great promise. Bettegowda and colleagues discovered that cfDNA was detectable in many different cancer types, and they were able to detect KRAS and EGFR mutations from cfDNA[6].

"TISSUE BIOPSY PROCEDURES REQUIRE SPECIALIST STAFF AND EQUIPMENT. DURING THE PANDEMIC MANY PEOPLE HAVE BEEN ANXIOUS TO VISIT HEALTHCARE ESTABLISHMENTS. LIQUID BIOPSIES WOULD HAVE BEEN HUGELY BENEFICIAL, WHEREBY A MEMBER OF STAFF WOULD HAVE BEEN ABLE TO CARRY OUT A DIAGNOSTIC TEST REMOTELY."

Likewise, Zill et al. found that liquid biopsies identified cancer mutations in 85% of all advanced tumours, of which nearly half were associated with targeted therapies[7]. Most liquid biopsy studies are limited by their cohort size, so to come to this conclusion by somatic genomic profiling of over 15,000 patients was an incredible advancement.

MEASURING TREATMENT RESPONSE AND PREDICTING PROGNOSIS

Another of the major benefits seen by oncologists of liquid biopsies is that, due to their non-invasive nature, repeated testing throughout treatment is not detrimental to the patient. Using this so-called "real-time" liquid biopsy to monitor the molecular changes the tumour is undergoing in real-time, would present a big step forward in the clinical journey and in clinical decision-making.

As most cancer therapies (such as chemotherapy) are unpleasant for the patient, being able to prevent the continuation of ineffective therapies would be of great benefit to avoid unnecessary side effects[8]. Studies have also been able to demonstrate that the number of CTCs detected at baseline, and throughout treatment, can be predictive of overall prognosis. Karachaliou and colleagues - as part of the Spanish Lung Cancer group - were able to demonstrate that EGFR status detected from cfDNA was prognostic of clinical outcomes in patients with non-small cell lung cancer[9]. As part of the study, the team found a shorter median overall survival time in patients with the L858R mutation in cfDNA than in those with the exon 19 deletion. The team concluded that the L858R mutation in cfDNA could be used as a prognostic marker.

Beyond prognosis, oncologists dream of being able to use repeated liquid biopsies to measure treatment response. Birkenkamp-Demtröder et al. used liquid biopsies to monitor treatment response and metastatic relapse in advanced bladder cancer[10]. Between 20-80% of patients who undergo a radical cystectomy will relapse, and so the researchers evaluated the use of ctDNA in plasma and urine to determine whether ctDNA levels would be indicative of relapse. The team exome sequenced tumour and germline DNA from patients with muscle-invasive bladder cancer and monitored ctDNA in subsequent liquid biopsies throughout the disease courses, and found that patients with metastatic relapse had significantly higher ctDNA levels compared with disease-free patients. Additionally, they also found that the mean positive lead time between ctDNA detection and relapse diagnosis was 101 days after cystectomy. The team concluded that liquid biopsies could serve as a highly sensitive tool to monitor patients and support clinicians in guiding treatment decisions.

MINIMAL RESIDUAL DISEASE

Minimal residual disease (MRD) can be defined by the presence of tumour cells that have disseminated from the primary tumour in distal organs in patients who currently lack clinical or radiological signs of metastasis or residual tumour cells left behind after local therapy. MRD is thought to be a cause of relapse in patients.

Many cancers relapse within five years of treatment. Hormonal receptor-positive breast cancer is well known for typically late relapses, sometimes up to twenty years after treatment, and thus it is thought that relapse is caused by micro-metastases or MRD that persists after what has been considered successful therapy[11].

While there are many studies that correlate CTC count at diagnosis and overall prognosis, there are less studies that support the prognostic relevance of liquid biopsy analysis for surveillance of MRD following treatment. However, an example of a study that did test the prognostic value of this was Trapp et al.[12] who studied the prognostic relevance of CTCs following chemotherapy in high-risk breast cancer patients. The CTC status was measured at baseline and at a follow-up two years after therapy. Of the 1087 patients enrolled in the study, 18.2% of them were CTC-positive at baseline, and it was found that the presence of CTCs two years after chemotherapy was associated with decreased overall survival and disease-free survival, indicating surveillance strategies for breast cancer survivors should be considered.

Studying MRD is currently part of the surveillance strategy for patients with haematological malignancies, but is not currently routinely done for patients with solid tumours. Current liquid biopsy technologies enable the detection of tumour-derived material and studies support the notion that the monitoring and surveillance of MRD could help identify patients who are at higher risk of relapse earlier, enabling clinical intervention.

Overall, liquid biopsies can be informative in various stages of tumour progression and can influence treatment decisions once more interventional trials have enabled them to be routinely used. ■

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GRAND CHALLENGES PREVENTING CLINICAL ADOPTION

TECHNICAL CHALLENGES AND (PRE)ANALYTICS

One of the challenges that repeatedly gets attention when addressing the future clinical utility of liquid biopsies is the need for a standardised pre-analytical protocol. The pre-analytical phase of testing includes the selection of an appropriate test, specimen collection and transport[1]. Of the entire clinical testing process, 46%-68% of errors occur in the pre-analytical phase, which negatively impacts the data quality for the following phases. This increases diagnostic costs and impacts clinical decision-making for the patient[2].

The most common pre-analytical mistakes include:

- The selection of inappropriate tests
- Use of inappropriate blood collection tubes
- Poor sample collection methods (e.g. haemolysis or insufficient volume)
- Incorrect sample storage

It is important to note that the pre-analytical phases can differ depending on the analyte of interest, such as cfDNA, CTCs and exosomes. There is no set definition of which stages are included in the pre-analytics, beyond "the steps before the analysis". The figure below is taken from Pinanzi et al.[3], which showcases some of the various methods involved in pre-analytics.

The biggest impact of pre-analytical mistakes is on patient outcomes. About 10% of patient deaths and 17% of adverse events are reported to be attributable to pre-analytic mistakes[4]. With such a heavy impact, a lot of effort has been put into standardising the protocols through global collaborations.

Highlighting the importance of setting up a standardised protocol, in 2018 Trigg et al.[5] demonstrated that in the review of 50 randomly

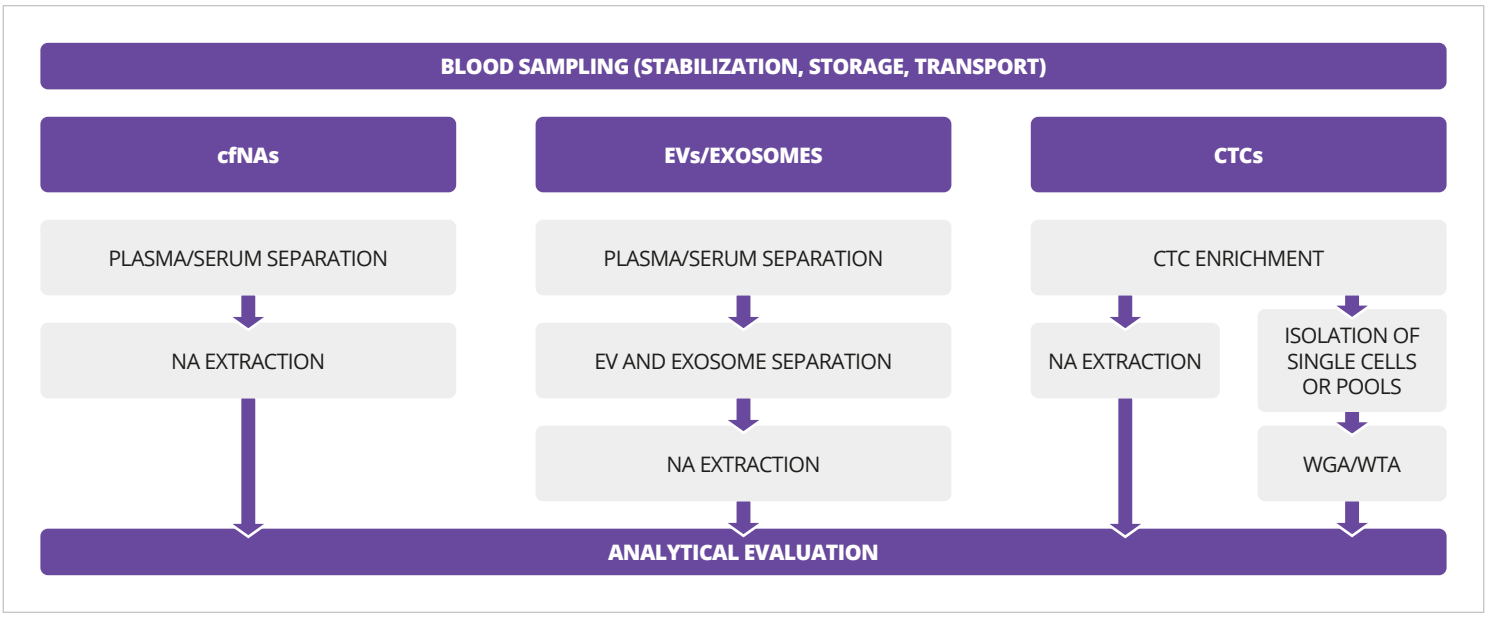


Figure 1: *Pre-analytic variables to consider*

> "THE BIGGEST IMPACT OF PRE-ANALYTICAL MISTAKES IS ON PATIENT OUTCOMES. ABOUT 10% OF PATIENT DEATHS AND 17% OF ADVERSE EVENTS ARE REPORTED TO BE ATTRIBUTABLE TO PRE-ANALYTIC MISTAKES. WITH SUCH A HEAVY IMPACT, A LOT OF EFFORT HAS BEEN PUT INTO STANDARDISING THE PROTOCOLS THROUGH GLOBAL COLLABORATIONS."

sampled papers that were published in 2015, one third did not provide information on the plasma or serum isolation, nineteen of them did not detail the centrifugation steps, and eighteen gave no information on the cfDNA quantification method.

As this is such an integral part of the liquid biopsy process, we will now review a few of the key considerations for each type of analysis, taken from Pinzani et al:

CFDNA ANALYSIS

When analysing cfDNA, pre-analytical considerations that need to be considered include:

- **Matrix** – most papers use plasma, but others use serum.
- **Blood Collection Tubes** – the choice of blood collection tube is one of the considerations that make a big impact on the analytical phases. The most common collection devices are the K2/K3EDTA- containing tubes. One of the limitations of this type of tube is that they require a short period between blood draw and sample processing, as leukocyte lysis causes an increase in DNA concentration over time, meaning that blood stabilisation is a pre-requisite for generating data that is reproducible.
- **Blood sample storage** – The conditions in which the blood samples are stored also present an issue for analysis, as this can influence cfDNA fragmentation.
- **Blood processing** – This is another pre-requisite for reliable results. According to a recent paper[6] that investigated where we stand on pre-analytical issues for cfDNA, the pre-analytical step that most people agree on is the preparation of cell-free bodily fluids by using two centrifugation steps. Additionally, it has been found that delayed processing harms the cfDNA, causing issues such as reduced detection for rare mutants.
- **Extraction method** – There are a wide variety of different methods, including phase isolation, silicon membrane spin columns and magnetic beads, none of which appear to out-perform the other. Despite this, this is an incredibly important step, as high sensitivity is needed due to the low quantity of cfDNA in a blood sample.

cfDNA enrichment strategy is an important step to improve the signal: noise conundrum many researchers face, where "noisy" NGS methods can lead to difficulties in distinguishing the signal from the cfDNA–leading to false-positive results. Although there is no universally agreed protocol to enrich cfDNA, size-selection technology can enable researchers to isolate only the DNA of interest and has the potential to strengthen the signal in their samples. You can read more on strengthening the signal in the noise with our exclusive interview with Hunter Underhill, starting on page 30.

Despite the many factors to consider and more research being needed to standardise procedures, there are two main things which are uniformly agreed upon when studying cfDNA:

1. **Preservative agents are required to stabilise cfDNA**
2. **Automated protocols tend to perform better than manual kits when isolating cfDNA**

CTC ANALYSIS

There have been many technologies developed to identify, count, and characterise CTCs in the bloodstream, presenting the problem of choosing the right technologies for the pre-analytical phases.

For CTC analysis, the following considerations should be considered:

1. **Blood collection before analysis**
   Most commonly, blood samples for CTC analysis are collected in K3EDTA tubes, which requires a very short interval of time between collection and sample processing. There are very few commercially available tubes that are designed for CTC analysis, with most of the tubes being specifically designed to stabilise cfDNA by preventing the release of nucleic acids from cells. So, the choice of the collection tube is impactful to the research, as many of the blood collection tubes with preservatives will also kill cells.

2. **Enrichment strategy**
   The enrichment strategy - as part of CTC analysis - involves the recognition of marker proteins and tumour cell properties. This will influence the results, as the proteins will select for different populations of cells. CTC enrichment strategies are broadly classified into those that are affinity-based or label-free. Label-free strategies are generally preferred.

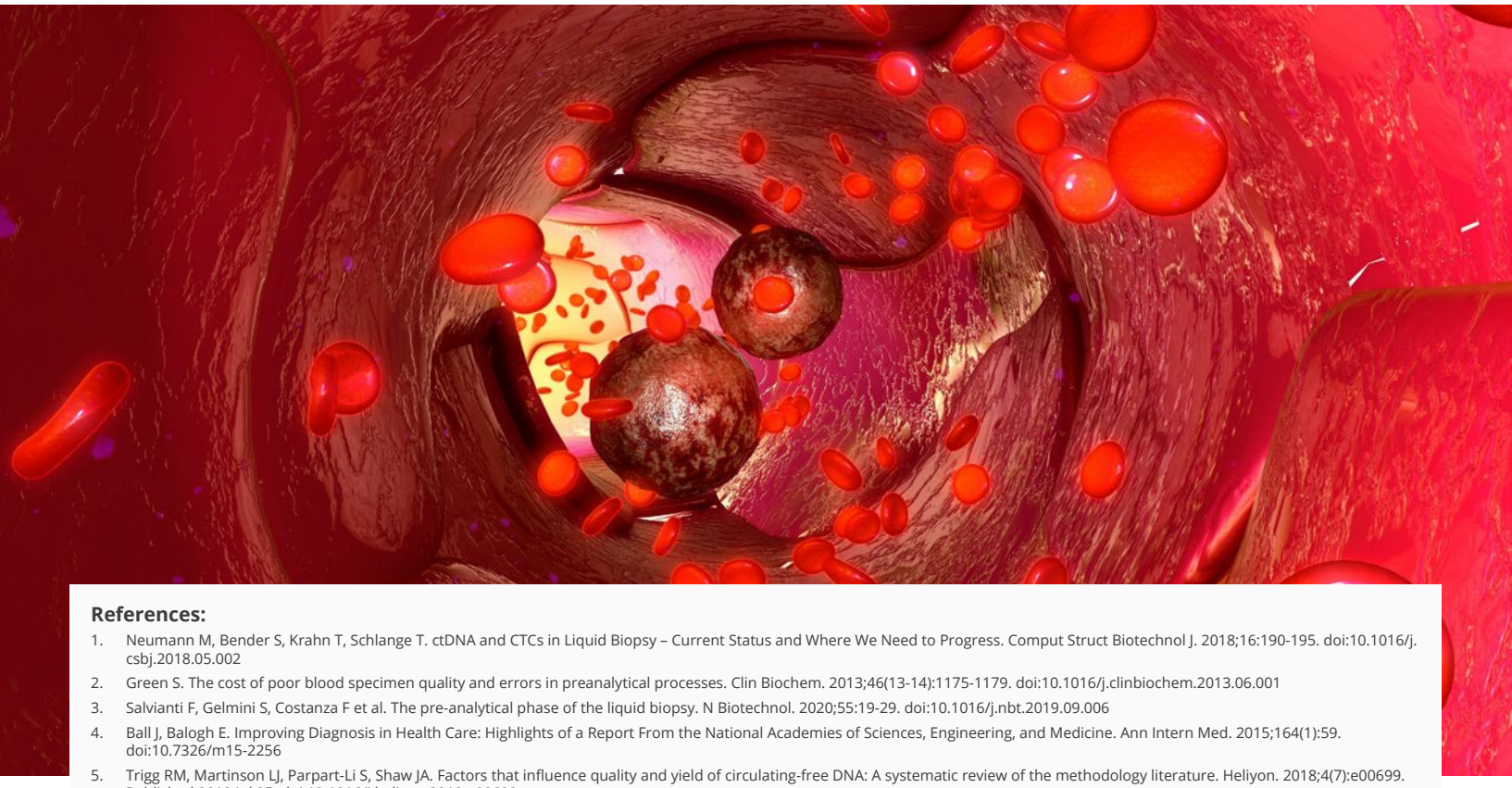
In addition to the lack of a robust, standardised pre-analytical protocol, there are still technical challenges to overcome when studying each analyte, which includes[7]:

1. **ctDNA sensitivity issues**
   NGS or PCR methods are used to detect and identify ctDNA. According to Diaz and Bardelli, 2014[8], PCR methods have a higher sensitivity than NGS methods.

2. **Bioinformatics tools**
   Liquid biopsy analysis requires computational tools, such as single-cell CTC variant callers, to quantify the cells. These methods tend to have many pitfalls, as they were originally developed for bulk-sequencing. Computational tools are also used to analyse the concordance in mutational profiles between cfDNA and tumour tissues.

These tools need improvements in order to be used for routine clinical adoption of liquid biopsy assays.

Finally, there are doubts relating to what extent liquid biopsy samples accurately reflect the heterogeneity of a tumour, the reproducibility of the results, and high false-positive rates. ■



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OTHER BARRIERS TO CLINICAL ADOPTION

The ultimate goal of liquid biopsy research is to eventually bring this technology closer to the patient and enable non-invasive molecular testing of circulating tumour biomarkers. Both scientific and social barriers are preventing the reimbursement and uptake of liquid biopsies.

Throughout this chapter, we highlight the social and scientific barriers to adoption, and some of the work the community is doing to help overcome this.

OVERCOMING THE SOCIAL BARRIERS TO CLINICAL ADOPTION

AN EXCLUSIVE INTERVIEW WITH **LAUREN LEIMAN,** THE EXECUTIVE DIRECTOR OF **BLOODPAC.**

The Blood Profiling Atlas in Cancer Consortium was launched on October 17th, 2016, to accelerate the development and validation of liquid biopsy assays to improve the outcomes of patients with cancer.

We recently caught up with Lauren Leiman, the Executive Director of BloodPac, to discuss the social barriers to clinical adoption, and the importance of data-sharing and open collaboration, after the criminal acts of Theranos knocked the public's confidence in the technology.

FLG: Could you introduce yourself, BloodPac and the work that you do?

Lauren: I am Lauren Leiman, I'm the Executive Director of BloodPac, the Blood Profiling Atlas in Cancer. I founded it back in 2016 when I was working as the Head of External Partnerships for White House Cancer Moonshot. It was originally a commitment of about 20 different organisations to help create standards and provide data to support those standards, with the sole mission to accelerate the development and approval of liquid biopsy technology for patient benefit.

"WE HAVE ACHIEVED THE CANCER MOONSHOT GOAL OF A DECADE OF PROGRESS IN LESS THAN HALF THE TIME"

Since then, we have become an independent organisation, our own non-profit in February 2017. And now we are a consortium made up of about 46 or 47 different organisations, ranging from all stakeholders in the field; from pharmaceutical companies, diagnostic companies, academics, clinicians, patient advocacy groups, foundations focusing on funding in the field, government agencies, regulatory agencies etc.

FLG: Could you give us a quick overview of the social barriers to clinical adoption of liquid biopsies and the work you have done at BloodPac to identify them?

Lauren: We have decided to call the challenges "barriers" or hurdles. We went through an exercise over the past quarter, while everyone was at home and travelling less, to take time out to think through the challenges within our working groups. We have about ten different working groups – these are the basis of how we operate in BloodPac. We were all asked by our co-chairs to discuss, based on what their groups were doing and their organisations, what were the barriers for clinical use of liquid biopsy availability for all patients. We then took those findings and brought them back to our scientific co-chairs, and had a presentation/discussion around what we found, what the main concerns are and how we can combine all these things. We had a fantastic list. There are a lot of challenges.

I narrowed it down to two:

Number 1 is reimbursement - making it feasibly available for patients to utilise. Obviously, you can get liquid biopsies developed, you can

have them approved by regulatory agencies; but at the end of the day, if they are not financially viable for patients they are not going to be utilised. That was at the top of everyone's list - not the 12-month out emergency barrier, but a 'further down the road' issue.

The second barrier, we haven't decided what to label it yet. But I think it's two things: (1) the boosting of confidence in the technology for the broader community, and (2) education. How we want to do that is still up in the air. Through education and through the collection and analysis of data to support the technology, you boost confidence in it. I think there are a lot of different stakeholders to educate, one of which is the payers, which goes back to the first hurdle.

And how do you go about educating physicians, nurses etc. on how to use this technology appropriately and how to advocate it for their patients appropriately? That is its own educational hurdle. How to educate patient advocacy groups about this. What language to use when educating and using a standardised language; these are all hurdles. We are educating different groups around different issues, all to accelerate liquid biopsies into the clinic for all patients and boost confidence in the technology, so that patients can access it regularly in the future.

FLG: We have seen a recent shift in payer policies regarding reimbursement, but what do you think is needed to shift it further? What else do you think payers need to see?

Lauren: As you know, there are multiple payers in the field that have different opinions. Some are much more progressive in their view of liquid biopsy, and they have medical officers who understand the benefits of the technology and are starting to move the needle on how to provide access and reimbursement on these tests.

On the other hand, there are a lot of medical officers who are a little slower in moving towards our end goal. So, I think each individual, not to mention each company, will give you a different answer on what exactly they are looking for and what exactly they need. And each researcher too, within each company, will have their own interpretation of what the medical officers and payers want. So, I think our approach has been, and my constant question to our working group has been, we as BloodPac have two things going for us that are beneficial:

Firstly, we are a collaboration of different organisations, not just one company coming to a payer, saying, "hey - don't you want to reimburse this test, here's my data?" We are a consortium who, - I would argue - for the past three and a half years, have achieved the cancer moonshot of a decade of progress in half the time. Faster than that. And through collaboration.

And therefore my question to our internal working group and the payers themselves has been, "how can you utilise us, use Bloodpac as a resource, as a group of individuals and organisations in the liquid biopsy space, who can come together and provide you with information, data, opinions, expertise around this technology to help educate, better understand and come up with a strategic plan to accelerate the reimbursement process?"

So, instead of being just one-offs, how do you use the consortium as well. I think that's really important.

"IF YOU HAVE 46 DIFFERENT ORGANISATIONS WORKING TOGETHER TO COLLECT DATA AND TO BE FULLY TRANSPARENT ABOUT THEIR TECHNOLOGY, AND BE TRANSPARENT ABOUT THE DATA THEY HAVE, TO OPENLY WORK TOGETHER, TO MEET, TO DISCUSS AND COME UP WITH GENERAL ANALYTICAL PROTOCOL TOGETHER – THAT'S AN EXTREMELY TRANSPARENT VERSION OF EVERYTHING."

Secondly, the other thing we can do to support this piece of the pie, is that we have a data commons. We are in the process right now of working to become a source of valid scientific evidence as an FDA data commons around specific questions. It's a project called 'Project Exhale'; looking at liquid tissue concordance in lung cancer. The idea is to demonstrate that it can actually be done and - far into the future - ask this question: Is it possible to have a data commons that could be a reliable source of scientific evidence for payers as well?

And even if that never becomes a reality, understanding the capacity that we have and understanding the needs of payers is really important to accelerate the timeline.

FLG: You mentioned the lack of confidence in the technology. What have you found, so far, are the main messages that you need to convey to people and what are their biggest concerns in that sense?

Lauren: Well, number one, I'd urge you to read OpEd that I read with Foundation Medicine, our opinion piece in STAT on Theranos, which – scarily – was only written three years ago.

I think that it is still in people's minds. I still get questions about it, especially from patients. The ordinary individual who is not in this field wants to know, "wasn't there this documentary I saw on HBO about that lady?!" And yes, you did.

So, I feel that BloodPac is one of the tools in our toolbox to combat the negativity around the technology and help to build confidence. Because if you have 46 different organisations working together to collect data and to be fully transparent about their technology, and be transparent about the data they have, to openly work together, to meet, to discuss and come up with general analytical protocol together – that's an extremely transparent version of everything. It is really what should be happening in the field and it benefits everyone, not just the companies who are participating. And it combats the secretive and criminal nature of what took place with Theranos. I always say that I think BloodPac is a tool to help promote this technology collectively.

I also think that because BloodPac has access to so many different individuals and companies within the field, we can explain why differences exist to help educate the public on the different performance characteristics of different tests. We can answer some of the "whys" in a transparent, user-friendly environment. We're able to explain why nuances exist in a very complex technology in a way that others sometimes have a difficulty doing. And I think that that will help address some of these concerns or the lack of understanding of what the technology can do today, what it will be doing tomorrow and the potential for the future.

FLG: How important is global collaboration and what can we learn from each other?

Lauren: I have been having this conversation with the FDA from the moment I proposed BloodPac to them until today. I have said this at multiple conferences. My hope for BloodPac is that we are just a giant demonstration project to show consortia like BloodPac can exist, can be successful, can be easily replicated, and should be utilised. We have done it within liquid biopsy, but I'd love to see other consortia develop in other diseases, in other technology spaces. You could even take it outside of healthcare.

But in my heart and soul, my mission has always been to demonstrate that we can achieve great things, better things, faster, more effectively and more efficiently, when we work together, than if we try to do them on our own.

I have a great platform, in that - within BloodPac - I found the right partners. We have come together and continued to demonstrate that with a narrow focus, and being very determined to come together to benefit patients, there are so many pre-competitive areas where we can come together to benefit from one another.

We have achieved the cancer moonshot goal of a decade of progress in less than half the time, and I urge everyone in all different spaces to think through how to create organisations like this. Specifically, within my personal mission, within the liquid biopsy space, I applaud everyone who is coming together and creating different organisations. BloodPac is a very focused organisation and we get a lot of proposals that come to us about forming different working groups, or taking on different issues. With a very diverse group of stakeholders there will always be a lot of specific interests from specific people, for specific companies etc. I always say that it's important to find the middle of the Venn diagram, where everyone's interests intersect to have the most benefit, for the majority of us in the organisation and the field in general. I think we have done that.

It's incredibly important, but BloodPac isn't necessarily the right organisation to lead that, and so I am grateful that there are so many organisations forming. Some that have existed long before us that are taking on different aspects of the work that are important.

I am also grateful that in liquid biopsy we have become a pretty tight-knit community, where the majority of us know one another and can collaborate across different organisations and discuss, so we don't waste time and duplicate efforts in terms of our work.

FLG: What has been, in your opinion, one of the biggest achievements that BloodPac has helped to make for the field of liquid biopsies?

Lauren: One of the things that BloodPac has been able to accomplish that has been pretty impressive (and again, I take no responsibility for it) is to have created the general analytical validation protocol which demonstrates that there is a need for everyone to get on the same page around how to analytically validate your assay.

We went through a process where we asked the FDA whether, if we came up with the generic protocol, would they be interested in reviewing it? The answer was yes. So, fifteen different organisations within BloodPac came together and drafted it over the course of two years. It went through multiple edits with the agency, and we came out with a document to serve the community as a whole and we published it. We have made it publicly available and accessible.

So, we went back to the FDA and asked how can we create a second version of this to further serve the community? Their answer was to create a version 2.0, to include minimal residual disease, which they are working on now.

I think that really touched on the whole spectrum of why consortia are important, and what we can achieve. We're filling a void, creating a product that is needed that can only be done with a huge group of people coming together to work on something. We're then making it openly accessible to the community. It has demonstrated what we are about. And then we have the data commons to support collecting data around trying to figure out whether we got it right or whether we got it wrong.

At our core, that is what BloodPac is trying to achieve. We want the resource to be used in the studies. You are welcome to submit the data to the BloodPac data commons to help find answers. So, this demonstrates the space that BloodPac fills. Again, it's a narrow scope but we don't verge outside of that process.

Staying focused is important. I don't think it's the same as the decade-long hurdles of reimbursement or education. But there are the 1-2-year short term issues that we can address, and then there are the long-term hurdles on how we can get the technology into clinical use for all patients. ■

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Scientific challenges

Despite near-constant advances in the technologies, some broader scientific challenges need to be overcome before we see liquid biopsies boom. This section of the chapter highlights a couple of the challenges, excluding the lack of standardised protocol in this space.

1. CLINICAL TRIALS

For liquid biopsies to be made routinely available in the clinic, their utility must be proven via robust clinical trials. Not only does the liquid biopsy need to be informative about the tumour and its progression, but trials need to prove that the test can inform the clinician with vital information that can alter treatment decisions for patient benefit.

Although there are not many large-scale clinical trials, recent years have seen a lot of progress and new trials testing the utility.

Here, we examine a large-scale European study: PADA-1

CASE STUDY: PADA-1 TRIAL[1]

Trial information: https://clinicaltrials.gov/ct2/show/NCT03079011

The PADA-1 trial is a study that is testing the clinical utility of circulating tumour DNA detection, searching for ESR1 mutations in metastatic breast cancer.

It was designed to assess whether changing treatment from aromatase inhibitors after ctDNA detection of ESR1 mutations could be beneficial for metastatic breast cancer patients.

Here, we share the trial design for PADA-1, and the first results.

ESR1 MUTATIONS

ESR1 mutations are associated with resistance to endocrine therapy in metastatic breast cancers and, ultimately, poor patient prognosis. According to the PADA-1 trial, the ability to detect these mutations and clearing cells with these mutations through early therapeutic intervention can greatly reduce the risk of recurrence.

The oestrogen receptor (ER) is a driver in approximately 70% of metastatic breast cancers. The ER can bind to DNA and activate survival signalling pathways and the transcriptome, once it has bound to oestradiol. Lowering oestradiol can prevent the oestrogen receptor from binding to the DNA, but ESR1 mutations can be formed as a mechanism of resistance, which can enable the oestrogen receptor to reactivate the pathway. There is a clear increase in the prevalence of ESR1 mutations after exposure to aromatase inhibitors (AI), but these mutations are still sensitive to selective oestrogen receptor degraders (SERS), such as fulvestrant (Ful). Usually after the detection of ESR1 mutations, patients will be given a more targeted therapy of Pal+Ful.

ESR1 mutations can be detected in ctDNA months before tumour progression. Thus, the safety and efficacy of testing for these mutations via liquid biopsy can offer a clinical benefit to the patient. This was integrated into the trial aims.

The trial designers developed a very sensitive assay for detecting ESR1 mutations using ddPCR.

CLINICAL TRIAL

The PADA-1 study enrolled 1,107 first-line metastatic breast cancer patients who were all sensitive to AI therapy, either because they have had no relapse under adjuvant AI or have had no previous exposure to AI. It was found that patients with ESR1 mutations at baseline had double the chance of disease progression compared to those who subsequently developed the mutations (wildtype). However, if the mutations were cleared early in treatment, it was found that the risk was roughly the same as the wild-type patients.

In the first step of the trial, all patients (N=800) were treated with Pal+AI, and their ctDNA was tracked using ddPCR at baseline, after 1 cycle of treatment and then after every other cycle. In the second step, when an ESR1 mutation is detected in the blood and the patient had progression at the same time, they were randomised between keeping the same treatment or being switched to Pal+ Ful, the targeted therapy. In the third step, patients that had been randomised to the standard regiment may crossover to targeted therapy following tumour progression. This crossover is important in proving the clinical utility and patient benefit.

PADA-1 is still ongoing, with around 150 randomised patients now at stage two. It is expected that data for the main study objective, which is to test the progression free survival from randomisation, will start to be collected next year.

2. ASSAY DEVELOPMENT AND REGULATORY CONSIDERATIONS

The development of robust liquid biopsy assays remains challenging, partially due to their unique regulatory challenges around needing validation. The primary limitation for assay development is the variability in the shedding rates of tumour-derived material. The shedding rate refers to how often the primary tumour will release material that will circulate the bloodstream and is detectable via liquid biopsies. Additionally, the assay needs to be informative of the tumour location to aid in clinical diagnoses, and natural biological processes such as clonal haematopoiesis of indeterminate potential mutations, can lead to false-positive results.

Figure 2 below, from Narayan P, Ghosh S, Philip R, et al. highlights the potential applications of ctDNA assays and regulatory considerations that agencies look for in approving them. ■

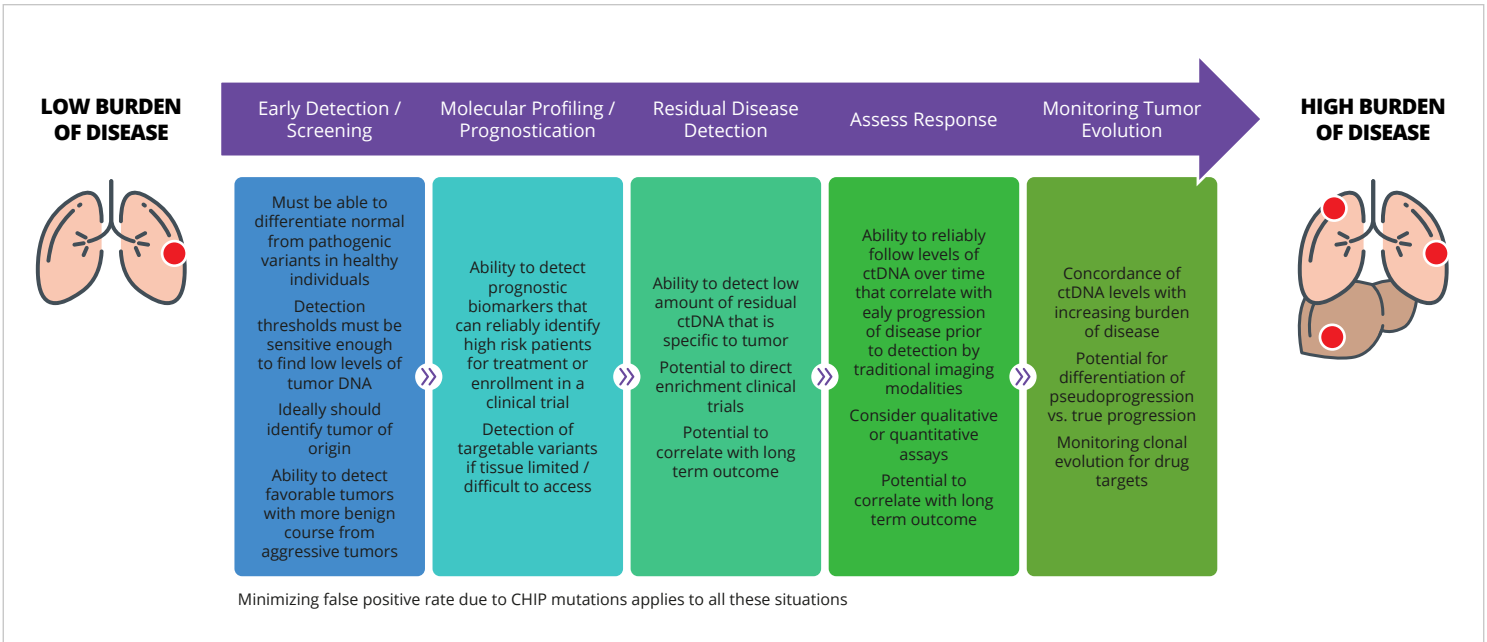

Figure 2: *Potential applications of ctDNA assays and regulatory considerations*[2]

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INTERVIEW WITH DR HUNTER UNDERHILL

SEEKING SIGNALS IN NOISE – USING SIZE-SELECTION RANGER TECHNOLOGY TO ENHANCE CFDNA DETECTION.

Dr Hunter Underhill is an Assistant Professor of Paediatrics within the medical genetics' division at the University of Utah School of Medicine and has a research lab at the Huntsman cancer institute studying the applicability of cfDNA detection for brain tumour surveillance and monitoring.

We worked with Matthew Nesbitt, CEO of Coastal Genomics, a Yourgene Health company, to interview Dr Underhill to better understand how his lab is seeking rare signals amongst noise using liquid biopsies for cancer detection.

Matthew Nesbitt: Please tell us a bit about yourself and your research?

Hunter: I'm in the Division of Medical Genetics in the Department of Paediatrics at the University of Utah. I also work at Huntsman Cancer Institute in the research domain. I see patients clinically and I have an active lab. I started in neurosurgery because I have a strong interest in brain tumours, and I left my residency early because I wanted to see if we could do something more than surgery to help patient outcomes. I left my residency early to pursue a PhD in bioengineering with an emphasis in MRI physics. As part of that research, I became very interested in DNA and the molecular aspects of tumours. After finishing my PhD I did a residency in medical genetics at the University of Washington. Then, I did a post-doctoral in tumour biology with Dr Rostomily, a neurosurgeon at the University of Washington, studying the invasive aspects of glioblastoma. Subsequently, I did a clinical fellowship in biochemical genetics at the University of Utah where I stayed on as faculty. I have been on the faculty at the University of Utah since 2015.

The move to Utah was interesting because prior to departing University of Washington we learned via an animal model that there might be a specific size discrepancy between the cell free DNA that comes off of a tumour versus the cell free DNA that comes off of healthy cells. When I came to the University of Utah, we were able to translate our animal findings to humans. We saw the same difference in size profile and we went on to publish that work and try to demonstrate from a molecular point of view that there is this distinct size difference between healthy and tumour-derived cfDNA.

We published our findings in 2016, which launched our laboratory efforts to more intensively study cfDNA as a diagnostic marker - ideally in early-stage and non-metastatic tumours.

Matt: Thank you, I think it is fair to say that your research and getting into the nuances of fragment size is at the cutting edge of what is going on in liquid biopsy in oncology. Where have you published your findings, and more specifically if you can, zero in on what your work is focused on?

Hunter: Our work is primarily focused on the personalisation of cfDNA. The molecular fingerprints of tumours can potentially be leveraged to not only detect recurrence or diagnose disease, but also to inform on response to therapy or dictate therapy changes. So, my lab has largely been focusing on trying to get it to the point where we can go in blind and see if we can identify ctDNA associated with the malignancy. By blind, I mean, not using a tumour-informed search. Tumour-informed searches are where you first sequence the DNA from the solid tumour, identify somatic mutations and then search for those specific mutations. We and others are trying to take it to the next step where we would no longer require looking at solid tumour DNA first to direct our searches. That's where we have been trying to focus and the key aspect to that is error - because NGS is very noisy and there has been a variety of methodologies to try to overcome that noise and potentially enhance the signal from ctDNA.

Our original work in 2016 showed that you can exploit that size difference and enrich for ctDNA. In a follow-up paper in PLOS One in 2018, we showed that you can enrich for ctDNA in a variety of different cancer types. I think one interesting and novel aspect to this work was that we found not only enrichment for ctDNA but that we could also push down NGS noise through the reduction of sample complexity enabling generation of larger family sizes. Larger family sizes can be used for in silico error correction leading to fewer false positive. I think that's important because getting the noise down without compromising sensitivity is a real challenge and size selection may be a way to improve that.

Matt: So, you're talking about blind vs. informed approaches to cancer detection. Most clinical implementations of liquid biopsies in the oncology space, at least that I'm aware of, take that informed approach in so far as they depend on prior knowledge of the cancer type or they are only enquiring as to a set of pre-determined mutations. Why would the field benefit from a blind approach, like the one you have been pursuing?

Hunter: I think there are two aspects to it. First, if you look at a tumour-informed search you are constrained to the mutations that are present in a very small piece of tissue DNA that may not reflect the entire tumour heterogeneity. Second, along those lines, when you only look at tissue DNA, you may miss mutations associated with metastatic lesions that may already be present. So, if you are not looking for something that is different or open to that possibility, then it may be missed, and these missed mutations might have therapeutic or treatment implications.

The other case is whether we can use this at all to survey an at-large population for the presence of a tumour. So, instead of doing colonoscopies or mammograms, or any other type of screening procedure, can you replace that with cfDNA diagnostics assuming you have no idea what the patient has - if anything?

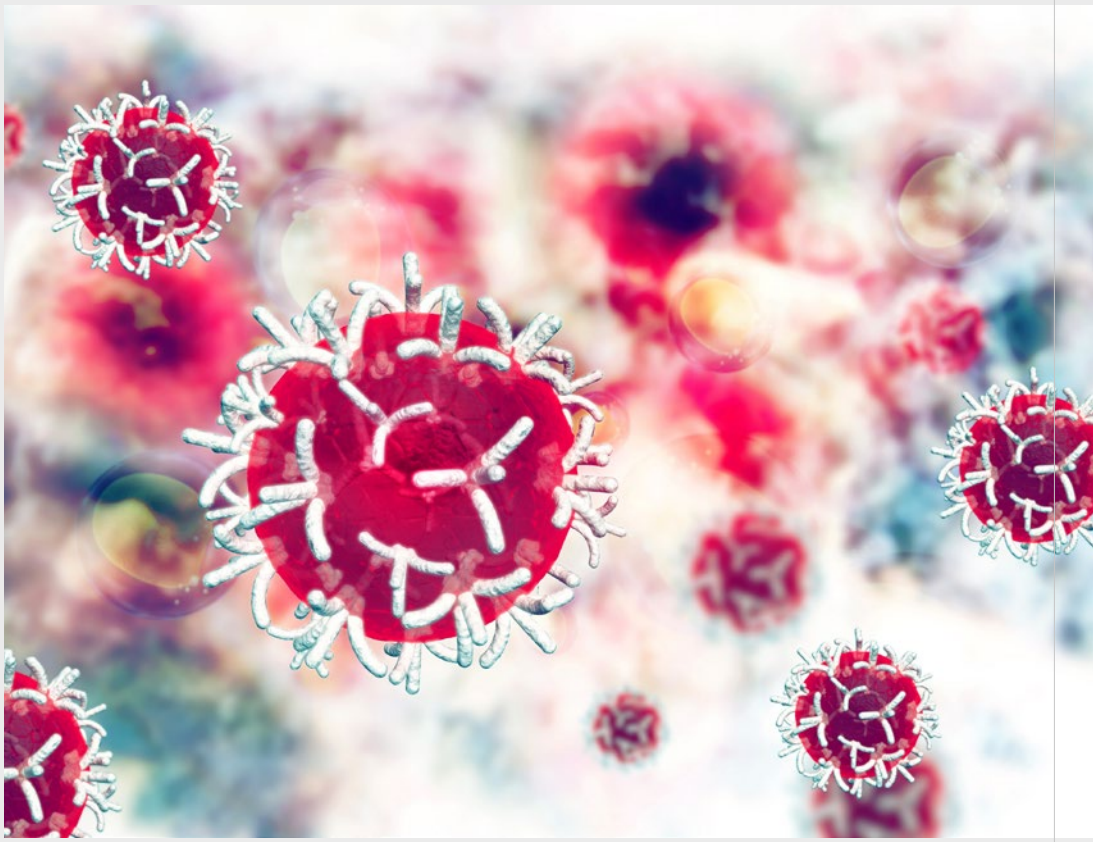
So, these blind searches are important in my mind because it allows you to better understand the molecular landscape in a patient with an existing tumour and also survey a population for earlier detection of tumours.

Matt: We have talked about the informed approach and you have highlighted the benefits of a blind approach, there are barriers or else we would know of more groups taking a blind approach in the clinic. What is preventing the widespread adoption of that approach?

Hunter: Well, it's signal and noise. The signal is weak, and the noise is too much. NGS, even though it has gotten cheaper and cheaper, it has gotten noisier and noisier to some extent. If you can't distinguish signal from noise, then you are going to have many false-positives. If you develop thresholds to suppress the noise, you may substantially alter your sensitivity and the challenge with cfDNA is that you have so little input. Particularly in patients who are seemingly healthy or have early-stage disease, the amount of cfDNA that you can get in plasma is low. So, if you are trying to detect something that is 1 part per million, you may not have enough signal to do that. I think the technical aspects are trying to achieve full error correction abilities without suppressing the signal that might actually be there, and I think there are various ways to do that. There are various in silico technologies, there are adapter technologies, there's size selection technologies and I think the likely methodology will have some integration of these different approaches to really do the error correction and signal detection.

Matt: Could you explain what your group is doing to develop solutions to these challenges with the signal and the noise?

Hunter: I think we have tried using duplex adapter technology with the idea being that instead of applying a molecular barcode to a single stranded piece of DNA and then following those amplicons as they get generated, you do the same thing, but you label double stranded DNA. So, what molecular barcoding allows you to do is collect all of the PCR amplicons with the same barcode and collapse them into a single sequence where you adjudicate each base position based on the consensus interpretation from all the PCR amplicons associated with that barcode. That would be step one.



Step two is to take each consensus sequence, identify its paired strand mate (dsDNA) and then do a second collapse, and that second collapse in theory would fully eliminate all early PCR errors or push the error rate down so low that it's manageable. In theory, the idea is perfect. In practice, we have had a really hard time finding the strand mates. In fact, we find it in less than 1% of our samples.

So, we use duplex adapters to reduce error but still rely on tumour-informed searches. To further improve error, we try to exploit *in vitro* size selection for the short fraction of cell-free DNA that is enriched with ctDNA. We've previously shown that the reduction of sample complexity associated with size selection generates larger family sizes, which leads to improved in silico error correction.

The key aspect of size selection is reproducibility - we showed in 2018 in a PLOS One publication that with automated Ranger Technology we can isolate the short fraction of cfDNA off the mono-nucleosome very reproducibly. In doing so, we were able to enrich for ctDNA and also push down the error in our samples. We confirmed ctDNA enrichment with both digital droplet PCR and NGS, which is important because we were able to validate that what we were seeing in NGS was real and not an artefact, as confirmed by ddPCR.

Matt: Thank you very much, going on a little bit more about Ranger Technology, you talk about the precision. What else about this option makes it suitable for your group and potentially other groups looking to adopt your solutions as they move towards a blind approach?

Hunter: Well, when we first started doing size selection, we were doing it using polyacrylamide gel electrophoresis (PAGE) – a technology that has been around for decades but requires handmaking each individual gel between two sheets of glass. We would have to do one sample at a time because once the gel ran, we would then use razor blades to cut out different fractions of the gel. We found that if you ran more than one sample, just because of the way the gel runs and the fluids moving around and the cutting, you were likely to get a contamination between samples. So, running one sample essentially took all day. In the original paper in 2016, we only did 4 samples along with negative controls.

What Ranger Technology allowed us to do was to scale up, so you had the ability to reproducibly isolate very finite sections of the mono-nucleosome without worrying about cross-contamination between lanes. When we originally used Ranger Technology it was scalable to 96 samples and the smaller LightBench platform[1] that we have in the lab now can run 8-12 samples. For a lab of our size, this is more than sufficient and can certainly be adapted by other groups to expand to a larger number if needed. It's all about scalability and reproducibility because we were getting very reproducible results using PAGE technology, but it is very time consuming and labour-intensive. Now, it's more a case of loading the cassette and letting the machine do all the work.

Matt: In terms of how you see the future of liquid biopsy for cancer detection progressing, do you have any forecasts? I know that people question when early detection is going to become a reality or when oncology will become a mainstream application in any capacity for early detection. Given your understanding of the challenges that are there, and the progress being made, including by yourself and others, what do you think the coming years look like for this field?

> "THE MOLECULAR FINGERPRINTS OF TUMOURS CAN POTENTIALLY BE LEVERAGED TO NOT ONLY DETECT RECURRENCE OR DIAGNOSE DISEASE, BUT ALSO TO INFORM ON RESPONSE TO THERAPY OR DICTATE THERAPY CHANGES."

Hunter: I think it's going to become more and more personalised. We will start seeking out patient-specific mutations rather than doing searches for common mutations. I think we will be able to look at more personalised mutations that might be unique to the individual or the cancer.

I think surveillance is a real challenge because the signal can be so very low. However, if we are able to harness both *in vitro* and in silico approaches to eliminate NGS noise without compromising signal detection, I think we may be able to make an important jump towards surveillance. Ultimately, some types of tumors may be more amenable to surveillance with cfDNA than other types of tumours. But you never know until you go looking. Regardless, I certainly expect cfDNA to be used for surveillance for at least some types of tumours in the not-too-distant future.

Matt: Thanks! We really appreciate you sharing some insights into your work and the way you see things. What can you tell us about the work you are doing so we can all be keeping our eyes open for what comes from your group?

Hunter: My lab is supported by the National Cancer Institute under an R37. We're actively researching detection of primary brain lesions using cfDNA. The goal of our lab is to diagnose these lesions before surgery is required. Surgery will still be a component down the road but obtaining a diagnosis before surgery may present the opportunity to provide neoadjuvant therapy and optimize a treatment regimen prior to surgery. We are also trying to show that we can detect recurrence and differentiate recurrence from pseudo-progression and radiation necrosis. Sometimes, in high grade brain lesions the treatment induces a response that looks very much like tumour recurrence. The only way to differentiate it from tumour recurrence is to take a biopsy or wait for the lesion to become larger. We are using cfDNA to discern these differences. Size selection for the short fraction of cell-free DNA is a key component of our strategy and we hope to share our findings with the scientific community soon. ■

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1. Lightbench (Yourgene QS250) is powered by Ranger Technology from Yourgene Health

CHAPTER 4

NON-BLOOD LIQUID BIOPSIES

ALTHOUGH THE TERM LIQUID BIOPSY MOSTLY REFERS TO STUDYING TUMOUR MATERIAL IN THE BLOOD, RECENT ATTENTION HAS BEEN TURNED TO OTHER LIQUIDS THAT CAN HOST TUMOUR MATERIAL, SUCH AS SALIVA, URINE, OR CEREBRAL SPINAL FLUID. THESE ALTERNATIVE BIO-FLUIDS CAN, IN SOME CASES, PROVE TO BE ADVANTAGEOUS EITHER BECAUSE OF THEIR NON-INVASIVE COLLECTION OR DUE TO THE PROXIMITY TO THE TUMOUR.

The most collected biofluids are urine and saliva, and thus, are completely non-invasive and provide an economic solution to collect the sample that does not require a medical professional, and can even be performed at home under instruction.

SALIVA

Saliva is one of the easiest-to-collect biofluids and is often used for diagnostic purposes. Saliva offers a promising source of biomarkers for cancer.

Mulvey et al.[1] studied saliva samples and plasma for somatic mutations and HPV in patients with squamous cell carcinomas, and found that saliva was preferentially enriched for tumour DNA in the oral cavity compared to plasma. This indicates that not only are other biofluid biopsies an option for exploration due to the ease of sample collection, they could, in some cases, also be a more reliable test.

Additionally, a recent meta-analysis explored the diagnostic value of salivary biomarkers and found salivary biomarkers had an 85% accuracy for non-oral tumours. This could potentially serve to be a huge area of interest, particularly in low-income countries where the ability to perform diagnostic tests yourself would be hugely advantageous[2].

URINE

For studying urinary tract cancers, urine could potentially be "liquid gold" due to a potential abundance of tumour material for biopsy[3]. As urine has direct contact with bladder tumours, you can potentially expert large tumour marker quantities in the urine. Additionally, the urine can be collected at several diagnostic stages to help in clinical decision making.

There currently are several urine biomarkers that are FDA-approved for detection and surveillance of urothelial malignancies. Urine biopsies have the potential to be both a predictive and prognostic biomarker.

However, despite the abundance, urine DNA is associated with a low mutant allele fraction, which is a significant challenge. In addition, tumour DNA in urine is prone to degradation due to the absence of proper storage during transportation for analysis. Thus, urine biopsies will likely require new preservative technologies to enable analysis.

Did you know that yellow can be the new red?

Urine: a promising sample type for cancer detection and monitoring

In comparison to blood, urine sampling is **easy, non-invasive**, available in **larger quantities** and not limited by the health status of the patient.

A high number of potentially informative **cancer biomarkers**, including DNA, RNA, proteins, extracellular vesicles and metabolites can be found in urine.

Urine can be used as a **liquid biopsy** for urogenital **cancers** such as bladder, prostate, colorectal, renal, ovarian, endometrial and cervical cancer. It also has potential in **other cancer types**, including thyroid, breast, lung and pancreatic cancers.

A **urine preservation medium** can improve sample stability and maintain the quality of the collected samples for storage and transport, allowing for home collection.

Visit our website for more information
https://novosanis.com/liquid-biopsy-urine-sample-type

Jordaens S, Mehta A, Van Avondt Q, Pasmans D, Beyers K, Vankerckhoven V - Urine as a liquid biopsy - is it the holy grail? September 2019.

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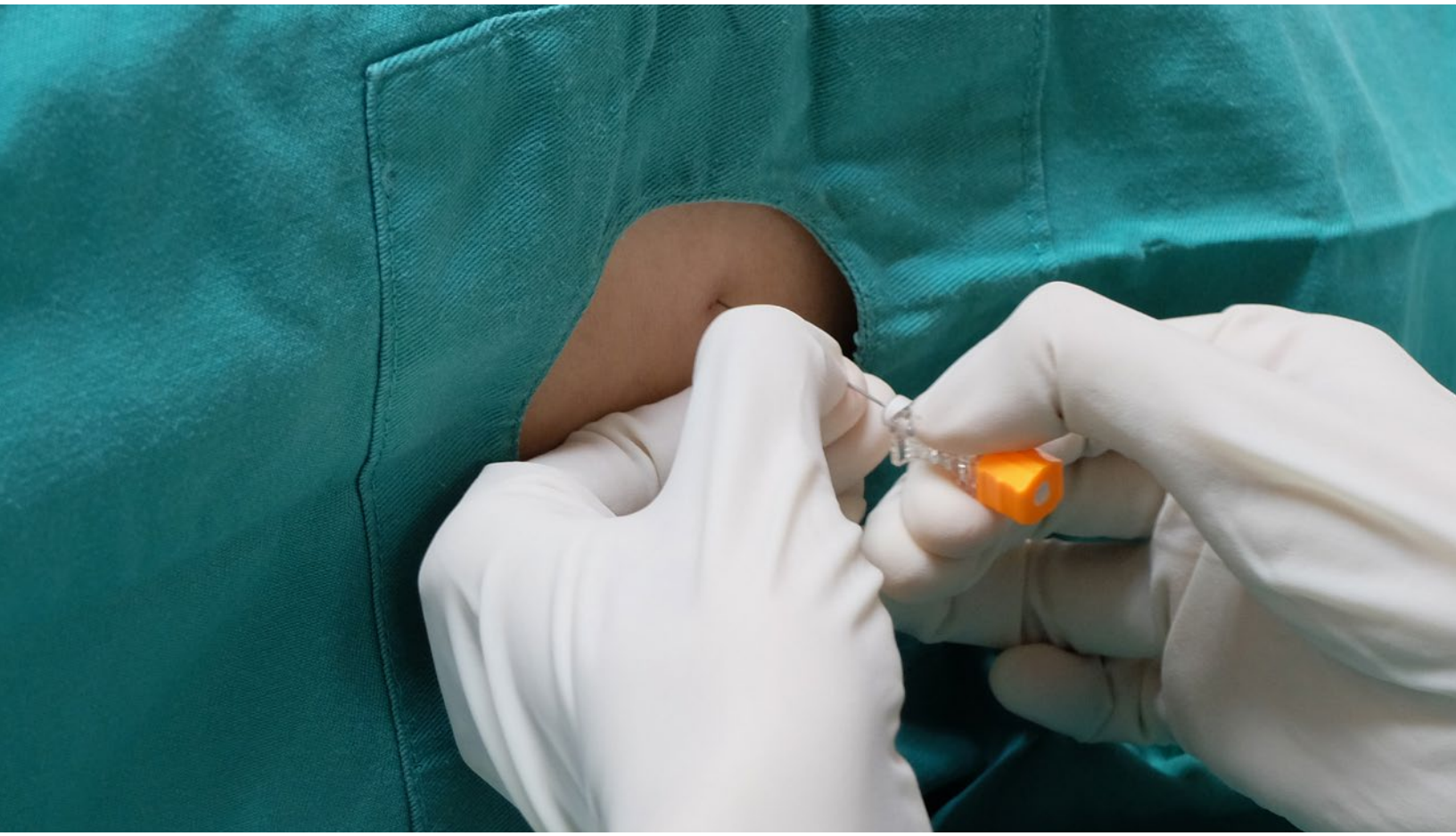

CEREBROSPINAL FLUID

An exciting area of non-blood biopsy research is the opportunity to study cerebral spinal fluid (CSF) for the presence of CTCs as, in healthy patients, the CSF would be free of cells. Malignancies of the central nervous system (CNS) tend to be associated with poor prognosis, likely due to the low rate of early diagnosis and limited understanding of tumour progression. Currently, the standard method for diagnosing and monitoring CNS tumours is via imaging techniques, but this does not provide molecular information and has limited applicability to inform on treatment response or disease progression. On the other hand, repetitive sampling of CNS tumours is usually highly invasive and, in some cases, difficult to obtain due to the tumour location.

For CNS tumours, the CSF is often in intimate contact with tumour lesions, and it can serve as an alternative fluid to monitor the molecular changes of tumour progression in patients. ■

"CURRENTLY, THE STANDARD METHOD FOR DIAGNOSING AND MONITORING CNS TUMOURS IS VIA IMAGING TECHNIQUES, BUT THIS DOES NOT PROVIDE MOLECULAR INFORMATION AND HAS LIMITED APPLICABILITY TO INFORM ON TREATMENT RESPONSE OR DISEASE PROGRESSION.

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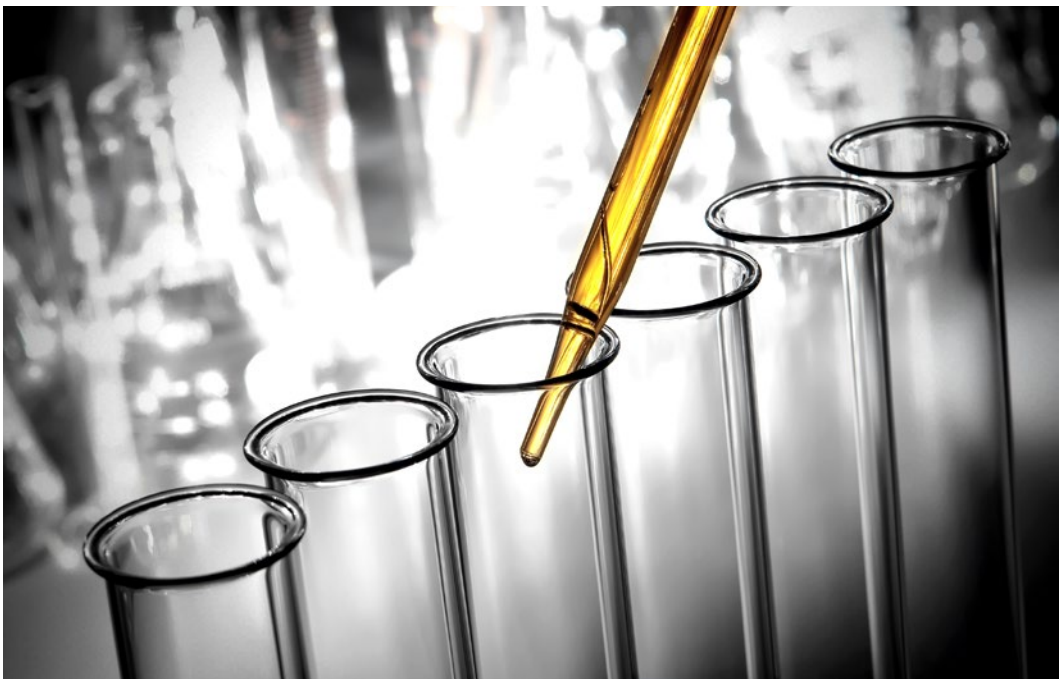
3. Satyal U, Srivastava A, Abbosh PH. Urine Biopsy-Liquid Gold for Molecular Detection and Surveillance of Bladder Cancer. Front Oncol. 2019;9:1266. Published 2019 Nov 19. doi:10.3389/fonc.2019.01266

FUTURE DIRECTIONS

THERE IS LITTLE DOUBT THAT LIQUID BIOPSIES HAVE THE POTENTIAL TO TRANSFORM THE WAY WE DIAGNOSE AND MONITOR CANCERS. SO, WHAT DOES THE FUTURE LOOK LIKE FOR LIQUID BIOPSIES?

It is becoming increasingly apparent that there will not be a clearly defined protocol for monitoring all cancers through a single liquid biopsy. This is due to the nature of diverse cancer markers that are, at times, tumour specific. Therefore, the future of liquid biopsies will likely involve the identification of multiple specific combinations of markers that inform clinicians about cancer's status, origin and progression[1]. In a recent Nature outlook article, Dr Alix-Panabieres recommended the development of an algorithm that can combine the data from circulating tumour biomarkers with other aspects of the circulating microenvironment that can guide treatment choices.



In addition, the monitoring of circulating tumour markers will probably need to be coupled with other biological markers, such as immune cells to monitor immunotherapy responses etc. A fuller and more comprehensive picture of tumour progression through these non-invasive sampling methods will help clinicians make better-informed decision-making.

From a collaboration perspective, much work is still needed to develop standardised pre-analytical and analytical protocols for liquid biopsies, and to meet the rigorous regulatory standards[2].

"FROM A COLLABORATION PERSPECTIVE, MUCH WORK IS STILL NEEDED TO DEVELOP STANDARDISED PRE-ANALYTICAL AND ANALYTICAL PROTOCOLS FOR LIQUID BIOPSIES, AND TO MEET THE RIGOROUS REGULATORY STANDARDS."

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1. Alix-Panabières C. The future of liquid biopsy. Nature. 2020;579(7800):S9-S9. doi:10.1038/d41586-020-00844-5
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