LIQUID BIOPSY

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Liquid biopsies are a general process of examining cells, RNA, DNA or exosomes that are in the blood and from this analysis ascertaining the presence or prognosis of a cancer. We examine some of the recent work in this field which extends what we did in the area of prostate sourced oncosomes. Copyright 2018 Terrence P. McGarty, all rights reserved.

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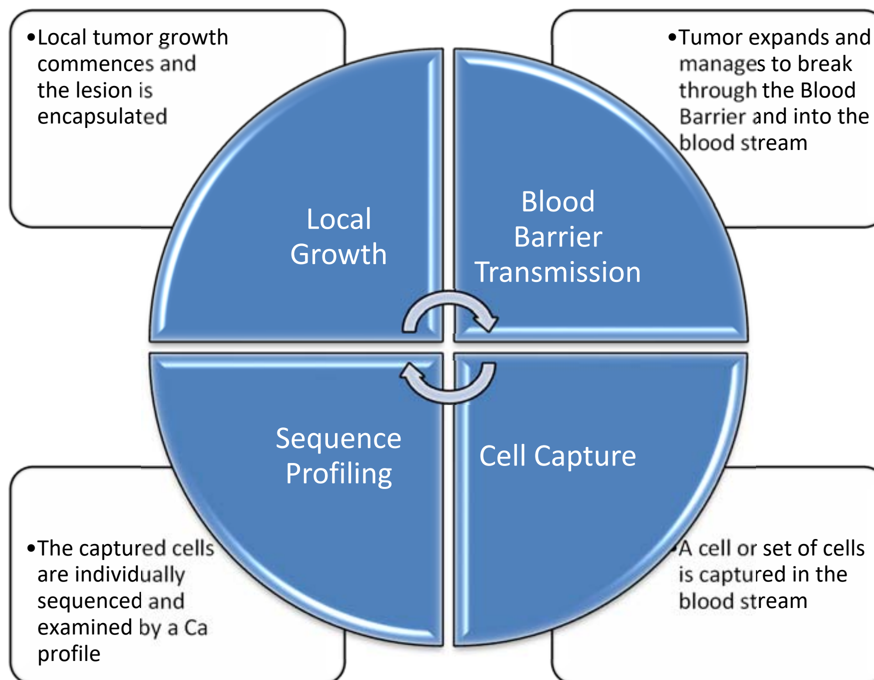
1 INTRODUCTION

Cancer has for years been diagnosed via a biopsy of the focal lesion tissues. Prostate cancer was diagnosed based upon biopsies of the prostate, often from samples taken in the "dark", namely needle biopsies guided by ultrasound, but little else. Melanoma was diagnosed by a skin biopsy, examining a lesion seen visually and then examined histologically. Some lesions are examined via immunological tests or other such tests. In general, this is done on an inspection of specific tissues.



It is also known that primary as well as metastatic lesions slough off cells, proteins, RNA or DNA, or exosomes of various types which end up in the blood stream. Primary lesions also use the blood stream as a means to metastasize, as well as the lymphatic system. Thus there has been an interest in using what is in the blood stream to see if there is a cancerous growth, to be used as a prognostic tool, and even to be used as a means to develop some form of precision therapeutic.

The advantage of sampling the blood is that it is readily accessible. The disadvantages are multiple:

1. We really have no idea where the cells or components have come from. Are they primary or a met, are they from a truly virulent cancer or just an incidental finding?

2. We always face the problem of a cancer stem cell or cell of origin. Thus what we analyze may be a progeny which is not the driving factor in the development of the cancer.

3. The "tool" problem is always there. Namely, do we have tool adequate to ascertain a single cell or cell component to achieve the desired specificity and sensitivity?

Many of these hurdles are being overcome and the examination of a individual based on sampling of the blood is now within reach. This is essentially the field of "liquid biopsy".

1.1 What is a Liquid Biopsy?

Some of the earliest work on cancer cell shedding was done in the early 1970s and reported by Butler and Gullino who noted:

The rate of tumor cell shedding into efferent tumor blood was measured in growing and regressing MTW9 rat mammary carcinomas. The hormone-dependent tumor, grown as an isolated preparation, permitted collection of all of the efferent blood. Regression was induced by reduction of mammotropin level in the host. Tumor cells were differentiated from normal leukocytes by indirect immunofluorescence. Growing tumors shed 3.2 x 10^6 and regressing tumors shed 4. 1 x 10^6 cells per 24 hr per g tissue.

Cell shedding rates of growing versus regressing tumors were not significantly different over a tumor size range of 2 to 4 g. The number of tumor cells in the arterial blood was 12-fold smaller than in the efferent tumor blood. It is concluded that: (a) cell shedding via blood probably plays only a minor role in the total cell loss by growing MTW9 carcinomas; (b) hormone-induced tumor regression does not depend on increased cell shedding; (c) tumor cells are rapidly cleared from circulating blood; and (d) a 2-g MTW9 carcinoma pours enough cells into the host circulation to transplant the tumor every 24 hr.

More recently Harris et al (2018) have noted[1]:

Circulating tumor cells (CTCs) are released at a rate of 3.2x10^6 cells/g tissue daily, however, <0.01% of these cells will initiate metastases. It remains unknown how CTCs initiate metastases due to an inability to examine the journey of CTCs from the primary tumor to the metastatic niche. A subset of CTCs postulated to drive metastasis are cancer stem cells (CSCs). The CSC theory postulates that a subpopulation of tumor cells remaining after resection drive recurrence, while the CTCs surviving the circulation and arresting at metastatic sites driving tumor growth are metastatic CSC. In either case, CSCs are capable of self-renewal and asymmetric division and may be able to recapitulate the initial tumor heterogeneity. Further, these CSCs are more resistant to most treatments. While multiple markers were proposed for prostate cancer CTCs, most have yet to be validated in the circulation of patients.

Thus there is a continual shedding of tumor cells and the above result has been verified many times over the past decades. But again, this shedding is from anywhere the tumor may have resided, and in its journey in the blood stream we would find it difficult to determine that. However we have argued elsewhere that it may very well be possible to determine the location of the cell by its surface markers which often are descriptive of from whence it came[2].

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[1] https://www.biorxiv.org/content/early/2018/04/23/256107

[2] https://www.researchgate.net/publication/271907544_Cancer_Cellular_Dynamics

As noted in the NCI site[3], the current working definition of "liquid biopsy" is as follows:

A test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor 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 tumor.

In a recent paper by Harris et al (2018) the authors note:

Cancer stem-like cells (CSCs) are associated with cancer progression, metastasis, and recurrence. CSCs may also represent a subset of tumor-initiating cells, tumor progenitor cells, disseminated tumor cells, or circulating tumor cells (CTCs); however, which of these aggressive cell populations are also CSCs remains to be determined. In a prior study, CTCs in advanced prostate cancer patients were found to express CD117/c-kit in a liquid biopsy.

Whether CD117 expression played an active or passive role in the aggressiveness and migration of these CTCs remained an open question. In this study, we use LNCaP-C4-2 human prostate cancer cells, which contain a CD117+ subpopulation, to determine how CD117 expression and activation by its ligand stem cell factor (SCF, kit ligand, steel factor) alter prostate cancer aggressiveness. CD117+ cells displayed increased proliferation and migration.

Further, the CD117+ cells represented a CSC population based on stemness marker gene expression and serial tumor initiation studies. SCF activation of CD117 stimulated increased proliferation and invasiveness, while CD117 inhibition by tyrosine kinase inhibitors (TKIs) decreased progression in a context-dependent manner. We demonstrate that CD117 expression and activation drives prostate cancer aggressiveness through the CSC phenotype and TKI resistance.

This article highlights several interesting areas and their convergence.

First, the reassessment of the cancer stem cell especially as applied to PCa.

Second, the use of CTCs to assess the progress of a disease and thus establish a reliable prognostic marker.

Third, the identification of CD117 as a specific marker for an aggressive form of PCa.

The authors provide an interesting platform for this convergence but it also allows for a window on all three of these areas. CTCs are receiving more attention. We know that cancer cells leave local sites, travel through the blood to distant sites. Likewise these distant sites also slough off

[3] https://www.cancer.gov/publications/dictionaries/cancer-terms/def/liquid-biopsy

cells or parts of cells. We have previously examined this for oncosomes in prostate cancer a while back[4].

Thus we may ask; why a liquid biopsy at all? Zhe et al give a fundamental answer:

Despite major advances in research and therapy, cancer continues to be the second cause of death in the United States, with 1 in 4 deaths due to cancer. Primary tumors rarely have deadly consequences, while metastatic disease accounts for around 90% of the mortality due to solid tumors. Therefore, the development of new sensitive methods that allow the detection of cancer dissemination, most notably in the common carcinomas, before full blown clinically detectable gross metastatic deposits are established is of tremendous utility to help physicians in treatment decisions.

Basically a liquid biopsy presages metastatic growth. Localized tumors, often termed "carcinoma in situ" are just that, local. The cells may have begun to proliferate but they do so locally and have not begun to wander into the blood or lymph system.

1.2 Definitions

Domínguez-Vigil et al present a recent update as to what can be the basis for detection of cancers via hematological sampling. They note:

Circulating tumor DNA (ctDNA): DNA is continuously released in fragments into the circulation through processes such as apoptosis and necrosis by both normal and cancerous cells. When released irrespective of cell of origin, it is typically referred to as cfDNA (cell-free DNA); but when released specifically by cancer cells, it is mostly referred to as ctDNA (circulating tumor DNA). Among the molecular characteristics of ctDNA are that it may harbor mutations, CNVs, methylation changes, or integrated viral sequences associated with the tumor

Circulating tumor cells (CTCs): CTCs have been discovered for Asworth in 1869 during an autopsy of a patient who had metastatic cancer. They are cancer cells that detach from a primary or metastatic tumor site and are present in the circulation. Clinical evidence indicates that patients with metastases have 1–10 CTCs per mL of blood and they are rarely found in clinically healthy people or in people with nonmalignant tumors. CTCs have been detected in different types of cancers, such as breast, ovarian, prostate, lung, colorectal, hepatocellular, pancreatic, head and neck, bladder, and melanoma

Exosomes: Exosomes are small round vesicles, 30–120 nm in diameter, and of endosomal origin carrying RNA, miRNAs, DNA, and proteins that are released by multiple cell types (including tumor cells) into the extracellular environment. Exosomes may mediate some form of communication between cells, being internalized by other cells

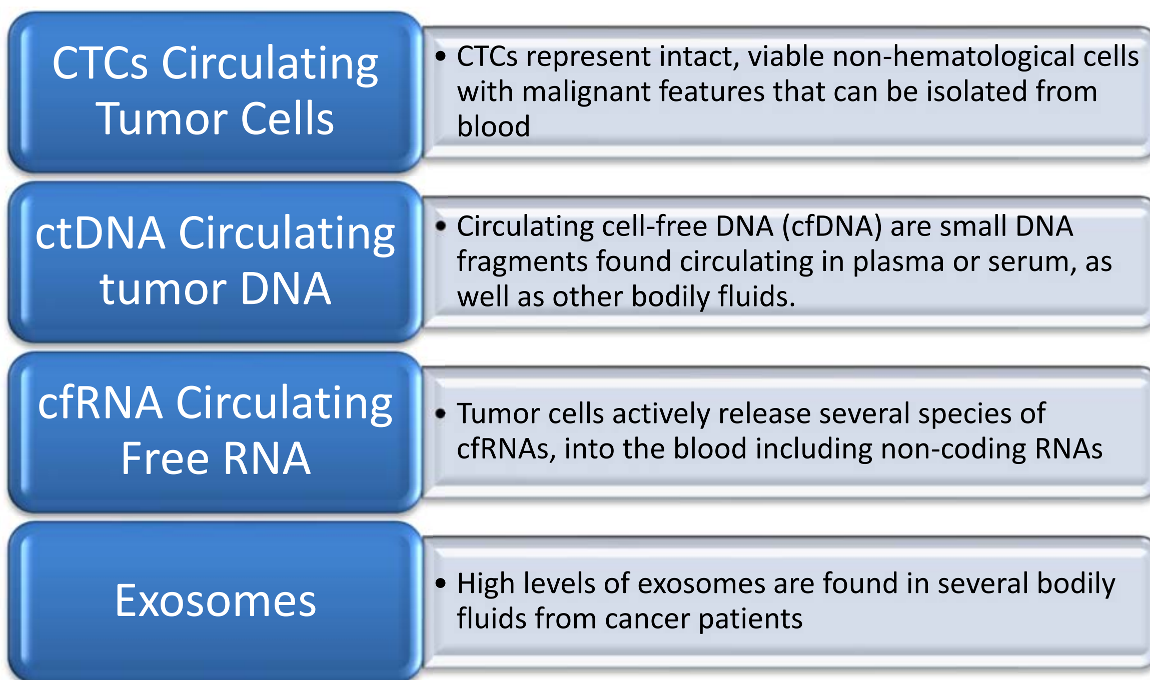
miRNAs: MicroRNAs or miRNAs are small molecules of non-coding RNA, between 19 and 24 nucleotides in length, that act as regulatory molecules of gene expression, exerting function by

[4] See McGarty, Oncosome, 2013.

hybridizing to inhibit the translation of mRNAs of its target genes. Differential expression of miRNAs in patients with cancer has been described.

1.3 VARIOUS OPTIONS

Liquid biopsies come in many different forms. We outline them in the figure below and then provide a brief discussion (see Abraham et al).



2 HEMATOLOGICAL SPREAD

The major question that must be posed is; how does a tumor in some solid tissue break through the vascular system and enter the blood stream? We know that it happens, it is the basis of metastatic spread. We further know that not only does it enter the blood stream but that it exits the blood stream in places most suited for its growth. Thus melanoma may spread to the lung and brain. In the brain the blood-brain barrier is quite well controlled but despite that the melanoma cells manage to escape and prosper.

2.1 CANCER CELL MOVEMENT THROUGH THE BLOOD

The movement of cancer cells from the initial site of development and to distant sites is a complex problem. We have previously provided a high level model for this process but it makes significant assumptions of the movement parameters which may not necessarily be reflective of the prime biochemical processes actually involved . In a recent paper by Chen et al the authors have modeled the movement from the blood stream into distant organs.

The metastatic process is a complex concatenation of loss of cell localization and cell survival and proliferation in new environments. Generally as cells mature into specific cell types they become localized to their environment, such as in melanocytes and the E cadherin binding . Cells also work within their own environment via communications with the extra cellular matrices .

The authors state:

We demonstrate tight endothelial cell–cell junctions, basement membrane deposition and physiological values of vessel permeability. Employing our assay, we demonstrate impaired endothelial barrier function and increased extravasation efficiency with inflammatory cytokine stimulation, as well as positive correlations between the metastatic potentials of MDA-MB-231, HT-1080, MCF-10A and their extravasation capabilities.

High resolution time-lapse microscopy reveals the highly dynamic nature of extravasation events, beginning with thin tumor cell protrusions across the endothelium followed by extrusion of the remainder of the cell body through the formation of small (1 mm) openings in the endothelial barrier which grows in size (8 mm) to allow for nuclear transmigration. No disruption to endothelial cell–cell junctions is discernible at 60 X, or by changes in local barrier function after completion of transmigration. Tumor transendothelial migration efficiency is significantly higher in trapped cells compared to non-trapped adhered cells, and in cell clusters versus single tumor cells.

Specifically the investigators have developed a mechanical model of the vasculature, one they can manipulate for analytical purposes, and then they demonstrate the movement of the malignant cells across the interface into a quasi-cellular environment.

As Chu states:

Now researchers at MIT have developed a microfluidic device that mimics the flow of cancer cells through a system of blood vessels. Using high-resolution time-lapse imaging, the researchers captured the moments as a cancer cell squeezes its way through a blood vessel wall into the surrounding extracellular matrix.

She continues:

As tumor cells make their way through the circulatory system, some "arrest," or pause at a particular location, adhering to a blood vessel's wall — the first stage of extravasation. Scientists have thought that this cell arrest occurs in one of two ways: A cell may send out sticky projections that grab onto the vessel lining, or it may be too big to pass through, literally becoming trapped within the vessel.

To investigate which possibility is more likely, the researchers grew a network of tiny blood vessels from a solution of human umbilical-cord endothelial cells. They injected a solution containing vascular cells into a small microfluidic device containing a reservoir of hydrogel, along with growth factors normally present in the developing circulatory system. Within days, an intricate system of microvessels took shape, with each about one millimeter long and 10 to 100 microns in diameter — dimensions similar to the body's small capillaries.

The group then pumped tumor cells through the vascular network, using a line of breast cancer cells known to be particularly invasive. Using high-resolution confocal microscopy, the team watched as tumor cells flowed through the miniature circulatory system. They observed that the majority of cells that arrested along a vessel did so due to entrapment — that is, they simply became stuck.

The observations are unfortunately in vitro and in a constructed environment and lack much of the biochemical elements that often make up for the transport. Although this is interesting in principle it fails to substantiate all of the elements which make up an in vivo process.

Then she concludes:

In addition to observing the extravasation of single tumor cells, the group also looked at the behavior of cell clusters — two or more cancer cells that accumulate in a blood vessel. From their observations, the researchers found that almost 70 percent of cell clusters broke through a blood barrier, compared with less than 10 percent of single cells.

But some cells that make it out of the circulatory system may still fail to metastasize. To see whether a cell's ability to extravasate correlates with its metastatic potential, the group compared the efficiency of extravasation of different cancer cell lines. The lines included breast cancer cells, cells from fibrosarcoma (a cancer of the connective tissue), and a line of nonmetastatic cancer cells.

Sure enough, the team observed that the most metastatic cells (fibrosarcoma cells) were also the most likely to extravasate, compared with breast cancer and nonmetastatic cells — a finding suggesting that targeting drugs to prevent extravasation may slow cancer metastasis.

The problem is that the cancer cells move in and out. There is a continual flow and at the same time they have the problem of mutating as well.

From the paper the authors note:

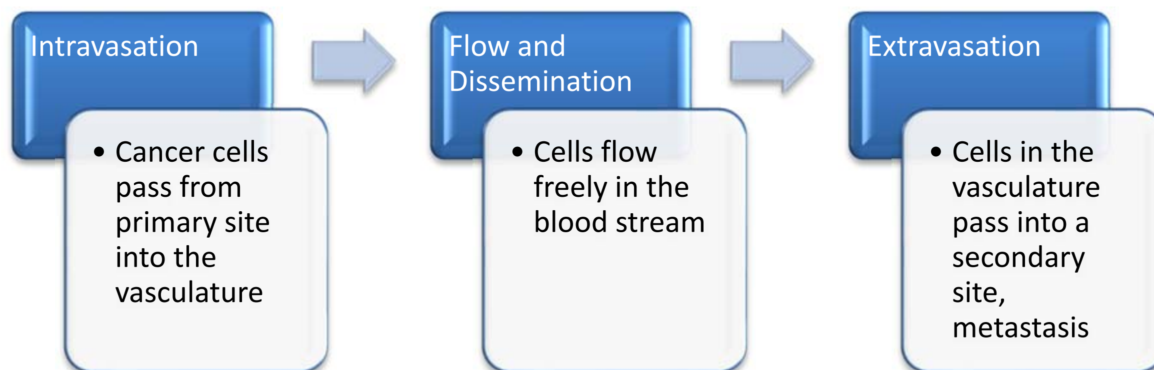
A deeper understanding of the mechanisms of tumor cell extravasation is essential in creating therapies that target this crucial step in cancer metastasis. Here, we use a microfluidic platform to study tumor cell extravasation from in vitro microvascular networks formed via vasculogenesis. We demonstrate tight endothelial cell–cell junctions, basement membrane deposition and physiological values of vessel permeability.

Employing our assay, we demonstrate impaired endothelial barrier function and increased extravasation efficiency with inflammatory cytokine stimulation, as well as positive correlations between the metastatic potentials of MDA-MB-231, HT-1080, MCF-10A and their extravasation capabilities. High-resolution time-lapse microscopy reveals the highly dynamic nature of extravasation events, beginning with thin tumor cell protrusions across the endothelium followed by extrusion of the remainder of the cell body through the formation of small (∼1 μm) openings in the endothelial barrier which grows in size (∼8 μm) to allow for nuclear transmigration. No disruption to endothelial cell–cell junctions is discernible at 60×, or by changes in local barrier function after completion of transmigration.

Tumor transendothelial migration efficiency is significantly higher in trapped cells compared to non-trapped adhered cells, and in cell clusters versus single tumor cells.

2.2 In and Out of the Blood

The very nature of cancers is their ability to spread almost at will. In spreading the use the hematological system, specifically the blood stream. They must get into the blood and then out of it. The blood system is generally a patent system, keeping random cells from entering.



As Hanahan and Coussens have noted:

The vasculature plays a similar role in metastatic seeding at distant sites, where an intact normal endothelium with intimate pericyte coverage can be envisaged to block cancer cell extravasation from the blood into normal parenchyma. Indeed, it is increasingly evident that metastatic primary tumors can precondition the vasculature in metastatic sites with factors such as VEGF, supplied systemically or produced locally by the disseminated cancer cells they spawn; the actions of VEGF on the endothelium at incipient metastatic sites ***facilitates both loosening of vessel walls for extravasation, and subsequent induction of angiogenesis to support metastatic tumor growth****. Still to be clarified is the identification and possible roles of factors produced by endothelial cells and pericytes that contribute to metastatic processes….*

Concentration gradients of growth factors established by leukocytes also coordinate tumor cell movement toward, and ***intravasation into, tumor-associated vasculature****. For example, macrophages are the primary source of EGF in the developing mammary gland and in mouse models of breast cancer. EGF promotes invasion/ chemotaxis and intravasation of breast carcinoma cells through a paracrine loop operative between tumor cells and macrophages that are required for mammary cancer cell migration via cofilin-dependent actin polymerization. Transcriptome profiling has revealed that the TAMs participating in this paracrine interplay represent a unique subpopulation that associates intimately with tumor vessels*

2.3 Metastasis

As Grivennikov et al note:

The process of metastasis can be grossly divided into four major steps.

1. The first step is represented by epithelial-mesenchymal transition, in which cancer cells acquire fibroblastoid characteristics that increase their motility and allow them to invade epithelial linings/basal membranes and reach efferent blood vessels or lymphatics. Loss of E-cadherin expression is envisioned as a key event in the epithelial-mesenchymal transition.

2. In the second step, cancer cells intravasate into blood vessels and lymphatics. Inflammation may promote this through production of mediators that increase vascular permeability.

3. This is followed by the third step, in which metastasis- initiating cells survive and travel throughout the circulation. It has been estimated that only about 0.01% of cancer cells that enter the circulation will eventually survive and give rise to micrometastases.

4. Next, integrin-mediated arrest allows the extravasation of circulating cancer cells. Finally, single metastatic progenitors interact with immune, inflammatory, and stromal cells and start to proliferate. Some of these cells may already be targeted to the premetastatic niche in response to tumor-generated inflammatory signals prior to the arrival of metastasis-initiating cancer cells. One of these inflammatory signals is the extracellular matrix component versican, which leads to macrophage activation and production of the metastasis promoting cytokine TNF-a. However, it has been difficult to determine whether versican production by metastatic cancer cells conditions the future metastatic site prior to their arrival.

3 BLOOD BORNE ANALYSIS

We now examine some of the elements associated with identification and detection of cells which have usefulness in cancer existence and progression.

3.1 CTC

We first start with the circulating tumor cells, CTC, the prime source of information on the tumor itself. One must recall that DNA and RNA segments are suggestive and exosomes are the same. The cell itself carries with it a wealth of information on the specific malignancy, its state of development and even its location. As Lianidou et al (2015) have noted:

Detection of Circulating Tumor Cells (CTCs) in peripheral blood can serve as a "liquid biopsy" approach and as a source of valuable tumor markers. CTCs are rare, and thus their detection, enumeration and molecular characterization are very challenging. CTCs have the unique characteristic to be non-invasively isolated from blood and used to follow patients over time, since these cells can provide significant information for better understanding tumour biology and tumour cell dissemination.

CTCs molecular characterization offers the unique potential to understand better the biology of metastasis and resistance to established therapies and their analysis presents nowadays a promising field for both advanced and early stage patients. In this chapter we focus on the latest findings concerning the clinical relevance of CTC detection and enumeration, and discuss their potential as tumor biomarkers in various types of solid cancers. We also highlight the importance of performing comparison studies between these different methodologies and external quality control systems for establishing CTCs as tumor biomarkers in the routine clinical setting.

As Castro-Giner et al note:

Circulating tumor cells (CTCs) are defined as those cells that detach from a cancerous lesion and enter the bloodstream. While generally most CTCs are subjected to high shear stress, anoikis signals, and immune attack in the circulatory system, few are able to survive and reach a distant organ in a viable state, possibly leading to metastasis formation. A large number of studies, both prospective and retrospective, have highlighted the association between CTC abundance and bad prognosis in patients with various cancer types.

Yet, beyond CTC enumeration, much less is known about the distinction between metastatic and nonmetastatic CTCs, namely those features that enable only some CTCs to survive and seed a cancerous lesion at a distant site. In addition, critical aspects such as CTC heterogeneity, mechanisms that trigger CTC intravasation and extravasation, as well as vulnerabilities of metastatic CTCs subpopulations are poorly understood. In this short review, we highlight recent studies that successfully adopted functional and computational analysis to gain insights into CTC biology. We also discuss approaches to overcome challenges that are associated with CTC

isolation, molecular and computational analysis, and speculate regarding few open questions that currently frame the CTC research field.

The author continues:

Molecular interrogation of CTCs has been made possible not only by the development of specialized CTC isolation technologies but also with the achievement of single-cell-resolution-sequencing protocols and single-cell-based assays. The application of these approaches to CTCs has already generated a number of exciting observations, many of which provide insightful information in regard to features of metastatic precursors, CTC heterogeneity, and patient stratification.

CTC isolation requires considerable technical capabilities. First it requires the detection of the cell, where there are very few CTCs in the blood to begin with. Then it requires the extraction of the cell, and finally the analysis of the cell. The latter point means that we have well understood markers for various malignancies. Thus we face a very complex task.

The recent approach, called CancerSEEK, has the following elements with regard to its processing (see Cohen et al):

1…designed a 61- amplicon panel, with each amplicon querying an average of 33 base pairs (bp) within one of 16 genes

2. ..this panel would theoretically detect 41% (liver) to 95% (pancreas) of the cancers …

3. On the basis of this analysis of the DNA from primary tumors, the predicted maximum detection capability of circulating tumor DNA (ctDNA) in our study varied by tumor type, ranging from 60% for liver cancers to 100% for ovarian cancers …

4. …used multiplex-PCR to directly and uniquely label each original template molecule with a DNA barcode. This design minimizes the errors inherent to massively parallel sequencing and makes efficient use of the small amount of cell-free DNA present in plasma.

5. .. divided the total amount of DNA recovered from plasma into multiple aliquots and performed independent assays on each replicate. In effect, this decreases the number of DNA molecules per well; however, it increases the fraction of each mutant molecule per well, making the mutants easier to detect.

6. Many proteins potentially useful for early detection and diagnosis of cancer have been described in the literature. We searched this literature to find proteins that had previously been shown to detect at least one of the eight cancer types described above with sensitivities >10% and specificities >99%.

7. We identified 41 potential protein biomarkers (table S3) and evaluated them in preliminary studies on plasma samples from normal individuals as well as from cancer patients.

8. We found that 39 of these proteins could be reproducibly evaluated through a single immunoassay platform, and we then used this platform to assay all plasma samples.

9. Eight of the 39 proteins proved to be particularly useful for discriminating cancer patients from healthy controls.

3.2 Other Detection Mechanisms

Zhe et al discuss methods for detection, They note:

The identification of CTCs is achieved through different techniques that are typically used in combination with CTC enrichment procedures (see next section). These methodologies, summarized in recent reviews, are essentially constructed on antibody-based or nucleic acid-based approaches.

In the first case, antibodies targeted to epithelial-specific antigens (e.g., epithelial cell adhesion molecule [EpCAM], or cytokeratins [CKs]), tissue-specific antigens (e.g., prostate specific antigen [PSA] in prostate cancer), or tumor- associated antigens (e.g., mammaglobin in breast cancer, or carcinoembryonic antigen [CEA] in colon cancer) are employed, since tumor-specific antigens have not been identified in most cancers.

As a consequence of this, antibody-based techniques usually have low specificity, and anti-CD45 antibodies are frequently used to distinguish potentially contaminating leukocytes. One of the advantages of these techniques is that they do not require cell lysis, allowing morphological characterization, enumeration, and molecular characterization (e.g., fluorescent in situ hybridization [FISH]) of CTCs.

However, these methods are hampered by the low concentration of CTCs in blood. Despite this drawback, flow cytometry and laser scanning cytometry … are currently being used to detect CTCs, since these methods are rapid, quantitative, can simultaneously analyze multiple parameters, such as size, DNA content, and specific antigens, and identify viable from nonviable cells. Despite their high specificity, and the advantage of LSC® over flow cytometry in allowing morphological analysis through automated fluorescence microscopy, both methods have low sensitivity, requiring large sample volumes to detect a few CTCs if no previous enrichment is done. The fiber-optic array scanning technology (FAST) is an ultraspeed technology that can scan up to 3x105 cells per second and can detect CTCs with fluorescently labeled antibodies directly on a slide, without CTC enrichment required.

Another antibody-based method potentially useful for CTC detection is EPISPOT (epithelial im-munospot), an assay based on the enzyme- linked immunosorbent spot (ELISPOT) assay, which is a common method for monitoring immune responses. EPISPOT is based on the identification of specific soluble proteins secreted by single viable epithelial tumor cells, such as cathepsin D and mucin-1 in breast cancer, and full-length CK19 in different cancers. This assay is usually used with enrichments methods, and allows the indirect identification of viable epithelial cancer cells cultured for up to 48 hours via specific immunocapture of the proteins released by

unlabeled antibodies immobilized on the bottom of the well, and then by addition of biotinylated or fluorochrome- conjugated antibodies.

Each immunospot detected by either immunohistochemistry or immunofluorescence, is considered the fingerprint left only by one viable cancer cell releasing the protein under analysis. Despite being the only assay available that can detect cancer cells on the basis of secreted/shed proteins, it has not been tested until now in large clinical trials.

3.3 Some Summaries

From Castro-Giner et al we have an interesting summary of the targets used in various testing procedures:

Molecular Assay	***Target***
Tracing fluorescently labeled cancer cells in circulation	Quantification of the metastatic potential of single CTCs and CTC clusters
Quantitative mass spectrometry of cultured CTCs	Detection of protein expression levels to identify differentially regulated proteins upon drug treatment
Quantitative RNA	Assessment of dynamically expressed transcripts upon drug treatment and identification of CTC subpopulations.
Direct xenograft transplantation of patient-derived CTCs	Phenotypic analysis of metastasis-initiating CTCs
Single-cell RNA sequencing	Detection of gene expression changes to identify differentially regulated genes and pathways in individual CTCs
Single-cell DNA sequencing	Identification of single nucleotide variants (SNPs), insertions, deletions, amplifications, and translocations to determine the genomic landscape of individual CTCs

In addition Alix-Panabières and Pantel have summarized the available techniques and focusing them on cells and DNA. They state:

1. CTCs

Recent progress has been made in the development of various devices to enrich and detect CTCs, including the discovery and validation of new CTC markers). It is important to note that the CTC field focused on the biology of tumor dissemination and in particular on EMT affecting tumor

cells with potential stem cell–like properties. As a consequence, many groups optimized new devices to select and detect CTCs that underwent EMT.

As reviewed recently, CTC assays usually start with an enrichment step that increases the concentration of CTCs by several log units and enables an easier detection of single tumor cells. Subsequently, CTC can be detected in different ways.

In principle, CTCs can be positively or negatively enriched on the basis of biologic properties (i.e., expression of protein markers) or on the basis of physical properties (i.e., size, density, deformability, or electric charges). Positive or negative CTC enrichment can also be achieved on the basis of a combination of physical and biologic properties in the same device. CTCs can then be detected using immunologic, molecular, or functional assays. In the past, many research teams focused on functional tests using CTC cultures/cell lines and xenografts. These in vitro and in vivo models can be used to test drug susceptibility.

However, in order to contribute to personalized medicine, the efficacy of establishing CTC cultures and xenografts needs to be enhanced. So far, hundreds of CTCs are needed to establish a cell line or xenograft, which limits this approach to few patients with advanced disease. The focus on new technical developments based on discoveries on CTC biology has slowed down the introduction of CTCs into clinical diagnostics.

However, new important insights into the biology of CTCs combined with various innovative technologies have been reported, and technical platforms for combined enrichment, detection, and characterization of CTCs are on the horizon.

2. ctDNA

Highly sensitive and specific methods have been developed to detect ctDNA, including BEAMing Safe-SeqS, TamSeq, and digital PCR to detect single-nucleotide mutations in ctDNA or whole-genome sequencing to establish copy-number changes.

In principle, technologies can be divided into targeted approaches that aim to detect mutations in a set of predefined genes (e.g., KRAS in the context of EGFR blockade by antibodies) or untargeted approaches (e.g., array-CGH, whole-genome sequencing, or exome sequencing) that aim to screen the genome and discover new genomic aberrations, e.g., those that confer resistance to a specific targeted therapy.

The strengths and limitations of these technologies have been recently discussed. In general, targeted approaches have a higher analytic sensitivity than untargeted approaches, despite strong efforts to improve detection limits. Recently, we have seen an emergence of ultrasensitive technologies able to detect the smallest amounts of ctDNA in the "sea" of normal cfDNA, which are needed for early detection of cancer or minimal residual disease.

Notwithstanding, there are no "gold" standards currently accepted. Also the above neglects the RNA and exosome elements.

4 APPLICATIONS

We now examine some of the work done on various cancers as reported in the literature. This is representative but not complete

4.1 PROSTATE

There are a multiple set of approaches to prostate cancer (PCa). For example, as Heitzer et al have noted (2013):

Patients with prostate cancer may present with metastatic or recurrent disease despite initial curative treatment. The propensity of metastatic prostate cancer to spread to the bone has limited repeated sampling of tumor deposits. Hence, considerably less is understood about this lethal metastatic disease, as it is not commonly studied. Here we explored whole genome sequencing of plasma DNA to scan the tumor genomes of these patients noninvasively. We wanted to make whole-genome analysis from plasma DNA amenable to clinical routine applications and developed an approach based on a benchtop high-throughput platform, i.e. Illuminas MiSeq instrument.

We performed whole-genome sequencing from plasma at a shallow sequencing depth to establish a genome-wide copy number profile of the tumor at low costs within 2 days. In parallel, we sequenced a panel of 55 high-interest genes and 38 introns with frequent fusion breakpoints such as the TMPRSS2-ERG fusion with high coverage. After intensive testing of our approach with samples from 25 individuals without cancer we analyzed 13 plasma samples derived from 5 patients with castration resistant (CRPC) and 4 patients with castration sensitive prostate cancer (CSPC).

The genome-wide profiling in the plasma of our patients revealed multiple copy number aberrations including those previously reported in prostate tumors, such as losses in 8p and gains in 8q. High-level copy number gains in the AR locus were observed in patients with CRPC but not with CSPC disease.

We identified the TMPRSS2-ERG rearrangement associated 3-Mbp deletion on chromosome 21 and found corresponding fusion plasma fragments in these cases. In an index case multiregional sequencing of the primary tumor identified different copy number changes in each sector, suggesting multifocal disease. Our plasma analyses of this index case, performed 13 years after resection of the primary tumor, revealed novel chromosomal rearrangements, which were stable in serial plasma analyses over a 9 months period, which is consistent with the presence of one metastatic clone.

The above fusion combination is a well known target. The issue is if this rearrangement persists as a driver of PCa after treatment as noted in the above. Namely can we see targetable gene profiles and yet not have a metastatically active cancer.

From Lianidou (2014)

In patients with advanced prostate cancer, CTC enumeration using the Veridex CellSearch system at baseline and posttreatment has been cleared by the FDA for quantifying the load of tumor cell dissemination. This test is prognostic of survival and is currently being implemented into routine clinical practice for estimating prognosis and monitoring treatment success58. The clinical utility of monitoring CTC changes with treatment, as an efficacy-response surrogate biomarker of survival, is currently being tested in large phase III trials with the novel anti-androgen therapies abiraterone acetate and MDV3100. Molecular determinants can be identified and characterized in CTCs as potential predictive biomarkers of tumor sensitivity to a therapeutic modality….

Since persistence of ligand-mediated androgen receptor (AR) signaling has been documented in CRPC, abiraterone acetate (AA), an androgen biosynthesis inhibitor, is used to prolong life in patients with CRPC already treated with chemotherapy. Miyamoto and colleagues have shown that measuring AR signaling within CTCs may help guide therapy in metastatic prostate cancer, highlighting the use of CTCs as a liquid biopsy. Leversha and colleagues have shown that FISH analysis of CTCs can be a valuable, non-invasive surrogate for routine tumor profiling in patients with progressive castration- resistant metastatic prostate cancer.

Recent results by Darshan and colleagues suggest that monitoring AR subcellular localization in the CTCs of CRPC patients might predict clinical responses to taxane chemotherapy. Moreover, coding mutations in the AR gene that represent a possible mechanism underlying the development of CRPC have been identified in tissue samples from patients with advanced prostate cancer, as well as in CTC-enriched peripheral blood samples from CRPC patients.

4.2 Melanoma

Melanoma is a highly aggressive cancer and has a strong tendency to metastasize. It frequently goes to the brain or lungs and such metastatic progression can be treated with a wide variety of therapeutics including the immunological ones. Thus monitoring a patient to assess metastasis would be advisable. From Lianidou (2014) we have the following:

Blood-based assays to detect melanoma progression by monitoring levels of CTCs and cfDNA can be used to evaluate progress and therapy response in melanoma patients. Advances in the molecular analysis of CTCs may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management. Mutated BRAF was detected in 81% of 21 assessed stage IV melanoma patients. Single, isolated CTCs from patients with melanoma have been subjected to BRAF and KIT mutational analysis. The BRAF sequences and KIT sequences identified in CTCs were inconsistent with those identified in autologous melanoma tumors, showing clonal heterogeneity. The expression of MART-1, MAGEA3, and PAX3 mRNA biomarkers has been evaluated by RT-qPCR in stage IV melanoma patients. CTC biomarker(s) detected in 54% of patients and were significantly associated with disease-free survival and overall survival.

4.3 Breast Cancer

Breast cancer in many ways is like prostate cancer. It has a high incidence and like PCa has a carcinoma in situ phase as well. The question is; haw aggressive is it and has it spread. From Hench et al (2018)

Detection of oncogenic driver mutations in early-stage presurgical breast cancer might tremendously impact on clinical management. Oncogenic driver mutations were screened for in 29 patients with early-stage BC (I-III) positive for 1/3 PIK3CA mutations (p.H1047R, p.E545K, p.E542K) before and after surgery. Mutant AFs in blood before surgery were low (0.01–0.07%) with the exception of one case with 2.99% who relapsed 26 months later. The same cfDNA mutations were found in 22% of 110 stage I–III BC patients, indicating a prognostic value for ctDNA AFs: higher values correlated with shorter RFS and OS which holds true for TNBC, too. ctDNA detection in stage II–III TNBC patients with residual disease after neoadjuvant chemotherapy predicts recurrence with high specificity, but moderate sensitivity (66), potentially due to low plasma volume (1 ml) and a non-optimized NGS approach. ctDNA content shows significant correlation with prognosis at early cancer stages, as opposed to conventional protein tumor markers.

Postoperative plasma ctDNA abundance after neoadjuvant chemotherapy and surgery, but not baseline levels predicted early recurrence. 50% of relapsing patients were ctDNA-positive postsurgically which increased to 80% during follow-up. None of the relapse-free patients had a ctDNA-positive blood sample. Importantly, ctDNA detection had a median lead time of 7.9 months over clinical recurrence diagnosis

4.4 LUNG CANCER

Lung cancer comes in a variety of forms. Unlike melanoma and even prostate cancer, there is a wide variation in lung carcinomas. Thus there is an increased level of complexity in diagnosis and prognosis. From Hench et al (2018)

Unfortunately, despite WHO-defined LC entities, several recent studies on liquid biopsies use the old, outdated SCLC and NSCLC categorization and lack precise distinction between LAC and LSCC, which in terms of tumor biology and targeted treatment options would be more informative. We tried to comply with the WHO classification wherever possible.

Lung cancer commonly presents at advanced stage due to the lack of screening. Therefore, many studies assessed cfDNA testing for early LC detection and for recurrence monitoring. Concordance rate between tumor DNA and ctDNA mutations in the pretreatment plasma at early-stage (I, II) NSCLC patients was 78.1% (positive predictive value 94.7%), making it an indicator of early-stage LC. 89.7% of 58 early-stage (I–II) NSCLC patients had increased cfDNA out of whom 60.3% were ctDNA-positive with tumor-specific mutations.

Others detected ctDNA in 100% of stage II–IV and 50% of stage I NSCLC patients. Reasons for this discordance are differences in detection technologies, small tumor size and molecular analysis of potentially non-representative tumor sections. 78% of 97 advanced-stage (IIIB, IV) NSCLCs featuring an EGFR variant in the primary had the same mutations in ctDNA; EGFR(p.L858R) in either tumor tissue or cfDNA predicted shorter OS and PFS. Changes in

ctDNA AFs were observed when comparing pre- and postoperative cfDNA: AFs drop 11.52% in stage Ia and 14.63% in stage Ib, but only 0.57% in stage IIa, and 0.13% in stage IIIa. This drop already occurs 2 days postoperatively (86). cfDNA may have a higher positive predictive value compared to serum protein tumor biomarkers for early-stage LC

4.5 Colorectal Cancer

Colon cancer is also a common cancer with high incidence but generally a slow growth rate. Unlike a melanoma which can metastasize very rapidly, colon cancer goes through phases that take time and this is the reason that the use of colonoscopies can dramatically reduce mortality. From Hench et al (2018) we have:

Approximately 50% of localized CRC patients will develop metastases. Although there has been dramatic decline in the number of cases due to screening, CRC incidence remains high. Therefore, cfDNA testing might further improve screening efficiency. Traditional serum protein biomarkers (e.g., CEA, CA19-9) lack high specificity and sensitivity. There is only a limited number of studies investigating cfDNA testing for early CRC detection.

A study on 170 subjectively healthy patients positive for occult fecal blood assessed the predictive power of plasma cfDNA levels and ctDNA KRAS mutations. Adenocarcinoma, but not intraepithelial precursor lesions, including HGIN, could be detected based on these values alone. Yet, the KRAS mutant AF was low (3%) compared to the AF in the tumor itself (45% in AC and HGIN), leaving the positive predictive value of the test questionable. Prospectively collected plasma cfDNA of 232 patients subjected to colonoscopy was analyzed for KRAS mutations which had previously been identified in the tumor tissues of 35 patients.

These mutations were detectable in cfDNA in 29 patients (81%). 39% of patients positive for a KRAS mutation in cfDNA had a KRAS mutant colorectal neoplasia, suggesting to add cfDNA testing for frequent CRC mutations in screening programmes despite restriction to well-known CRC genetic aberrations. CEA is the only routine tumor marker for estimation of tumor burden and progression monitoring despite low sensitivity and specificity, being elevated in 40% of CRC cases only. Several studies found that cfDNA performed better than CEA…

4.6 Head and Neck

Head and neck cancers are less common but quite virulent. Despite their location, potentially being evident to patient and physician, they often go undetected until a mets has occurred. From Lianidou (2014)

According to a prospective clinical follow-up study of patients with squamous cell carcinoma of the head and neck (SCCHN) undergoing surgical intervention, patients with no detectable CTCs had a significantly higher probability of DFS109. The same group has shown recently that in patients with SCCHN, the presence of CTCs correlates with worse disease-free survival. This conclusion was based on the results obtained after isolation of CTCs by a purely negative enrichment methodology which does not depend on the expression of surface epithelial markers.

According to another prospective multicentric analysis that studied the possible role of CTC identification in locally advanced head and neck cancer (LAHNC), CTCs were frequently identified in oro- and hypopharyngeal cancer and in sinonasal undifferentiated carcinoma (SNUC). A decrease in the number of CTCs or their absence throughout the treatment also seemed to be related to non-progressive disease, after either complete or incomplete remission, and with the proportion of patients alive and no evidence of disease

4.7 SUMMARY

We can now summarize the above markers and target malignancy in a table based upon Lianidou et al (2014) where we have the following:

Organ	*Markers*
Colon	CK-19, EpCAM, CEACAM5, PLS3, CEA/CK/CD133, KRAS mutatons
Liver	Cytokeratins, EpCAM, ASGPR1, N-cadherin, vimentin
Pancreas	(CK, CD45)
Head and Neck	CK, vimentin, EGFR, CD44, N-cadherin
Prostate	PSA, KLK2, AR, PSCA, ERG, AR and PTEN, AR mutations, MYC, BRCA1 allelic imbalances TMPRSS2-ERG rearrangements
Breast	CK-19, HER2, ER, PR, EpCAM, MUC1, EMT markers, StemCell markers, DNA methylation
Ovary	EpCAM-, MUC-1-, HER-2, PPIC, CCNE2, DKFZp762E1312, EMP2, MAL2, SLC6A8
Lung	EpCAM, CK-19, CK-7, MYC1, vimentin, cadherins hTERT, TTF-1, EGFR mutations, ALK rearrangements
Bladder	CK-8, survivin
Melanoma	TYR, MUC-18, p97, MART-1, MAGE-A3, MLANA, MITF, GalNAc-T, BRAF mutations

5 OBSERVATIONS

The area of "liquid biopsies" is an active and exciting area of research. However it still presents a multiplicity of challenges. We briefly discuss some these here.

5.1 ISSUES

This is an interesting and strongly visual result. However, there are several observations:

Mutations: Cancer cells are continually mutating. Mutations results oftentimes in new surface receptors due to the changes in the internal proteins. The cell surface receptors may respond differently to the passage through such a cellular membrane. Thus this model may be reflective of itself but not of reality.

Localization: One of the most intriguing things about cancers is the localization of the metastases. Why, for example, do we so often see prostate cancer go to the bone, melanoma to the brain, across the blood brain barrier, and the same with so many other cancers. There is a predisposition to transfer at specific sites. How does this approach deal with such localization effects?

Stem Cells: Stem cells are a potential significant factor in understanding metastasis. One question is; do stem cells move as easily as others or more so? Or, are stem cells just active wherever they are and their products are carried through the blood stream to sites where they can continue cellular proliferation, and possibly induce a new set of stem cells there?

In and Out Flows: One of the questions one must ask when looking at cancer cells in the blood, as has been done recently, is if the cell is coming or going? Namely is the cell going from a source site, a primary, to a remote or metastatic site, or from an already metastatic site to another new one? The tagging of such cells would be important. The understanding of the genetic changes would also be of critical importance.

Biochemical Drivers: The nature of cell surface markers, receptors and the like, often dominate how the cells behaves, interacts with the ECM, and can move to the blood system and exit from it as well. We have argued that the cancer cell just flows and diffuses in the blood system and that there is no growth. That is just gross speculation but it is open to debate. Moreover the interaction of the cell with the cell way, and the localization effects of the cell way by organ specificity may be an attractive basis for organ specific metastasis. Or possibly not. But, having all these elements at play in vivo is better than in vitro.

Immune System: Then also is the impact of the immune system as the cells flow through the vessels. The cells are in a massive amount of immune system interactions, and how does this impact the cells?

These are but a few of the unanswered questions elicited by this paper. The simulation is well worth looking at the paper, but taking its results as fact is stretching it a bit too far.

5.2 CURRENT PROGRESS

It would appear that a great deal of progress has been made yet there are still many significant challenges. As Kaiser has recently noted:

A team of researchers has taken a major step toward one of the hottest goals in cancer research: a blood test that can detect tumors early. Their new test, which examines cancer-related DNA and proteins in the blood, yielded a positive result about 70% of the time across eight common cancer types in more than 1000 patients whose tumors had not yet spread—among the best performances yet for a universal cancer blood test. It also narrowed down the form of cancer, which previously published pan-cancer blood tests have not. The work, reported online today in Science, could one day lead to a tool for routinely screening people and catching tumors before they cause symptoms, when chances are best for a cure. Other groups, among them startups with more than $1 billion in funding, are already pursuing that prospect.

The new result could put the team, led by Nickolas Papadopoulos, Bert Vogelstein, and others at Johns Hopkins University in Baltimore, Maryland, among the front-runners. "The clever part is to couple DNA with proteins," says cancer researcher Alberto Bardelli of the University of Turin in Italy, who was not involved in the work. The researchers have already begun a large study to see whether the test can pick up tumors in seemingly cancer-free women.

Yet there are reservations. Kaiser concludes:

For those who test positive twice, the next step will be imaging to find the tumor. But that will bring up questions raised by other screening tests.

Will the test pick up small tumors that would never grow large enough to cause problems yet will be treated anyway, at unnecessary cost, risk, and anxiety to the patient?

Papadopoulos thinks the problem is manageable because an expert team will assess each case. "The issue is not overdiagnosis, but overtreatment," he says. Still, others working on liquid biopsies say that it will take time to figure out whether widespread screening of healthy people with a universal blood test can reduce cancer deaths without doing harm. "If people expect to suddenly catch all cancers, they'll be disappointed," says cancer researcher Nitzan Rosenfeld of the University of Cambridge in the United Kingdom. "This is exciting progress," he says. "But evaluating it in the real world will be a long process."

Namely Kaiser does reflect the reality of assuming that liquid biopsies are a pending reality. As we have noted, there may be a chance for prognostic use in already metastatic disease. Will liquid biopsies identify indolent cancers, will its use result in putative diagnoses that result in costly tests and procedures but to no avail.

Overall as a physician, one would like to know what lesion is where and how large and how aggressive. Then using the therapeutic tool box one could possibly treat the lesion.

5.3 Limitations

As noted above there is considerable interest in "liquid biopsy" approaches but yet there are reservations as well. As Fouad and Aanei have noted:

Once in circulation, circulating tumor cells (CTCs) are exposed to harsh selective conditions and must devise adaptive techniques. Examples include platelet coats shielding from shear forces and immune-clearance, and metabolic rewiring blunting oxidant stress. Serving as a "liquid biopsy", isolated CTCs could provide means for cancer screening, estimation of metastatic relapse risk, identification of targetable components, exploring tumor heterogeneity, and monitoring therapeutic response. Multiple challenges still stand in the way and will need to be addressed before clinical utilization.

CTCs can be a powerful marker. Yet they leave many questions unanswered.

6 REFERENCES

1. Abraham et al, Liquid Biopsy – Emergence of a new era in personalized cancer care, App Cancer Res 2018Alix-Panabieres and Pantel, Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy, May 2016, Cancer Discovery, 479
2. Aparicio and Caldas, The Implications of Clonal Genome Evolution for Cancer Medicine, NEJM, 368, 9, Feb 28 2013.
3. Armstrong and Wildman, Extracellular Vesicles and the Promise of Continuous Liquid Biopsies, Jrl Path and Trans Medicine, 2018
4. Butler and Gullino, Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma, Cancer Research 35, 512-516, March 1975
5. Castro-Giner et al, Beyond Enumeration: Functional and Computational Analysis of Circulating Tumor Cells to investigate Cancer Metastasis, Frontiers in Medicine, Feb 2018.
6. Chen, M., et al, Mechanisms of tumor cell extravasation in an in vitro microvascular network platform, Integ Bio, 2013, http://pubs.rsc.org/en/content/pdf/article/2013/ib/c3ib40149a
7. Chu, J., Watching tumors burst through a blood vessel, MIT News, http://web.mit.edu/newsoffice/2013/watching-tumors-burst-through-a-blood-vessel-0920.html
8. Cohen et al, Detection and localization of surgically resectable cancers with a multi-analyte blood test, Science 359, 926–930 (2018)
9. Dawson et al, Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer, NEJM, 368, 13, March 28 2013.
10. Ding et al, Perspective on Oncogenic Processes at the End of the Beginning of Cancer Genomics, Cell 173, 305–320 April 5, 2018
11. Domínguez-Vigil et al, The dawn of the liquid biopsy in the fight against cancer, Oncotarget, 2018, Vol. 9, (No. 2), pp: 2912-2922
12. Fouad and Aanei, Revisiting the hallmarks of cancer, Am J Cancer Res 2017;7(5):1016-1036
13. Grivennikov et al, Immunity, Inflammation, and Cancer, Cell March 2010, pp 883-899
14. Gupta and Massagué, Cancer Metastasis: Building a Framework, Cell November 17, 2006
15. Hanahan and Coussens, Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, Cancer Cell 21, March 20, 2012
16. Harris and Kerr, Prostate Cancer Stem Cell Markers Drive Progression, Therapeutic Resistance, and Bone Metastasis, Stem Cells International, Volume 2017, Article ID 8629234, 9 pages
17. Harris et al, CTC Marker CD117/c-kit Represents a Prostate Cancer Stem-Like Subpopulation Driving Progression, Migration, and TKI Resistance, bioRxiv preprint first posted online Apr. 23, 2018; doi: http://dx.doi.org/10.1101/256107

18. Heitzer, et al (2013), Tumor associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing Genome Medicine 2013, 5:30
19. Heitzer et al (2015), Circulating Tumor DNA as a Liquid Biopsy for Cancer, Clinical Chemistry 61:1 112–123 (2015)
20. Hench et al, Liquid Biopsy in Clinical Management of Breast, Lung, and Colorectal Cancer, Frontiers in Medicine, Jan 2018.
21. Hu et al, Circulating Tumor Cells in Prostate Cancer, Cancers 2013, 5, 1676-1690
22. Kaiser, 'Liquid biopsy' for cancer promises early detection, 19 January 2018 Science, Vol 359, Issue 6373
23. Lianidou et al, Circulating tumor cells as promising novel biomarkers in solid cancers, Critical Reviews in Clinical Laboratory Sciences, 2014
24. Lianidou et al, The Role of CTCs as Tumor Biomarkers, Adv Exp Med Biol. 2015;867:341-67.
25. Lou et al, MicroRNAs in cancer metastasis and angiogenesis, Oncotarget, 2017, Vol. 8, (No. 70), pp: 115787-115802
26. McGarty, T., Exosomes and Cancer, 2013, Telmarc www.telmarc.com
27. Vizio, et al, Large Oncosomes in Human Prostate Cancer Tissues and in the Circulation of Mice with Metastatic Disease, The American Journal of Pathology, Vol. 181, No. 5, November 2012
28. Wang et al, Cancer stem cell targeted therapy: progress amid controversies, Oncotarget V 6, No 42, 2015
29. Wang et al, A luminal epithelial stem cell that is a cell of origin for prostate cancer, Nature 2009.
30. Zhe et al, Circulating tumor cells: finding the needle in the haystack, Am J Cancer Res 2011;1(6):740-751